

### University of Catania

PhD Degree in Telecommunications Engineering

## Modeling of cell-to-cell communication using Extracellular Vesicles as information packets

Supervisor: Prof. A. Lombardo

*PhD Student:* Fabrizio Pappalardo

Advisors: Prof. G. Morabito Prof. N. Iraci

## Department of Electrical, Electronic and Informatic Engineering

# $\mathbf{XXXVI}^{th} \ \mathbf{Cicle}$

Academic Year 2023/2024

"Lo scienziato nel suo laboratorio non è solo un tecnico, è anche un bambino davanti a fenomeni della Natura che lo affascinano come un racconto di fate."

Marie Curie

# Contents

Contents			
$\mathbf{Li}$	st of	Figures	$\mathbf{v}$
$\mathbf{Li}$	st of	Tables x	iii
1	Abb	previations	1
<b>2</b>	$\mathbf{List}$	of Publications	4
3	$\mathbf{Abs}$	tract	7
4	Som	nmario	9
5 6	<b>Intr</b> 5.1 5.2 5.3 <b>Biol</b> 6.1	Oduction         Molecular Communication         Biological System Modeling         5.2.1         System Modeling         Microfluidics applied to biology         5.3.1         Microfluidics System         Additional System Modeling         System Modeling         Microfluidics applied to biology         5.3.1         Microfluidics System         Microfluidics System         Communication in Biology through the exchange of Extracellular Vesicles         6.1.1       EV biogenesis         6.1.2       EVs uptaking strategies         Cell cultures used	<ol> <li>11</li> <li>11</li> <li>13</li> <li>13</li> <li>14</li> <li>15</li> <li>17</li> <li>17</li> <li>18</li> <li>20</li> <li>22</li> </ol>
	6.2	Cell cultures used	22 22 23 24
	6.3	<ul> <li>Methodology</li> <li>6.3.1 Scanning Electron Microscopy (SEM) Processing</li> <li>6.3.2 Histological Processing</li> <li>6.3.3 EVs Isolation and Characterization</li> <li>6.3.4 EV Negative Staining for Transmission Electron Microscopy (TEM)</li> </ul>	<ul> <li>25</li> <li>26</li> <li>27</li> <li>27</li> <li>28</li> </ul>

		$\begin{array}{c} 6.3.5 \\ 6.3.6 \\ 6.3.7 \\ 6.3.8 \\ 6.3.9 \end{array}$	EV Immunogold Labelling for Transmission ElectronMicroscopy (TEM)SH-SY5Y Culture, Differentiation, and TreatmentsEV LabellingHigh-Resolution Respirometry (HRR)Statistical Analysis	28 29 31 32 33
7	Woi	rk pro	pose	35
8	Partion 8.1	t I - S chan I <sup>st</sup> Ke	tudy and characterization of a cellular communica- nel through an exchange of EVs ey Study: Extracellular Vesicles Communication in neu-	37
		ronal	context	37
		8.1.1	Small Extracellular Vesicles secreted by Nigrostriatal AS preserve vitality and mitochondrial function in Parkin- son's Disease	38
		8.1.2	AS from the Nigrostriatal System Secrete Small EVs in a Region-Specific Manner	39
		8.1.3	Both VMB- and STR-AS-Derived Vesicles Are Enriched in sEV Markers	42
		8.1.4	Both VMB- and STR-AS-Derived Vesicles Are Inter- nalized by SH-SY5Y Cells	44
		8.1.5	EVs from CCL3-Activated AS Prevent $H_2O_2$ -Induced Caspase-3 Activation in Differentiated SH-SY5Y Neurons	46
		8.1.6	Both VMB- and STR-AS-Derived EVs Preserve the Activity of Mitochondrial Complex I in Differentiated SH-SY5Y Neurons Injured by the Neurotoxin $MPP^+$ .	48
		8.1.7	Only EVs Secreted by VMB-AS Ameliorate ATP Pro- duction in Differentiated, <i>MPP</i> <sup>+</sup> -Injured SH-SY5Y Neu-	40
	89	I Ind	Tons	49 52
	0.4	<u>11</u> 891	Extracellular Vesicles as molecular packets improve coll	52
		0.4.1	to-cell communication	52
		8.2.2	Key study: EVs vs packets molecules	$52 \\ 52$
		8.2.3	Model	53
		8.2.4	Numerical results	55
	8.3	$III^{rd}$	Key Study: EVs Fusion uptake	56

	8.3.1	Modeling Extracellular Vesicle Fusion to the Plasma	50
	0 2 9	Membrane of the Target Cell	. 50 57
	8.3.2	Fusion Model     Model colution	. 01 61
	0.0.0 0.0.1	Nodel Solution	. 01 65
	0.3.4 0.2.5	Solution of the normal Adel ODE of the second kind.	. 05
	8.5.0	Evaluation of EV Fusion Process Parameters at the	67
	0 9 G	Modeling of the fusion process	. 01 69
	$\begin{array}{c} 0.3.0 \\ 0.2.7 \end{array}$	Initial conditions	. 00 71
	0.0.1	Analyzia of the ODE solutions	. 11 72
	0.0.0	Temporal evolution of FP and FV concentrations	. 75 75
	8.3.9 8.3.10	Import of model parameters on the concentration of	. 75
	0.3.10	internalized EVs	78
	8311	Internalization Bate	. 70 79
	8.3.12	Parameter inference	. 15 79
	8.3.13	An Analytical Model for the generic internalization of	. 15
	0.0.10	EV	. 82
	8.3.14	Uptake Model	. 83
	8.3.15	Model solution	. 85
	8.3.16	Experimental case study	. 87
	8.3.17	Model application	. 88
	8.3.18	From the model to lab experiments	. 89
	8.3.19	Model parameter inference	. 90
	8.3.20	Forecasting and Design	. 93
8.4	$IV^{th}$ K	Key Study: EVs Endocytosis Uptake	. 94
	8.4.1	Evaluation of reaction rates for the endocytosis phe-	
		nomena in Extracellular Vesicles cell-to-cell Communi-	
		cations	. 94
	8.4.2	System Model	. 96
	8.4.3	Simulation Results	. 98
	8.4.4	Modeling EVs interactions into a tumor microenviron-	
		ment	. 101
	8.4.5	System Model	. 105
	8.4.6	Analytical Solution for EV-based Cell Interactions	. 108
	8.4.7	Frequency Response of EVs Internalization	. 108
	8.4.8	Release and Internalization Functions for a Closed-loop	
		Cell to Cell Interaction	. 111
	8.4.9	Simulation Results	. 113

		8.4.10	Tumor-immune Unilateral Cell Communication	114
		8.4.11	Tumor-immune Bilateral Cell Communication	117
	8.5	$V^{in}$ K	ey Study: Evaluation of EVs uptaking rates in silico	
		approa	nch	121
		8.5.1	Estimation of Chemical Reaction Rates in Extracellu-	
			lar Vesicle Signaling through <i>in silico</i> approach	121
		8.5.2	System Model	125
		8.5.3	Computational Approaches	127
		8.5.4	Iterative Approach	129
		8.5.5	Michaelis-Menten Approach	131
		8.5.6	Linear Model	132
		8.5.7	Particle-based Simulation (PBS)	132
		8.5.8	Numerical Results	134
		8.5.9	Parameter Selection	134
		8.5.10	Parameter Studies	136
		8.5.11	Computational Complexity Analysis	143
0	D			
9	Par	t 11 - C	naracterization of a centuar communication chan-	1 4 F
	ner	Duran la	marked inposomes into a micronuldic systems	145
	9.1	Dropie	st_speed-snift keying: a modulation scheme for instan-	145
		taneou	Is micronulaic communications $\dots \dots \dots \dots \dots$	140
		9.1.1	Droplet speed shift keying (DSSK)	140
		9.1.2	Experimental setup	148
		9.1.3	Experimental results	150
		9.1.4	Limitations and potential improvements	154
		9.1.5	Modeling of droplet speed shift keying in microfluidic	154
		010	Communications	154
		9.1.0	Droplet Speed Shift Keying a new way to send infor-	155
		017	$ \begin{array}{c} \text{mation} \\ \dots \\ $	155
		9.1.7	Microfluidic Setup	150
		9.1.8	Experimental results and interpretation	157
		9.1.9	DSSK from theory to practice	161
10	Con	clusior	ns and Future prospective	165
11	Ack	nowled	lgments	167
Re	efere	nces		168

# List of Figures

1	Main Concepts in Molecular Communication. This di-	
	agram illustrates the key milestones in the concept of Molec-	
	ular Communication. Information packets, which are organic	
	molecules containing information, are secreted by a cell (the	
	transmitter). They travel through the extracellular space (the	
	channel) and reach the target cell (the receiver). To facili-	
	tate their study and predict their effects, researchers can cre-	
	ate mathematical models or design versatile tools with various	
	applications.	12
2	Methodology. This is an example of a biological system	
	modeling approach in which we begin with a culture of cells	
	(depicted in green), and we concentrate on a cellular com-	
	munication method, attempting to model it using non-linear	
	differential equations	14
3	Microfluidic Experimental Set-up.	17
4	Structure of a Generic EV: An EV is composed of a mem-	
	brane with a phospholipid bilayer and multiple membrane pro-	
	teins involved in internalization processes. Inside, one can find	
	various types of molecules, including DNA, RNA, proteins,	10
-	metabolites, etc. (source: $[1]$ )	19
9	EVs Uptake Strategies: This figure illustrates the most	
	well-known strategies for EVs internalization by target cells,	00
C	alongside new EVs synthesis	23
0	Microscope cells images. a) Undifferentiated human SH-	
	SY5Y cell line observed using optical microscopy; b) Differen-	
	thated numan SH-SY5Y cell line exposed to $10\mu$ mol of retinoic	
	acid as seen through optical microscopy; c)AS captured under	
	nuorescence microscopy with cell bodies shown in green, cell	95
	nuclei in blue, and microglia cells in red	25

7 Brain region influences the rate of secretion of ASsEVs and responsiveness to CCL3 treatment. A) NTA analysis for size distribution displays a peak  $\simeq 100$  nm. Error bars represent SD from n = 3 independent replicates. B) EV concentration, determined by NTA, was normalized over the number of cells. The mean of particles  $/10^6$  cells shows that AS from VMB region secret more EVs than STR and  $\Delta VS$ regions. Data are presented as floating bars with line at mean from n = 3 independent replicates, indicated with different symbols. One-way ANOVA with Tukey's multiple comparison \*p < 0.05 (VMB-AS-EVs versus STR-AS-EVs; VMB-AS-EVs versus  $\Delta VS$ -AS-EVs). C) TEM ultrastructural analysis reveals the presence of sEVs secreted by AS in every condition. Scale bars: 100 nm. D) In all AS-EV samples the average diameter is  $\simeq 60/70$  nm. Raw data (diameter values) are presented as scatter dot plots with line at median  $\pm$  SD from n = 5 (for VMB- and STR-AS-EVs) and n = 3 (for  $\Delta VS$ -AS-EVs) independent experiments. E) Quantitative analysis from TEM showed that AS from VMB secrete more EVs than STR and  $\Delta VS$  regions; the treatment with CCL3 stimulates VMB-AS to release more EVs. Data are normalized considering the number of starting cells, the resuspension volume after ultracentrifugation, the volume used in the microscope grid, and the area  $(\mu m^2)$  of each field in the grid. Data are presented as floating bars with line at mean plus individual data points based on 50 images over 5 independent replicates (for VMB- and STR-AS-EVs) and on 30 images over 3 independent replicates (for  $\Delta VS$ -AS-EVs), indicated with different symbols. One-way ANOVA with Tukey's multiple comparison: in (B) \*p < 0.05 (VMB-AS-EVs versus STR-AS-EVs and versus  $\Delta VS$ -AS-EVs; in (E) \*p < 0.05 (VMB-AS-EVs versus VMB-CCL3-AS-EVs), \*\*\*p < 0.0001 (VMB-AS-EVs) versus STR-AS-EVs and  $\Delta VS$ -AS-EVs), ns: not significant.

42

43

46

9 PKH26-labelled AS-EVs are internalized by differentiated, TH-positive SH-SY5Y neuronal cells. A) Max projection and orthogonal views of representative fields show the uptake of both VMB-AS- and STR-AS-PKH26-labelled EVs by differentiated SH-SY5Y. Each max projection is composed of a stack of 15 individual z planes, acquired every 0.4  $\mu m$  along the z axis. Scale bar 10  $\mu m$ . Plane a and Plane b orthogonal views represent, respectively, two selected planes located above and below the cellular nuclei (along the z axis), as represented by the cellular schematic. In all panels PKH26 is in red, TH in green, whereas nuclear DAPI counterstain is in blue. Confocal images show that PKH26 labelled EVs are present within the cellular bodies of SH-SY5Y target cells upon 6 h of incubation. B) Representative images from IFC of differentiated SH-SY5Y treated with PKH26-AS-EVs for 2, 6, and 24 h. Magnification  $20 \times$ , scale bar 20  $\mu$ m. C) IFC analysis of differentiated SH-SY5Y cells treated with PKH26-AS-EVs and PKH26-dye-only at different time points. Data are expressed as fold change of the mean fluorescence intensity  $\pm$  SD over CTRL set to 1 for comparison (dotted line), from n = 3 independent experiments, indicated with different symbols. One-way ANOVA with Tukey's multiple comparison versus CTRL. \*\*p < 0.01, \*\*\*p < 0.0001, ns: not significant.

vii

10 AS-EVs significantly reduce apoptosis in differentiated SH-SY5Y neurons challenged with  $H_2O_2$ . A) IF staining for MAP2 (in green), c-Casp-3 (in red), and DAPI (in blue), on differentiated SH-SY5Y exposed to AS-EVs and treated with 35  $\mu$ m  $H_2O_2$ . Scale bars: 50  $\mu$ m. B) Quantification of the c-Casp-3 staining in (A). The fluorescent intensities were normalized over the cell number. Data are expressed as mean  $\pm$  SD over CTRL set to 1 for comparison, from n = 3 independent replicates, indicated with different symbols. Oneway ANOVA with Tukey's multiple comparison \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001 versus CTRL, ns: not significant;  $\sharp\sharp$ 

48

11 AS-EVs recover mitochondrial functions in differentiated SH-SY5Y neurons challenged with  $MPP^+$ . A) Representative oxygraphic trace in untreated differentiated SH-SY5Y (control) cells alongside the specific protocol used. First, in intact cells, the physiological  $O_2$  consumption, corresponding to ROUTINE state, was measured. Second, adenylates were forced to leave the cells by a mild plasma membrane permeabilization in order to analyze the LEAK state. Third, the contribution of CI to the OXPHOS respiration was assayed in the presence of the previous addition of the appropriate substrates (pyruvate, malate, glutamate) and a saturating ADP concentration. Then, addition of succinate allowed the activation of CII (CI + CII) and the achievement of total OXPHOS respiration. 5012 Comparison between ligand-receptor uptaking and EVs **fusion.** On the left there is a cell with 2 different receptors for AGO-miRNA-1/-1'/-2/-2'. On the right there is a cell that interact with EVs with inside miRNA 1 and miRNA 2. . . . 5313**Numeric results.** (a) PDFs of miRNA-1 and miRNA-2 ligand svs.  $N_1$  and  $N_2$  and (b) crosstalk entropy at alpha parameter variation. 5414Extracellular Vesicle communication system. On the left there is the transmitter (donor cell) and on the right there is the receiver (target cell). Between them, there is the channel where EVs diffuse. 58

15	EV Fusion to the Plasma Membrane of the Target Cell This is a simple scheme that shows fusion process be-
	tween EV and membrane of the target cell
17	Concentration of FPs and EVs for different values of
	<b>parameters.</b> In those graphs the parameter $b_u$ remain 0,
	while others parameters change from 0.1 to 1000
18	Concentration of $\mathcal{V}_e(t)$ for different values of parameters. 76
19	Internalization rate for $V_0 = 10$
20	Numerical results. Likelihood of the model parameter val- ues given the Internalization Bate 81
21	Numerical results. (a) Comparison between the experimen-
	tal data, curve fitting and model solution, for the case <i>Exp2</i> .(b)
	Comparison between the experimental data and the EVs in-
	ternalization forecasted by the model
22	<b>Experimental set-up of EVs treatment.</b> Isolated EVs are
	added into the well in which the target cells are cultured 95
23	Numerical results. Obtained number of EVs in environment
	and bound to the cell with respect to time for a single PBS
	realization and ODE solution of $(90)$ and $(91)$ . The considered
	parameters are given in Table 5
24	Error estimation graph respect EVs number. Normal-
	ized mean squared estimation error for the reaction rates with
	respect to initial number of EVs in the environment. The
<u>م</u> ۲	considered parameters are given in Table 5 100
25	Error estimation graph about estimation of EVs num-
	<b>Der.</b> Normalized mean squared estimation error for the over-
	estimation (negative relative estimation error) and underesti-
	initial EVs in the well 101
26	Error estimation graph about reaction rate estima-
20	tion Normalized mean squared estimation error for the reac-
	tion rates with respect to time step size in PBS. The considered
	parameters are given in Table 5
27	Error estimation graph about reaction rates estima-
•	tion and time. Normalized mean squared estimation error
	for the reaction rates with respect to reaction rate value. The
	considered parameters are given in Table 5

28	Relationship between T-cells, Tumor cells and EVs into the ECM environment. The tumor microenvironment is made of an acellular part based on proteins and gel, called extracellular matrix (ECM), and a cellular part based on im- mune cells (T-cells) and tumor cells. These cells release differ- ent types of molecules, including extracellular vesicles (EVs), into the extracellular space (ES) that are involved in inflam- matory processes. The same processes of release and uptake
20	of EVs happen in both cells type
29	Block diagram of a biological closed-loop system for
	the cell to cell EV-based interaction. The natural re- lease function of the donor and the target cells is given by $R_0^{\rm D}(j\omega)$ and $R_0^{\rm T}(j\omega)$ , respectively. Overall release functions as the combination of the natural release and the induced release are also denoted by $R^{\rm D}(j\omega)$ and $R^{\rm T}(j\omega)$ . The induced releases are related to the overall internalization functions $I^{\rm D}(j\omega)$ and $I_0^{\rm T}(j\omega)$ through the release factors $k_{\rm re}^T(j\omega)$ and $k_{\rm re}^D(j\omega)$ in the frequency domain. Internalization functions are also expressed in terms of internalization frequency responses $H_{na}^{(.)}$ and $H_{ind}^{(.)}$ , respectively, due to the natural and induced release, given by
	(124)-(127)
30	Schematic representation of the system model for immune-
	tumor interaction
31	Numerical results. Internalization impulse response for tu- mor and immune cells when they are stimulated by a point source located at the other cell's center (for natural release) or membrane (for induced release)
32	Numerical results. IRF at tumor and immune cells for dif- forent cell sizes
33	Numerical results. IRF at the tumor and immune cells for
2.4	different diffusion coefficients of TDEVs and IDEVs
34	<b>Numerical results.</b> IRF at the tumor and immune cells with different internalization, binding, and recycling rates.
35	Numerical results. IBF at the tumor and immune cells
00	for different induced release coefficients of cell activation and inhibition

36	Numerical results. Maximum overall release (natural re-
00	lease + induced release) of TDEVs in terms of distance be-
	tween the cells membrane (d) and induced release coefficient
	for (a) pathway activation process and (b) pathway inhibition
	process
37	Numerical results. Maximum overall release (natural re-
	lease + induced release) of IDEVs in terms of distance between
	the cells membrane (d) and induced release coefficient for (a)
	pathway activation process and (b) pathway inhibition process, 123
38	EV uptake by the recipient cell can be driven by many
	different pathways: endocytosis, fusion and juxtacrine.
	This illustration is created using BioRender.com 124
39	Flowchart of the iterative approach
40	Numerical results. Obtained number of EVs in environ-
	ment and bound to the cell with respect to time for a single
	PBS realization and ODE solution of $(136)$ and $(137)$ . The
	considered parameters are given in Table 7 and $N=0.1.$ 136
41	Numerical results. Normalized mean squared estimation
	error for the reaction rates with respect to number of binding
	sites of the cells. The considered parameters are given in Table 7.137
42	Numerical results. Normalized mean squared estimation
	error for the reaction rates with respect to initial EV quan-
	tity in the medium. The considered parameters are given in
	Table 7. Since $N$ is fixed, the dimensional number of binding
4.0	sites changes accordingly
43	Numerical results. Normalized mean squared estimation er-
	ror for the overestimation (negative relative estimation error)
	and underestimation (positive relative estimation error) of the
	mitial EV quantity in the well. The considered parameters are
4.4	Numerical results. Normalized mean squared estimation or
44	ror for the reaction rates with respect to additive white Caus
	sian noise variance. The considered parameters are given in
	Table 7 141
45	Numerical results. Normalized mean squared estimation
10	error for the reaction rates with respect to reaction rate value
	The considered parameters are given in Table 7
	1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

46	Numerical regulta. The every number of required iters
40	tions of the iterative approach with respect to number of hind
	ing sites of the cells. The considered parameters are given in
	Table 7 142
17	Droplet gread shift keying DSSK modulator 147
41	Microfluidia components used in our experiments: T
40	iunction 140
40	Numerical results Droplet speed measured at the receiver
49	vs the droplet sequence number for two pairs of mass flow
	vs. the droplet sequence number for two pairs of mass now
50	Probability distributions of droplet stroom velocities
50	Fach distribution is calculated based on the mean and variance
	of the detect on which a normal distribution is derived 152
51	Dreplet Greed Chift Version DCCV and deleter 156
51	Droplet Speed Shift Keying, DSSK, modulator 100
52	Numercial results. Droplet speed measured at the receiver
	vs. the droplet sequence number for two pairs of mass now
50	rates
53	Probability distributions of droplet stream velocities.
	The PDFs report the number of samples for each flow veloc-
	ity class. Each distribution is calculated based on the mean
	and variance of the dataset, on which a normal distribution is
~ .	derived
54	Numerical results. Exemplary evolution of the droplet speed
	versus time in the case when the flow rates change from the
	pair 5-3 $\mu$ l/min to the pair 16-12 $\mu$ l/min
55	System response when the flow rates change from 5-3
	$\mu$ l/mi to 16-12 $\mu$ l/min and curve obtained by applying
	the fitting procedure
56	Exemplary RLC circuit

# List of Tables

1	List of antibodies used in WB
2	Coefficients of Equation (19) 62
3	Unit of measurement of model parameters
4	(a) Parameters for the best fitting curve in Fig. 21a - (b) Model
	parameter values according to (86)
5	Default simulation parameters, which are applied throughout
	the numerical results, if not stated otherwise
6	Parameter values of the system model in Fig. 30
7	Default simulation parameters, which are applied throughout
	the numerical results, if not stated otherwise. Parameters with
	a bar denote dimensional parameters
8	Statistical characteristics of the droplet speeds for different
	pairs of mass flow rates
9	Threshold, $\sigma_{Th}$ , and theoretical and experimental error prob-
	abilities, $p_E$ , for different associations between symbols and
	mass flow rates pairs. $\ldots \ldots 152$
10	Statistical characteristics of the droplet speeds for different
	pairs of mass flow rates
11	Threshold, $\sigma_{Th}$ , and theoretical and experimental error prob-
	abilities, $p_E$ , for different associations between symbols and
	mass flow rates pairs

# 1 Abbreviations

ABCA 1	ATP Binding Cassette Transporter 1
ALIX	Apoptosis-Linked Gene 2 Interacting Protein
$\alpha$ -syn	$\alpha$ -synuclein
ARF6	AdenoDiPhosphate-Ribosylation Factor 6
ARRDC1	Arrestin Domain-Containing Protein 1
AS	Astrocytes
ASCT-2	Neutral Amino Acid Transporter 2
AS-EVs	astrocyte-derived extracellular vesicles
ATP	Adenosine TriPhosphate
CCL3	Chemokine (C-C motif) Ligand 3
CMI	Confocal Microscopy Imaging
CNS	Central nervous system
CTRL	control
DAergic	dopaminergic
DCM	Donor cell membrane
$\Delta \text{VS-AS}$	AS from VMB
DLP	Digital Light Processing
DNA	DeoxyriboNucleic Acid
DSSK	Droplet_Speed Shift Keying
ECM	Extracellular matrix
ERK	Extracellular Signal-Regulated Kinase
ESCART	Endosomal Sorting Complex Required for Trasport
EV(s)	Extracellular Vesicles
$\exp(1/2)$	experiment $1/2$
FBS	Fetal Bovine Serum
FPs	Fusogenic Proteins
GTP	Guanine TriPhosphate
HRR	High-Resolution Respirometry
IDEV	immune cell-derived EVs
IF	ImmunoFluorescence
IFC	ImmunoCytoFlow
ILVs	IntraLuminal Vesicles

IRC	Induced release coefficient
IRF	Internalization rate function
LBs	Lewy bodies
LNs	Lewy neurites
LoC	Lab-on-a-Chip
MEM/F12	Minimum Essential Media $+$ F-12
miRNA	micro-RNA
MLC	Phosphorilates Myosin Light Chain
MLCK	Phosphorilates Myosin Light Chain Kinase
MSFD2a	Major Facilitator Superfamily Domain-Containing Protein 2a
MPTP	1-methyl, 4-phenyl, 1,2,3,6 tetrahydropyridine
MVBs	MultiVesicular Bodies
NEDD4	Neural Precursor Cell Expressed, Developmentally Downregulated 4
NMSEE	normalized mean squared estimation error
NTA	Nano Tracking Analysis
ODE	Ordinary Differential Equations
PBS	particle based simulations
PD	Parkinson's disease
PEN	Penicillin antibiotic
PFA	paraformaldehyde
PDFs	probability density functions
PDMS	PolyDimethylSiloxane
PLD	Phospho-Lipase D
PMMA	PolyMethyl MethAcrylate
PR	Plate Reader
PS	PhosphatidylSerine
PSAP/PPXY	Protein motives of ARRDC1
RA	Retinoic Acid
Re	Reynolds numbe
RNA	RiboNucleic Acid
RX	receiver

SEM	Scanning Electron Microscopy
SH-SY5Y	Neuroblastoma cell line
SLA	StereoLithography Apparatus
SNpc	substantia nigra pars compacta
SNT	Supernatant
STR	Striatum
STREP	Streptomycin antibiotic
T cells	T lymphocyte cells
TCM	Target cell membrane
TDEVs	Tumor-derived EVs
TEM	Transmission Electron Microscopy
$\mathrm{TH}$	tyrosine hydroxylas
TME	tumor microenvironment
TSG101	Tumor Susceptibility Gene 101
ТΧ	transmitter
VMB	Ventral MidBrain
WB	Western Blot

# 2 List of Publications

## Journal Articles

- Leggio, L., L'Episcopo, F., Magrì, A., Ulloa-Navas, M. J., Paternò, G., Vivarelli, S., **Pappalardo, F.**, ... & Iraci, N. (2022). Small Extracellular Vesicles Secreted by Nigrostriatal Astrocytes Rescue Cell Death and Preserve Mitochondrial Function in Parkinson's Disease. Advanced healthcare materials, 11(20), 2201203.
- Damrath, M., Zoofaghari, M., Lekić, M., Rudsari, H. K., Pappalardo, F., Veletić, M., & Balasingham, I. (2023). Computational estimation of chemical reaction rates in extracellular vesicle signaling. Nano Communication Networks, 37, 100455.
- Lombardo, A., Morabito, G., Panarello, C., & **Pappalardo, F.** (2023). Intercellular Chemical Communication Through EV Exchange: Evaluation of the EV Fusion Process Parameters at the Receiving Cell. IEEE Transactions on Molecular, Biological and Multi-Scale Communications.
- Zoofaghari, M., **Pappalardo, F.**, Damrath, M. & Balasingham, I. (2024). Modeling Extracellular Vesicles-Mediated Interactions of Cells in the Tumor Microenvironment. IEEE Transactions on Nanobio-science, 23(1), 101109.
- Leggio, L., Paternò, G., Vivarelli, S., L'episcopo, F., Tirolo, C., Raciti, G., Pappalardo, F., ... & Iraci, N. (2020). Extracellular vesicles as nanotherapeutics for Parkinson's disease. Biomolecules, 10(9), 1327. (Review Article)

### Under Submission

- Galluccio L., Lombardo A., Morabito G., Panarello C., Pappalardo, F. (2023). Modeling of droplet speed shift keying in microfluidic communications. Submitted to IEEE Transactions on Molecular, Biological, and Multi-Scale Communications.
- Iraci, N., Leggio, L., Lombardo, A., Morabito, G., Panarello, C., **Pappalardo, F.**, & Paternó, G. Modeling the EV concentration in different

internalization phases. 8th Workshop on Molecular Communications, Oslo, NO. (Abstract)

• Pappalardo, F., Calì, F., Tuccitto, N. The Role of Receiver Population Variability in MC Involving Living Insects. 8th Workshop on Molecular Communications, Oslo, NO.

### **Conference** Articles

- Lombardo, A., Morabito, G., Panarello, C., & **Pappalardo, F.** (2022, October). Modeling biological receivers: the case of extracellular vesicle fusion to the plasma membrane of the target cell. In Proceedings of the 9th ACM International Conference on Nanoscale Computing and Communication (pp. 1-6).
- Zoofaghari, M., Damrath, M., Rudsari, H. K., Pappalardo, F., Veletić, M., & Balasingham, I. (2022, October). Reaction rates estimation for the endocytic reception in extracellular vesicles-mediated communications. In Proceedings of the 9th ACM International Conference on Nanoscale Computing and Communication (pp. 1-6).
- Iraci, N., Leggio, L., Lombardo, A., Panarello, C., **Pappalardo, F.**, & Paternó, G. (2023, September). An Analytical Model for the Inference of the EV Reception Process Parameters in Cell-to-Cell Communication. In Proceedings of the 10th ACM International Conference on Nanoscale Computing and Communication (pp. 136-141).
- Galluccio, L., Lombardo, A., Morabito, G., **Pappalardo, F.**, & Quattropani, S. (2023, September). Droplet speed-shift keying: a modulation scheme for instantaneous microfluidic communications. In Proceedings of the 10th ACM International Conference on Nanoscale Computing and Communication (pp. 110-115).

### Poster

• Pappalardo, F., Iraci, N., Leggio, L., Lombardo, A., & Morabito, G. (2021, September). How molecular packets improve cell-to-cell communication. In Proceedings of the Eight Annual ACM International Conference on Nanoscale Computing and Communication (pp. 1-2).

Leggio, L., L'Episcopo, F., Magri, A., Ulloa-Navas, M. J., Paterno, G., Vivarelli, S., Pappalardo, F., ... & Iraci, N. (2021, July). Astrocytes from distinct nigrostriatal brain regions secrete extracellular vesicles able to mediate neuroprotection in cellular models of Parkinson's disease. In GLIA (Vol. 69, pp. E300-E302). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: WILEY.

#### Peer Review

- Salinity and Droplets: First Realization of Microfluidic Multiplexing for the 10th ACM International Conference on Nanoscale Computing and Communication (2023).
- HetMM: A Michaelis-Menten model for non-homogeneous enzyme mixtures for iScience (2023).
- The Intensity Parameter Estimation of Generalized Inverse Gaussian when Using Neuron as Communication Medium for the IEEE Transactions on Molecular, Biological, and Multi-Scale Communications (2023).

## 3 Abstract

Throughout the course of evolution, cells have developed various forms of communication, which are based on specific molecular interactions and tailored to convey specific messages. The primary cellular communication strategies are mediated through receptor binding or direct contact with the target cell's membrane. Communication based on the exchange of Extracellular Vesicles (EVs) has emerged as a focal point in the research of the scientific community due to the important stability and versatility of the vesicular structure, but also as a possible use in innovative and effective therapeutic therapies protocols. EVs are essentially information-rich "packages" containing DNA, RNA, proteins, and various molecules. They play a critical role in stimulating and regulating molecular pathways within target cells. In this doctoral work, various strategies are introduced, in which a model based on Ordinary Differential Equations (ODE) assesses the enhanced efficiency of cell-cell communication through EVs when compared to traditional ligand-receptor strategies. These findings have the potential to revolutionize drug delivery systems, especially for complex diseases. This interdisciplinary approach treats the biological entity of the cell as both transmitter and receiver, emphasizing the internalization of EVs through fusion with the plasma membrane of the target cell. However, the paucity of information regarding these processes hinders the full potential of EV-based therapies. To bridge this knowledge gap, this thesis work presents a mathematical methodology, marking a fundamental first step towards making a significant contribution to the study of EV-mediated communication.

Parallel to this, a mirror approach can be imagined in which the information is no longer the content of the single EV but the vesicle itself. The artificial vesicle (liposome) can then be marked with a dye and follow its path over time. This strategy is applied in the field of microfluidics where a new approach is introduced that involves the instant manipulation of dye droplets within a continuous oil phase, forming small droplets, to induce changes in flow properties. Applications range from medicine to biodefense and drug delivery. This thesis not only presents a new coding methodology, but also establishes a model to better understand and predict the dynamics within microfluidic channels, with the potential to revolutionize various fields.

The thesis begins with an introductory section that addresses the topic of general Molecular Communication (Section 5.1), its applications in biology (Section 6.1), and in microfluidics (Section 5.2). A background paragraph is

also reported in Section 6, in order to help understand the works reported in the thesis.

The purpose of the research is reported in Section 7, while the section dedicated to the results of the research work is structured into two independent parts. The section dedicated to the results of the research work is structured into two independent parts. Part I in Section 8 addresses the first issue, i.e. different modeling approaches of cellular communication based on real experimental data in which it is demonstrated that EVs can have a crucial and important role in the recovery of neuronal degeneration in Parkinson's disease. This strategy is performed through the use of linear and nonlinear models, which reconstruct and describe the main internalization strategies of EVs by a target cell. Furthermore, it focuses on mathematical strategies to derive the necessary parameters for such models starting from laboratory experimental data or *in silico* simulations. Part II in Section 9 introduces the possibility of transmitting information in a different way, since the information of interest is no longer the content of the EV but the lipid envelope of which it is formed. A microfluidic system is used to transmit useful information by varying the "sliding" speed of these artificial EVs.

Finally, Section 10 reports the conclusions and future perspectives of this research work and Section 11 contains acknowledgments.

## 4 Sommario

Nel corso dell'evoluzione, le cellule hanno sviluppato diverse forme di comunicazione basate su specifiche interazioni molecolari e mirate a trasmettere messaggi specifici. Le strategie principali di comunicazione cellulare avvengono mediante il legame con recettori o il contatto diretto con la membrana delle cellule bersaglio. La comunicazione basata sullo scambio di Vescicole Extracellulari (EVs) è emersa come punto focale nella ricerca della comunità scientifica, grazie alla stabilità e versatilità della struttura delle vescicole e alla possibilità di utilizzarle in terapie innovative ed efficaci. Le EVs sono essenzialmente "pacchetti" ricchi di informazioni contenenti DNA, RNA, proteine e diverse molecole, e svolgono un ruolo critico nella stimolazione e regolazione delle vie molecolari nelle cellule bersaglio.

In questa tesi di dottorato vengono presentate diverse strategie in cui un modello basato su Equazioni Differenziali Ordinarie (ODE) valuta l'efficienza superiore della comunicazione cellula-cellula attraverso le EVs rispetto alle tradizionali strategie ligando-recettore. Questi risultati hanno il potenziale per rivoluzionare i sistemi di somministrazione di farmaci, specialmente per le malattie complesse. Questo approccio interdisciplinare considera l'entità biologica della cellula come mittente e ricevente, enfatizzando l'internalizzazione delle EVs tramite fusione con la membrana plasmatica della cellula bersaglio. Tuttavia, la scarsità di informazioni su questi processi ostacola il pieno potenziale delle terapie basate sulle EVs. Per colmare questa lacuna di conoscenza, questa tesi presenta una metodologia matematica, rappresentando un primo passo fondamentale verso un significativo contributo allo studio della comunicazione mediata dalle EVs.

Parallelamente a ciò, è possibile immaginare un approccio speculare in cui l'informazione non è più il contenuto singolo delle EVs, ma la vescicola stessa. La vescicola artificiale (liposoma) può essere quindi contrassegnata con un colore e seguire il suo percorso nel tempo. Questa strategia è applicata nel campo della microfluidica, dove si introduce un nuovo approccio che coinvolge la manipolazione istantanea di gocce di colorante all'interno di una fase oleosa continua, formando piccole gocce, per indurre variazioni nelle proprietà del flusso. Le applicazioni spaziano dalla medicina alla biotutela e alla somministrazione di farmaci. Questa tesi non solo presenta una nuova metodologia di codifica, ma stabilisce anche un modello per comprendere e prevedere meglio le dinamiche all'interno dei canali microfluidici, con il potenziale di rivoluzionare vari campi. La tesi inizia con una sezione introduttiva che tratta dell'argomento della Comunicazione Molecolare generale (Sezione 5.1), delle sue applicazioni in biologia (Sezione 6.1) e in microfluidica (Sezione 5.2). Viene inoltre fornito un paragrafo di contesto nella Sezione 6, per aiutare a comprendere i lavori riportati nella tesi.

Nella Sezione 7 viene riportato lo scopo della ricerca, mentre la sezione dedicata ai risultati del lavoro di ricerca è strutturata in due parti indipendenti. La Parte I nella Sezione 8 affronta la prima problematica, ossia le diverse strategie di modellazione della comunicazione cellulare basata su dati sperimentali reali in cui si dimostra che le EVs possono svolgere un ruolo cruciale e importante nel recupero della degenerazione neuronale nella malattia di Parkinson. Questa strategia viene realizzata attraverso l'uso di modelli lineari e non lineari che ricostruiscono e descrivono le principali strategie di internalizzazione delle EVs da parte di una cellula bersaglio. Inoltre, si concentra sulle strategie matematiche per derivare i parametri necessari per tali modelli a partire dai dati sperimentali di laboratorio o dalle simulazioni *in* silico. La Parte II nella Sezione 9 introduce la possibilità di trasmettere informazioni in un modo diverso, poiché l'interesse non è più il contenuto delle EVs, ma l'involucro lipidico di cui sono composte. Un sistema microfluidico viene utilizzato per trasmettere informazioni utili variando la "velocità di scorrimento" di queste EVs artificiali.

Infine, la Sezione 10 riporta le conclusioni e le prospettive future di questo lavoro di ricerca, mentre la Sezione 11 contiene gli ringraziamenti.

# 5 Introduction

### 5.1 Molecular Communication

In the 21st century, modern technology and telecommunication systems enable us to simplify our lives and maintain constant communication through the use of computers, telephones, and the internet [2]. One might ponder whether human innovation in designing antennas and receivers was inspired by nature, as the underlying mechanism bears similarities to the communication methods employed by Prokaryotic and Eukaryotic cells. The key distinction lies in the transmission of information through chemical molecules rather than bits or bytes, with cell receptors serving as the equivalent of receivers [3].

Molecular Communication constitutes a branch of science that seeks to explore how cells communicate with one another, employing an 'engineering' approach to model and predict potential cellular responses to prior inputs [4]; in Fig. 1, depicts the main milestones in the field of Molecular Communication. Chemical molecules are employed for communication on both microscopic and macroscopic scales. Depending on the nature of the chemical molecules involved, Molecular Communication can be categorized into three classes: i) hydrophilic [5], ii) hydrophobic [6] and vesicular [7]. Various modeling applications aim to calculate transmitted information [8], "molecular noise" [9] or the regulation of metabolism [2]. These distinct strategies will be further elucidated in the following paragraphs. One of the primary goals of Molecular Communication is to transmit information encoded within 'information packets.' These packets travel within the extracellular matrix or the bloodstream through free diffusion. It is essential to underline that it is often complex to confirm that the sending and/or reception of specific "information packets" by the target cell has occurred or is responsible for the biological response under examination. This inherent limitation results in a high probability of errors. Furthermore, the understanding of the various molecular pathways is not always clear or comprehensible. Thus, at present, it is not possible to decode the transmitted information; instead, we can only calculate the extent of "molecular noise" or the disturbance in received information created during this process [9]. These signals are biocompatible and demand minimal energy for generation and transmission, rendering them ideal for various applications where the use of electromagnetic signals is impractical or undesirable. While chemical signals occur naturally



Figure 1: Main Concepts in Molecular Communication. This diagram illustrates the key milestones in the concept of Molecular Communication. Information packets, which are organic molecules containing information, are secreted by a cell (the transmitter). They travel through the extracellular space (the channel) and reach the target cell (the receiver). To facilitate their study and predict their effects, researchers can create mathematical models or design versatile tools with various applications.

and are used by microorganisms, such as bacteria, for communication and detecting other microorganisms, it has only recently been proposed to engineer a microscopic-scale communication system based on chemical signals [10, 11]. Another potential application of Molecular Communication involves simulating the transmission, diffusion, and reception of 'information packets' through the creation of computer simulations and mathematical models [12].

In addition to the 'biological' application of Molecular Communication, it can find applications in the field of chemistry. This includes the use of fluorescent molecules as information carriers [13], or artificial vesicles known as liposomes, which can transport specific molecules or trigger controlled chemical reactions [14]. Molecular Communication is a resource that is garnering significant interest within the scientific community due to its substantial potential for applications in diagnosis, pharmacology, and the study of cellular physiology.

### 5.2 Biological System Modeling

The modeling of biological systems can be traced back to the 17th century with the publication of the first physical laws by Isaac Newton. However, it was only with the advent of modern computers that this discipline began to develop significantly. In the 1950s and 1960s, many researchers started using the first computers to create mathematical models of biological systems, such as neural networks or physiological systems [15].

### 5.2.1 System Modeling

To date, the importance of modeling biological systems has significantly increased, representing an interdisciplinary discipline involving biology, mathematics, computer science, and engineering [16]. The advance of computer technology and the increase in the availability of biological data, thanks to the advent of the new "-omics" sciences [17], have contributed to an exponential growth in the applications of modeling biological systems in various fields, including medicine [18, 19], agriculture [20] and environment [21]. Bioinformatics tools are useful for deepening our understanding of organism physiology at the molecular level, shifting the research focus to the analysis of molecular networks [22]. From this line of research, Molecular Communication was born and evolved as a science that studies biological networks using mathematical tools and models [23].

Thus, a new definition of the concept of a biological system emerged, representing the subject of study and the context in which it exists [24]. Depending on the subject to be analyzed, it can be described as i) a linear dynamical system [25], ii) a nonlinear dynamical system [26] or iii) population dynamics [27].

In reality, all biological systems are complex and unpredictable. Modeling requires significant IT resources and knowledge of all parameters that regulate molecular processes involved. While many of these parameters are impossible to measure directly, they can be calculated indirectly through *in silico* simulations [12, 28].

Unlike classic laboratory strategies where the study of physiological or pathological phenomena can be explored in depth using cell cultures *in vitro* or animal models *in vivo*, models based on biological systems introduce a new frontier of study. It is possible to use digital simulations or mathematical reconstructions based on previous data and/or knowledge from the literature;



Figure 2: Methodology. This is an example of a biological system modeling approach in which we begin with a culture of cells (depicted in green), and we concentrate on a cellular communication method, attempting to model it using non-linear differential equations.

this approach is called *in silico* [29]. Thanks to this strategy, large quantities of data can be obtained, reducing costs and the waste of energy and materials used in experiments, and often being the only feasible approach in complex research.

In addition to the parameters and modeling dynamics, a component of "molecular noise" must be included; it describes the randomness of events in biological systems and their intrinsic non-linear properties [30]. "Molecular noise" is found in the variation of the concentration of molecules, the variation in the synthesis or reception of these molecules, or even in the arrangement of cellular receptors.

This thesis work will present different examples of biological system modeling in Section 8, as shown in Fig. 2, and discuss the role of "molecular noise" in these contexts. Moreover, several strategies for evaluating the parameters involved in the model and how to extrapolate extrapolate parameters from experimental data will be presented.

### 5.3 Microfluidics applied to biology

In parallel to the modeling of the biological system there is another strategy to study molecular communication which is microfluidics. Microfluidics and its applications introduce the possibility of transmitting information in a different way, not through electrical pulses or simple diffusion in the matrix but by exploiting the flow variation in the system, deriving from one or more external pumps [31, 32].

Such a strategy can be applied to biology using dye-labeled liposomes or dye droplets that can emulate them [33]. The information of interest is no longer the content of the single EV but becomes the lipid envelope from which it is composed, transmitting precious information as the "scrolling" speed of these artificial EVs varies. The core of such an approach could help track the movement of EVs in the body, study their effects in detail, and obtain the parameters involved in the secretion, diffusion, and internalization of such EVs, so that clinical trials or drug treatments can be planned and improved.

#### 5.3.1 Microfluidics System

Microfluidics enables the manipulation of fluids and the study of specific reactions in controlled environments. Consequently, a new research field has emerged that combines microfluidics with clinical biology, medicine, and physics [34].

The small size of chambers and channels, on the order of micrometers, along with the physical properties resulting from fluid confinement in such narrow spaces, allows numerous chemical and biological processes to be conducted using minimal volumes of liquids [35]. Moreover, at these dimensions, molecular consumption is significantly reduced compared to traditional macroscale experiments.

Microscale experiments offer several advantages. Due to their small size, they maintain low values of the Reynolds number (Re) [36]. In microfluidic devices  $(10^{-3} > \text{Re} > 10^{-5})$ , this value allows for the creation and stability of laminar flow, which has a significant impact on the precise control of critical reaction parameters, including temperature, fluid mixing speed, and reactant and product concentrations [37, 38]. Capillary forces enable fluid movement within the channels, but one or more pumps are necessary to generate and maintain specific pressures or replace reagents in the devices [39, 40].

The primary classes of microfluidic devices can be categorized as follows: paper, silicon, glass, polymer, and more recently, fiber. Paper devices, which use paper substrates (including nitrocellulose), are employed in medical clinical analyses and diagnoses through colorimetric assays [41, 42]. Silicon and glass are the original substrates for 3D microfluidic device development, creating microfluidic designs (microchannels, microchambers, etc.) [43, 44, 45].

Three-dimensional chips manufactured from polymers like polydimethyl-

siloxane (PDMS) through photolithography, or transparent resin using 3D printers, have become prevalent in recent years [46, 47]. 3D printing allows for the creation of microfluidic chips through a single fabrication process, eliminating potential errors or misalignments present in PDMS-based fabrication processes, which could affect their performance. Recent advancements have achieved excellent printing resolutions on the order of micrometers, and the plasticity of materials allows for the production of micrometric channels, T-junctions, and housing chambers for the study of cell or organ pathologies and testing new drugs [48, 49], as illustrated in Fig 3.

The geometry and design structure of a three-dimensional chip, along with printing resolution StereoLithography Apparatus (SLA) defined by the laser beam or pixel size in the case of Digital Light Processing (DLP) SLA technology, significantly affect fabrication [50]. However, current 3D printing methods are limited in modeling complex and "empty" micrometric channels, as they tend to collapse or deform [51]. Strategies to overcome these issues include the use of DLP projection resins, removal with high-pressure air or water jets, or the use of polymethyl methacrylate (PMMA) or glass substrates [52].

Fiber devices are utilized for the colorimetric detection of biomarkers in urine and artificial plasma samples, eliminating the need for hydrophobic barriers and possessing a natural microchannel structure with good flexibility. Networks can be easily formed into knots and tangles, creating microfluidic loops [53].

In this thesis work, in Section 9 an example of the application of microfluidics for the coding of information will be presented through the perception of speed variations of color droplets, which simulate liposomes circulating in a blood capillary.



Figure 3: Microfluidic Experimental Set-up.

# 6 Biological Background and Methodology

This section will briefly introduce some fundamental concepts of molecular communication in the biological context in 6.1, we will also introduce cell cultures in 6.2 and methods in 6.3 used in all the experiments that were the basis and the refutation of the models that will be discussed in my work and will help to understand the motivations mentioned in the previous section. For further specifications and insights see [54, 55].

## 6.1 Communication in Biology through the exchange of Extracellular Vesicles

To better understand the potential of Molecular Communication, it is necessary to introduce some concepts related to cell biology and their communication strategies. The cell is the simplest unit capable of sustaining its own life and reproducing completely independently. Based on their cellular structure, all organisms can be classified into two large categories: Prokaryotes, in which we find Bacteria and Archea, and Eukaryotes, divided into Plants, Animals, Fungi, Protozoa and Monera. Both cell types share the presence of a double-layer lipid membrane, different in protein, carbohydrate and lipid components based on the specific organism in question, which separates the external environment from the internal environment of the cell [56, 57]. Although their structures are specifically different, the cell membrane is highly amphipathic with very narrow pores and serves as a sieve for molecules that could transmit information to the cell.

As introduced in the preceding paragraph, evolution has driven organ-

isms to develop diverse communication strategies based on the nature of the molecules involved and the type of information that needs to be transmitted. If the "information packet" is a protein or a hydrophilic vitamin, it requires a receptor, typically a protein, capable of triggering the required cellular response [5]. When the "information package" is a lipid or a hydrophilic drug, it can independently cross the cell membrane, thereby transferring the information directly to the target cell [6]. Additionally, these individual molecules can be enclosed and protected within a single spherical structure known as a vesicle [58].

Extracellular vesicles (EVs), as shown in Fig. 4, are nanometer-sized spherical structures enclosed by a double lipid membrane. They can transport various molecules, including proteins, hormones, genetic material (RNA/ DNA), or metabolites between cells [59]. EVs are secreted by all cell types in vivo and in vitro and can be found in all body compartments [60]. EVs have emerged as a significant strategy for intercellular communication, and researchers have classified them based on their size and biogenesis. Although they constitute a heterogeneous group of vesicles with different functions, properties and origins, they can be classified into three main groups based on biogenesis: exosomes (30-150 nm), microvesicles (50 nm to 2  $\mu$ m), and apoptotic bodies (>50nm) [61]. Unfortunately, current technologies make it difficult to comprehensively isolate and separate the aforementioned groups of vesicles, which is why they are more generically classified into *small vesi* $cles (< 200 \,\mathrm{nm})$  and medium-large vesicles  $(> 200 \,\mathrm{nm})[62]$ . small vesicles have garnered increased attention due to their potential applications and unique characteristics supported by substantial scientific evidence in recent years [63].

Liposomes are artificial vesicles recreated in the laboratory, enriched only with the molecules intended for transport, often used in planning clinical trials [64]. Some anti-tumor therapies, based on the use of liposomes, are already on the market [65, 66].

#### 6.1.1 EV biogenesis

The biogenesis of EVs is a highly complex and dynamic process that is crucial for intercellular communication. These vesicles are released by cells into the extracellular space and play fundamental roles in various physiological and pathological processes. The process of EV formation typically begins with the inward budding of the endosomal membrane, leading to the formation



Figure 4: Structure of a Generic EV: An EV is composed of a membrane with a phospholipid bilayer and multiple membrane proteins involved in internalization processes. Inside, one can find various types of molecules, including DNA, RNA, proteins, metabolites, etc. (source: [1]).

of multivesicular bodies (MVBs). Within MVBs, proteins, lipids, and nucleic acids are packaged into small vesicles called intraluminal vesicles (ILVs) through a process involving the Endosomal Sorting Complex Required for Transport (ESCRT) protein family [67].

The EV formation process is different depending on the type of vesicle, whether *small* or *large*. The biogenesis of *small* vesicles typically begins with inward budding of the endosomal membrane, leading to the formation of multivesicular bodies (MVBs). Inside MVBs, proteins, lipids, and nucleic acids are packaged into small vesicles called intraluminal vesicles (ILVs) through a process involving the endosomal sorting complex required for transport (ES-CRT) family of proteins [67], there are also independent ESCRT biogenesis processes [68].

MVBs can then migrate to the plasma membrane and fuse with it, releasing their contents into the extracellular fluid as *small* vesicles. These *small* vesicles contain a variety of biologically active molecules, including proteins, lipids and nucleic acids, which can be taken up by target cells and influence their function. Alternatively, other types of EVs, such as microvesicles, can form directly through outward budding of the plasma membrane [69], as shown in Figure 5.

The biogenesis of *large* vesicles involves cytoskeletal components, the floppase ATP binding cassette transporter 1 (ABCA1) plays a key role by facilitating the translocation of phosphatidylserine (PS) to the outer side of the membrane, inducing an asymmetrical distribution of phospholipids [70, 71, 72]. Moreover, the binding of GTP to a member of the small GT-Pase family, ARF6, triggers a downstream signaling cascade that leads to the contraction of actin-myosin, enabling the scission of ectosomes from the plasma membrane. Specifically, GTP binding to ARF6 activates phospholipase D (PLD), facilitating the recruitment and activation of extracellular signal-regulated kinase (ERK) at the plasma membrane. ERK then phosphorylates myosin light chain kinase (MLCK), which, upon activation, phosphorylates myosin light chain (MLC), inducing actin-myosin contraction [73]. Notably, the machinery involved in the formation of exosomes, known as the ESCRT (endosomal sorting complexes required for transport), also contributes to ectosome biogenesis. In the case of arrestin domain-containing protein 1 (ARRDC1)-mediated ectosome formation, TSG101 of ESCRT-I and apoptosis-linked gene 2 interacting protein (ALIX) associate with AR-RDC1 through its PSAP and PPXY motifs, respectively. ARRDC1 also interacts with neural precursor cell expressed, developmentally downregulated 4 (NEDD4) via the PPXY motif during ectosome biogenesis, suggesting a mechanism based on ubiquitin for cargo sorting [74]. Subsequently, ALIX interacts with the ESCRT-III complex to facilitate membrane scission, leading to the secretion of ectosomes into the extracellular matrix [75].

The biogenesis of EVs is highly regulated and involves multiple proteins and protein complexes. A detailed understanding of these mechanisms is still the subject of active research, as it plays a fundamental role in numerous physiological and pathological processes, including development, the immune system, and the transmission of cellular signals [67, 69].

#### 6.1.2 EVs uptaking strategies

Once generated and released from donor cells, EVs follow different fates. Some EVs release their cargo into the extracellular space due to the dissolution of their membrane. Another fraction of EVs undergo long-term navigation in extracellular fluids, and can establish various types of interactions with target cells. Three noteworthy types of interactions include endocytosis in various forms, juxtacrine signaling, and fusion with the plasma membrane of the target cell [76, 77, 78], as illustrated in Figure 5.

Fusion is a well-known natural phenomenon that occurs when two separated membrane portions come into close contact and merge. Factors such as temperature, environmental acidity, specific lipids like cholesterol, membrane curvature, and the presence of proteins or receptors can activate this process [79]. The fusion between the membranes of EVs and cells appears to be activated by specific Fusogenic Proteins (FPs). High-affinity binding of at least two pairs of surface FPs (one from the EVs and the other from the target cell's plasma membrane) is expected to be required for this process [77]. However, the identity of the FPs involved in the fusion process is still under investigation. These surface binding and fusion processes, similar to the fusion between two cells, are mainly understood from the study of viruscell fusion, which involves four classes of proteins [80]. Proteins from two of these classes, such as syncytin-1, syncytin-2, the receptor Major facilitator superfamily domain-containing protein 2 (MSFD2a), and the neutral amino acid transporter ASCT-2, have been discovered on the plasma membrane of various cell types, such as placental cells, human gametes, blood cells, and tumor cells. These proteins participate in the cell-to-cell fusion process [81, 82, 83, 82, 84, 85]. Notably, these proteins were also found on placental trophoblast exosomes (a specific type of EV) destined to bind and fuse with blood cells, suggesting their possible involvement in the binding process preceding EV fusion with target cells [82, 77].

It's important to note that this process should not be confused with intracellular fusion between vesicles and membrane-bound cell compartments and organelles, such as lysosomes. Intracellular fusion has been extensively studied and documented in the literature [86, 87, 76]. In contrast, evidence of surface binding and fusion of EVs with target cells has only been described recently [77, 88], and many details remain to be clarified.

After the bond between the FPs is established, their hydrophobic segments begin to merge into the plasma membrane, followed by the molecular rearrangement (pre-folding and post-folding) of the bound proteins and the reorganization of the closely attached membrane portions of both the EV and the target cell. This process continues until dissolution occurs at the fusion site, with the EV membrane being inserted into the plasma membrane, creating a continuous membrane.

Unlike other simultaneous internalization processes that occur across the cell membrane, such as receptor-mediated endocytosis, in which EVs are internalized with their membrane and then broken down to metabolize their contents, fusion between the EV and the membrane cell or endosome mem-
brane, releases the contents of the vesicle directly into the cytosol.

Another possible interaction between EVs and target cells is mainly based on protein interactions, involving receptors, tetraspanins, integrins, clathrin, caveolin, etc. [89]. These interactions mediate both juxtacrine signaling and EV internalization, which includes mechanisms classified as (i) endocytosis (clathrin dependent and independent), (ii) macropinocytosis, and (iii) phagocytosis [89, 90, 91, 92].

In more detail, all these strategies follow a similar sequence of events, distinguished by the types of proteins involved and the response of the target cell membrane, leading to EV internalization. The process begins with the high-affinity binding of one or two pairs of surface proteins (one from the EVs and the other from the target cell"s plasma membrane), triggering cascading reactions that alter the target cell membrane. This leads to complete envelopment and internalization of the EV in an endosome, where it may undergo various fates [76, 93].

Juxtacrine signaling is a different strategy in which the target cell processes information bound to the EV. The main difference between endocytosis and juxtacrine signaling is that EVs bind with high affinity to the cell"s surface, initiating a specific molecular process [94]. This strategy is not included in our model, as the experimental data we have do not show an accumulation of EVs on the surface of the target cell, but only their internalization over time. We will explore this possibility further in future experiments.

The identity of the proteins involved in all of these processes is only partially known (caveolins, clathrins, receptors), and several proteins and mechanisms are still under investigation. This complexity poses challenges in developing efficient pharmacological treatments for long-term inhibition or selective uptake pathways in *in vitro* experiments, as inhibiting these pathways may also affect the uptake of other molecules, resulting in side effects on cell viability. Therefore, current experimental measures of the EV internalization rate in target cells do not distinguish among the possible uptake mechanisms.

### 6.2 Cell cultures used

#### 6.2.1 Cells cultures

For this thesis project, we utilized the SH-SY5Y neuroblastoma cell line as the target for EV treatment, with primary Astrocytes (AS) cultures serving



Figure 5: EVs Uptake Strategies: This figure illustrates the most well-known strategies for EVs internalization by target cells, alongside new EVs synthesis.

as the EV donors, as shown in Figure 6. Both cell types were maintained in culture within an incubator at a constant temperature of  $37^{\circ}$ C, in a humidified atmosphere with 5%  $CO^2$ . The culture medium was replaced every 2-3 days.

#### 6.2.2 SH-SY5Y cell line

The SH-SY5Y cell line is widely used cellular model for research in Parkinson"s disease due to its characteristic features of dopaminergic neurons. Furthermore, differentiation of this cell line can be induced by using retinoic acid (RA) and gradually depleting fetal bovine serum (FBS), leading to a dopaminergic neuronal phenotype. After differentiation, SH-SY5Y cells cease to proliferate, and the cell number remains constant [95]. Undifferentiated SH-SY5Y cells were cultured using a mixed culture medium of MEM/F12 (Minimum Essential Media + F-12) in a 1:1 ratio, supplemented with 1% glutamine and 10% FBS. To prevent bacterial contamination, 1% Penicillin and Streptomycin antibiotics (PEN/STREP) were added. Once the cell confluence reached 80%, the cells were detached and diluted to maintain them in culture. Cell splitting was performed by aspirating and preserving the supernatant (SNT), followed by a wash with 1X PBS (phosphate-buffered saline) to remove cellular debris and any remaining medium residues, following the protocol reported in [54].

#### 6.2.3 Primary cultures of murine midbrain AS

Primary cultures of AS and the corresponding treatments were conducted using wild-type C57BL/6 mice obtained from Charles River (animal experimentation approved by the Italian Ministry of Health, authorization number 442/2020-PR). The primary astroglial cell cultures were prepared according to the method described in [54]. Briefly, AS were derived from mice at postnatal days P2–P4 and isolated from the ventral midbrain (VMB) and striatum (STR) brain regions, as well as from brains lacking these two regions ( $\Delta VS$ ). These brain regions are involved in Parkinson's pathology, in particular. The substantia nigra is a gray laminar region, so called due to the presence of neurons rich in the pigment neuromelanin, which gives it its typical dark color. It is composed of a region dedicated to the inhibition of movement (pars reticulata) and a region that facilitates movement (pars compacta) [96]. The loss of pigmentation is mainly due to the degeneration of the cell bodies of the dopaminergic neurons in the SNpc in the ventral region of the midbrain, of their axons that afferent to the region of the striatum and of the noradrenergic neurons in the locus coeruleus [97], responsible for the origin of muscle movement.

The AS were cultured in DMEM (Sigma Aldrich, D6046) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810), 2 mm L-glutamine (Sigma Aldrich, G7513), 2.5  $\mu$ g/mL amphotericin B (Sigma Aldrich, A2942), and 1% penicillin/streptomycin (Sigma Aldrich, P0781) at 37°C with 5% CO2 for a period of 13-17 days in 10 cm dishes specifically designed for primary cultures (Corning, 353 803), following the protocol reported in [54].

Subsequently, cells were treated with CCL3 at a concentration of 300 ng/mL (R&D, 450MA050) or left untreated, in DMEM medium supplemented with 10% exosome-depleted FBS (System Biosciences, EXO-FBS -250A-1), following the protocol reported in [54]. Cells were maintained in this medium for 24 hours before collecting the supernatant for extracellular vesicle (EV) purification, described in detail in [54].

The choice of the CCL3 protein is not random, because several studies have highlighted a significant increase in the chemokine CCL3 (chemokine (C-C motif) ligand 3), in Parkinson's disease, as an index of acute inflammation. However, a high level was also observed in recovery processes both



Figure 6: Microscope cells images. a) Undifferentiated human SH-SY5Y cell line observed using optical microscopy; b) Differentiated human SH-SY5Y cell line exposed to  $10\mu$ mol of retinoic acid as seen through optical microscopy; c)AS captured under fluorescence microscopy with cell bodies shown in green, cell nuclei in blue, and microglia cells in red.

in *in vitro* and in *in vivo* models of Parkinson's disease. For example, AS treated *in vitro* with CCL3 increase their intrinsic protective action against dopaminergic neurons subjected to oxidative damage, and stimulate the differentiation of Neuronal Stem Cells [98].

#### 6.3 Methodology

For immunofluorescence (IF) analyses, AS were labelled with rabbit anti-GFAP antibody (Dako, Z0334), while microglial cells were stained with goat anti-Iba1 antibody (Novus, NB100-1028). AS proliferation was evaluated by 5-Bromo-2"- deoxyuridine (BrdU) incorporation assay. The day before fixa-

tion, BrdU 5  $\mu$ m (Sigma Aldrich, 19–160) was added to cells for 24 h. Proliferative cells were stained with mouse anti-BrdU antibody (Sigma Aldrich, B8434). Donkey Alexa fluor secondary antibodies were used, and nuclei were stained with DAPI (Sigma Aldrich, 32670–5MG-F). IF images were acquired using a Leica microscope (DM5500) and analyzed with Fiji Image J software 1.51n. For cytotoxicity analysis, 10  $\mu$ L of AS supernatants were collected and analyzed by LDH-Cytotoxicity Assay Kit (Fluorometric) (Abcam, ab197004), following the instruction provided by the kit. For viability analysis, CellTiter Blue reagent (Promega, G8080) (diluted 1:4 with PBS  $1\times$ ) was added to each well of 96 well plates and incubated at 37 °C for 4 h. Then, for both kits, the fluorescent signal was measured by Varioskan flash plate reader (Thermo Fisher). RNA was isolated from AS using the miRNeasy Mini Kit (Qiagen, 217 004). Total RNA quantity and purity were assessed with the NanoDrop ND-1000 instrument (Thermo Scientific) and cDNA synthesis was performed using the High-capacity cDNA reverse transcription kit (Applied Biosystem, 4 368 814). Gene expression was studied via qPCR with PowerUp SYBR Green Master Mix (Applied Biosystem, A25742), using the following primers:

Ccr1-forward: 5"-AGGTTGGGACCTTGAACCTTG-3", Ccr1-reverse: 5"-ACAGTGAGTCTGTGTGTTTCCAGA; and Ccr5-forward: 5"-TGAGACATCCGTTCCCCCTA -3", Ccr5-reverse: 5"-GCTGAGCCGCAATTTGTTTC-3". mRNA levels were normalized relative to Gusb: Gusb-forward: 5"-CCGACCTCTCGAACAACCG-3", Gusb-reverse: 5"- GCTTCCCGTTCATACCACACC-3".

Samples were tested in triplicate on a QuantStudio 3 Real-Time PCR System (Applied Biosystem) and expressed as  $\Delta Ct$ .

#### 6.3.1 Scanning Electron Microscopy (SEM) Processing

Cells were fixed in 3% Glutaraldehyde (Sigma Aldrich, G5882) for 1 h. Samples were then postfixed in 1% osmium tetroxide for 45 min at 4 °C. Samples were washed with deionized water and partially dehydrated in increasing concentrations of ethanol up to 100% ethanol. Subsequently, critical point drying and sputtering with gold/palladium alloy was performed at the Central Service for Experimental Research of the University of Valencia. SEM images were obtained on a Hitachi S4800 microscope.

#### 6.3.2 Histological Processing

Cells were fixed in 3% glutaral dehyde for 1 h, then they were post fixed with 2% osmium tetroxide (Electron Microscopy Sciences) for 2 h. Sections were then washed in deionized water, and partially dehydrated in 70% ethanol. Afterward, the samples were contrasted in 2% uranyl acetate (Electron Microscopy Sciences) in 70% ethanol for 2 h at 4 °C. The samples were further dehydrated and infiltrated in Durcupan ACM epoxy resin (Sigma) at room temperature overnight, and then at 60 °C for 72 h. 1.5  $\mu$ m sections were obtained using an Ultracut UC7 ultramicrotome (Leica Biosystems). Sections were stained with 1% Toluidine Blue. Images were taken with an i80 Nikon Microscope.

#### 6.3.3 EVs Isolation and Characterization

AS supernatants were collected and immediately centrifuged at  $1000 \times g$ at 4 °C for 15 min in order to pull down residual cells/cell debris. Next, the supernatants were subjected to ultracentrifugation in a Sorvall WX100 (Thermo Scientific). The first ultracentrifugation was performed at 100 000 g at 4 °C for 75 min, in ultra-cone polyclear centrifuge tubes, each containing the supernatant deriving from  $\approx 15 \times 10^6$  astrocytes (Seton, 7067), using the swing-out rotor SureSpin 630 (k-factor: 216, RPM: 23 200). Then the pellet was washed with cold PBS  $1 \times$  and ultracentrifuged again at the same speed for 40 min in thick wall polycarbonate tubes (Seton, 2002), using the fixed-angle rotor T-8100 (k-factor: 106, RPM: 41 000). The resulting pellets, containing AS-EVs, were resuspended in PBS  $1\times$  (for NTA, EM and functional experiments), in RIPA buffer (for WB characterization), or in Diluent C (for PKH26 staining). Nanoparticle Tracking Analysis (NTA): AS-EVs were diluted in PBS  $1 \times$  and analyzed for particle size distribution and concentration on a Nanosight NS500 (Malvern Instruments Ltd, UK) fitted with an Electron Multiplication-Couple Device camera and a 532 nm laser. The sample concentration was adjusted to 108–109 particles/mL and measurements were performed in static mode (no flow) at an average temperature of  $21 \pm 1$  °C. A total of 3 to 5 videos of 60 s were recorded for each independent replicate, loading a fresh sample for each measurement. Videos were processed on NTA software v3.2 and a detection threshold of 8 was used. The remaining settings were set to automatic. Total particle concentration for each EV sample was determined by NTA and used to calculate the number

of EVs released per  $10^6$  cells.

#### 6.3.4 EV Negative Staining for Transmission Electron Microscopy (TEM)

ASEVs were fixed with 2% paraformaldehyde (PFA) (Sigma Aldrich, P6148) in PBS 1× for 30 min. 200 mesh formvar and carbon coated nickel grids were glow-discharged to make the surface grid hydrophilic. Fixed samples were placed on the grids for 7 min, samples were washed with ultrapure water and stained with 2% uranyl acetate for 7 min and examined at 80 kV on a FEI Tecnai G2 Spirit (FEI Company, Hillsboro, OR) transmission electron microscope equipped with a Morada CCD digital camera (Olympus, Tokyo, Japan). To obtain the number of vesicles in EM, 10 random fields (from 60 000 × magnification) were counted, each from a different square of the 200-mesh grid, per each condition [99]. The results were normalized taking into account the following parameters: the number of starting cells, the resuspension volume after ultracentrifugation, the volume used in the microscope grid, and the area ( $\mu m^2$ ) of each field in the grid.

#### 6.3.5 EV Immunogold Labelling for Transmission Electron Microscopy (TEM)

To increase the hydrophobic properties of the grids 200 mesh formvar and carbon coated nickel grids were glow-discharged. Grids were placed on a 10  $\mu L$  drop of each sample for 7 min and washed with PBS 1×. Nonspecific reactions were avoided using blocking solution containing 0.3% BSA for 30 min. Then, samples were washed in 0.1% BSAc (Aurion, Wageningen, the Netherlands) in PBS 1×. The samples were incubated in 10  $\mu$ L of 1:50 primary antibody (rat anti-CD9 or rat anti-CD63, see Table 1) in 0.1% BSAc (Electron Microscopy Sciences) for 1 h. After, the samples were washed in 0.1% BSAc and incubated in 1:20 goat anti-rat 6 nm gold particles (Abcam, ab105300) in 0.1% BSAc for 1 h in the dark. Grids were rinsed with 0.1% BSAc and fixed with 2% glutaraldehyde for 5 min and washed with ultrapure water. Finally, negative staining with 2% uranyl acetate was performed for 5 min. The samples were examined at 80 kV on a FEI Tecnai G2 Spirit (FEI Company, Hillsboro, OR) transmission electron microscope equipped with a Morada CCD digital camera (Olympus, Tokyo, Japan). Western Blotting: AS and EVs extracts were processed as in [100, 101]. Briefly, AS and EVs

Antibody	Diluition	Brand	Catalog number
Rat monoclonal anti-CD63	1:5000	MBL	D263-3
Rat monoclonal anti-CD9	1:5000	Pharmigen	553758
Mouse monoclonal anti-Pdcd6ip	1:5000	BD transduction lab	611620
Mouse monoclonal anti-SDHA	1:1000	Abcam	ab14715
Rabbit polyclonal anti-Canx	1:10 000	Abcam	ab22595
Mouse monoclonal anti-GM130	1:1000	BD transduction lab	610823
Mouse monoclonal anti- $\beta$ -actin	1:10 000	Sigma Aldrich	A1978
HRP-conjugated anti-mouse secondary antibody	1:10 000	Dako	P0447
HRP-conjugated anti-rabbit secondary antibody	1:10 000	Invitrogen	31460
HRP-conjugated anti-rat secondary antibody	1:10 000	Invitrogen	31470

Table 1: List of antibodies used in WB.

were lysed in RIPA buffer (10 mm Tris HCl pH 7.2 (Fisher Scientific, BP152); 1% sodium deoxycholate (Sigma Aldrich, 30 970); 1% Triton X-100 (Sigma Aldrich, T8787); 0,1% (for cells) or 3% (for EVs) SDS (Sigma Aldrich, 71 736); 150 mm NaCl (Sigma Aldrich, S7653); 1 mm EDTA pH 8 (VWR chemicals, E177-100ML); 1 mm phenylmethanesulfonyl fluoride solution (PMSF, Sigma Aldrich, 93 482); 1× Complete Protease inhibitor cocktail (Roche, 0 469 311 6001), 1× Halt Phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78 420), and protein concentration was measured with DC Protein Assay (Biorad, 500- 0116), using BSA (Pierce, 23 210) as standard (AS-EV protein yield: 0.5– 1.5  $\mu$ g/106 cells). The same amount of cell or EV lysates was then loaded into 4–12% Bis-Tris plus gels (Invitrogen, NW04125BOX) in reducing or non-reducing conditions. Afterward, proteins were transferred onto PVDF membrane. All primary and secondary antibodies are listed in Table 1.

#### 6.3.6 SH-SY5Y Culture, Differentiation, and Treatments

SH-SY5Y cells were purchased from ICLC (Interlab Cell Line Collection, accession number ICLC HTL95013; obtained from depositor European Collection of Authenticated Cell Cultures [ECACC]) and cultured and differentiated as described in [102]. Briefly, cells were maintained in MEM/F12 medium (Biochrom GmbH, F0325 and Sigma Aldrich, N4888). For cell differentiation, MEM/F12 was replaced with DMEM/F12 and 10  $\mu$ m retinoic acid (Sigma Aldrich, R2625), and cultivated for 8 days with gradual serum deprivation until 0.5% FBS. At the end of differentiation, cells were detached and seeded at the density of 3 × 10<sup>5</sup> cells/cm2 in 12-well (for IFC analysis, see Section 5.10 for EV labeling), 96-well (for dose response curve), 24-well (for c-Casp-3 IF staining), or 6-well (for HRR analysis, see Section 5.11 in [54]) plates. For all the experiments where EVs were applied on target cells, the authors used the ratio 5:1 (i.e., EVs derived from five AS used to treat one SH-SY5Y cell). For IFC analysis, labelled AS-EVs were applied on differentiated and undifferentiated SH-SY5Y cells (see below) seeded on 12 well plates. Internalization was evaluated at different time points (i.e., 2, 6, and 24 h) at  $20 \times$  magnification by using the Amnis FlowSight Imaging Flow Cytometer (Luminex). At the end of each time point, cells were trypsinized and collected in 1 mm EDTA + 1% BSA. For all passages cells were kept on ice. Fluorescence intensity of PKH26 was measured by using 488 nm laser. Flow cytometric gating was used to select focused single cells and the mean fluorescence intensity of treated cells was compared with that of untreated cells. For normalization, the authors analyzed the first 1000 single cells, in order of acquisition, with an optimal focus, using IDEAS software version 6.2 183.0 (Amnis, part of Luminex). Two dose-response curves, one for  $H_2O_2$ (Sigma Aldrich, H1009) and one for MPP+ (Sigma Aldrich, D048), were performed at 24 h, using CellTiter Blue (Promega, G8080), as described in the Primary Astrocyte Cultures and Treatments section. For IF, cells were seeded on poly-L-lysine coated glass coverslips. After two days, EVs were applied on target cells. As a control, the vesicles eventually present as contaminants in the medium used to culture AS (cont-EVs) were also tested following the same experimental steps used for AS-EVs. Following ultracentrifugation, cont-EVs were resuspended in PBS  $1 \times$  and used to treat SH-SY5Y maintaining the same ratio with the starting volume of medium, as for the purification of AS-EVs. 6 h later, cells were treated with 35  $\mu$ m  $H_2O_2$  for a further 24 h. Coverslips were fixed with 4% PFA and stained with rabbit polyclonal anti-c-Caspase-3 (Cell Signaling, 9664) primary antibody and with mouse monoclonal anti-map2 primary antibody (Merck Millipore, MAB3418). The secondary antibodies used were the anti-Rabbit Alexa Fluor 546 (Thermo Fisher Scientific, A10040), and the anti-Mouse Alexa fluor 488 secondary antibodies (Thermo Fisher Scientific, R37114). Nuclei were counterstained with DAPI. IF images were acquired using a Leica microscope (DM5500) and analyzed with Fiji Image J software. The intensity of the c-Casp-3 signal was measured by using the following steps in ImageJ software: i) analyze; ii) measure; and iii) integrated density, as in [103]. Integrated density was normalized for the number of DAPI+ nuclei. As a further control, the chemokine CCL3 (at 30 and 300 ng mL-1) was added directly to SH-SY5Y cell cultures on 96-well plate 6 h before  $H_2O_2$  exposure. Cell viability/death was evaluated 24 h after the  $H_2O_2$  treatment with CellTiter Blue and Caspase-Glo 3/7 Assay (Promega, G8091). Undifferentiated SH-SY5Y cells were seeded at a density of  $1 \times 10^5$  cells/cm2 in 96, 12 and 6-well plates. For apoptosis analysis, cells were seeded in 96-well plates. Two days after, cells were treated with AS-EVs, then after 6 h with 35  $\mu$ m  $H_2O_2$  and finally analyzed with the Caspase-Glo 3/7 Assay after a further 24 h. For IFC and HRR analysis (see below), cells were seeded in 12 and 6-well plates, respectively, and processed like differentiated SH-SY5Y cells.

#### 6.3.7 EV Labelling

EV internalization was analyzed with two different approaches of labelling. First, AS were treated with the lipophilic dve PKH26 (Sigma Aldrich, MINI26-1KT), following the protocol suggested by the manufacturer. After 3 days cells were washed, and medium changed with DMEM supplemented with 10%FBS depleted of exosomes. EVs were isolated from AS supernatants after 24 h by ultracentrifugation. The resulting EVs were applied on differentiated SH-SY5Y cells seeded onto polyL-lysine (Sigma Aldrich, P9155) coated glass coverslips in 24 well plates. Target cells were stained with  $\alpha$ -TH primary antibody (Millipore, AB152) as in [102]. Imaging was performed using the confocal laser scanning microscope Leica TCS SP8. Image acquisitions were performed through LAS X software (Leica Microsystems). Image analyses were done using the open-source Java image processing program Fiji is Just ImageJ (Fiji). 3D reconstruction was done with the Fiji 3D Viewer dedicated plugin. For the second approach, EVs were directly labelled with the same lipophilic dve, following the protocol suggested by the manufacturer. with some modification. Briefly, EVs derived from 90 mL of AS supernatant were ultracentrifuged, and the resulting pellets were resuspended in 0.3 mL of Diluent C plus 4  $\mu$ L of dye, and incubated at room temperature for 5 min, mixing every 30 s. The labeling was quenched by adding 1% BSA in PBS  $1\times$ and again ultracentrifuged. The resulting pellet, containing the labelled EVs, were resuspended in 100  $\mu$ L PBS 1×. Residual PKH26 was eliminated into the Exosome Spin Column (Thermo Fisher Scientific, 4 484 449) according to the manufacturer's recommendations. Again, eluted EVs were applied on differentiated SH-SY5Y cells seeded onto glass coverslips in 24 well plates. At the end of the treatment cells were fixed with 4% PFA. As a control, PBS  $1\times$  with the same concentration of PKH26 dye was centrifuged under the same conditions and added to target cells. IF images were acquired using a

Leica microscope (DM5500) and analyzed with Fiji Image J software 1.51n. For IFC analysis see the SH-SY5Y Culture, Differentiation, and Treatments section.

#### 6.3.8 High-Resolution Respirometry (HRR)

The capacity of different respiratory states in differentiated or undifferentiated SH-SY5Y cells was assayed by High-Resolution Respirometry (HRR) using the  $O_2$ k-FluoRespirometer (Oroboros Instruments). Cells were seeded in 6-well plates and, after two days, AS-EVs were applied on the top of SH-SY5Y cells, as before. As control, 30% of ACM or supernatant (ACM after ultracentrifugation, SNT) were applied on target cells. 6 h later, cells were treated with MPP+ 1 mm and analyzed after further 24 h. All the experiments were performed in mitochondrial respiration buffer Mir05 (Oroboros Instrument, 60101-01) at 37 °C under constant stirring (750 RPM). A specific Substrate-UncouplerInhibitor Titration (SUIT) protocol was used for the determination of the  $O_2$  consumption in each specific respiratory state, as detailed in [102]. Briefly, respiration in the presence of endogenous substrates or ROUTINE was measured in intact cells. The mild-detergent digitonin (Sigma Aldrich, D5628) was added at the final concentration of 4  $\mu$ m in order to obtain the permeabilization of plasma membrane without compromising the mitochondrial membranes' integrity. The  $O_2$  consumption after permeabilization or LEAK was determined in the presence of 5 mm pyruvate (Sigma Aldrich, P2256) and 2 mm malate (Sigma Aldrich, M1000), but not adenylates. The contribution of complex I to the OXPHOS respiration was achieved by the addition of 10 mm glutamate (Sigma Aldrich, G1626) in the presence of a saturating concentration of ADP (2.5 mm, Sigma Aldrich, 117 105). The OXPHOS respiration was then stimulated with the addition of 10 mm succinate (Sigma Aldrich, S2378). The uncoupled maximal capacity of the electron transport system (ETS) was obtained after titration with 0.5  $\mu$ m of uncoupler carbonyl cyanide 3 chlorophenylhydrazone (CCCP, Sigma Aldrich, C2759) up to the complete dissipation of the proton gradient. Finally, the residual  $O_2$  consumption or ROX was obtained upon addition of 2  $\mu$ m rotenone (Sigma Aldrich, R8875) and 2.5  $\mu$ m antimycin A (Sigma Aldrich, A8674). The  $O_2$  consumption in ROUTINE, LEAK, OXPHOS, and ETS capacity was corrected for the ROX. Values were then expressed as Flux Control Ratio (FCR) of the maximal respiration, using ETS capacity as a reference state. [161] The  $O_2$  flux related to ATP synthesis was determined by correcting ROUTINE and OXPHOS for the LEAK respiration. Coupling efficiencies were calculated by correcting each state for LEAK respiration and expressing it as a percentage of the capacity in that specific state [104]. Instrumental and chemical background fluxes were calibrated as a function of the  $O_2$  concentration using DatLab software (version 7.4.0.1, Oroboros Instruments).

#### 6.3.9 Statistical Analysis

Pre-processing of data are described in each figure legend. The statistical analyses were performed with GraphPad Prism software (version 9.2.0). For all the analyses, differences among groups were analyzed by one-way ANOVA followed by a Tukey's multiple comparisons test. The values are expressed as mean  $(\pm \text{SD})$  and a p < 0.05 was accepted as significant. For IF on AS, data were obtained from n = 4 (for VMB- and STR-AS) or n = 3 (for  $\Delta VS$ -AS) independent biological replicates (from 4 to 10 images for each replicate). For cell viability/cytotoxicity on AS, data were obtained from n = 3 independent replicates. For NTA, data were obtained from n = 3 independent biological replicates (a total of 3 to 5 videos of 60 s recorded for each biological replicate). For EM, data were obtained from n = 5 (for VMB-and STR-AS) or n = 3 (for  $\Delta VS-AS$ ) independent biological replicates (10 fields for each replicate). For qPCR, data were obtained from n = 3 independent biological replicates. For IFC analysis on SH-SY5Y cells data were obtained from n = 3 independent biological replicates. For the dose-response curve of H2O2 and MPP+, data were obtained from n = 3 independent biological replicates were analyzed by nonlinear regression, dose-response-inhibition ([Inhibitor] versus response—variable slope [four parameters]). For IF on SH-SY5Y, data were obtained from n = 3 independent biological replicates (from 4 to 8 images for each biological replicate). For cell viability and apoptosis on differentiated and undifferentiated SH-SY5Y cells, data were obtained from at least n = 2 independent biological replicates. For HRR measurement on differentiated SH-SY5Y treated with AS-EVs, the following independent biological replicates have been performed: n = 4 for CTRL and MPP+, n = 3for +/-VMB-AS-EVs, n = 2 for +/-STR-AS-EVs. For HRR measurement on differentiated SH-SY5Y treated with ACM/SNT, the following independent biological replicates have been performed: n = 3 for CTRL and n = 2for MPP+, VMB-ACM/SNT, STR-ACM/SNT. For HRR measurement on undifferentiated SH-SY5Y treated with AS-EVs, the following independent

biological replicates have been performed: n = 3 for CTRL and n = 2 for MPP+, VMB-AS-EVs, and STR-AS-EVs. All relevant data of the experiments are available at the EV-TRACK knowledgebase (EV-TRACK ID: EV220106).

# 7 Work propose

The aim of this research is to study and characterize cellular molecular communication based on the exchange of EVs between two different cells, using an approach rooted in mathematical modeling, typical of engineering applied to biology. The aim of this research is to study and characterize cellular molecular communication based on the exchange of EVs between two different cells, using an approach rooted in mathematical modeling, typical of engineering applied to biology.

The work presented here is based on my master's thesis research conducted under the guidance of Prof. N. Iraci and Dr. L. Leggio, as cited in [105] and [54]. In these studies, EVs derived from murine astrocytes were isolated and characterized, with detailed descriptions available in [54].

These investigations have revealed the nature of EVs and their neuroprotective effects in the context of Parkinson's disease, along with their potential use as biomarkers for this pathology.

Once released from a donor cell, these EVs diffuse into the extracellular space and are taken up by some target cells through various potential uptake mechanisms [89]. These attributes make natural or synthetically engineered EVs suitable for use as carriers in the treatment of specific diseases by delivering drugs to affected cells [105, 106]. To this end, formalizing the exchange of EVs between donor cells and target cells, in accordance with information and communication theory paradigms, improves our understanding of the dynamics and characteristics of such cellular communication. This, in turn, provides precise support for the development of innovative and effective therapeutic protocols.

Based on these results and hypotheses, Part I of this thesis attempts to model the mechanisms by which target cells (neurons) absorb EVs. Initially, we will explore receptor-mediated entry through endocytosis and fusion, as detailed in Section 8. The main hypothesis is to formally describe the main internalization mechanisms of electric vehicles and experimentally verify that our models are efficient for future applications. Our goal is therefore to calculate the parameters involved in these processes to develop models that can be applied to real data. Through the use of cell culture and EV treatments, we have created several models and strategies to describe EV endocytosis and fusion, quantified uptake, and worked on possible therapeutic applications.

Part II focuses on a strategy that leverages microfluidics for innovative therapies involving artificial EVs, known as liposomes, as discussed in Section 9. In more detail, in Part I, we focused on the natural interactions between EVs and target cell with particular emphasis on the possible content. In Part II the focus becomes the lipid envelope of the EVs themselves, which can be phosphorescently labeled and follow its path within a microfluidic system that can mimic blood capillaries and could even carry pharmacologically useful molecules. The information is then transmitted by the very movement of the liposomes in the microfluidic chip and by the variation in their speed. We developed a system capable of creating colored drops, as if they were liposomes, and calculated the probability of error in attempting to transmit information by varying the speed of the drops. Let's therefore try to exploit a new microfluidic system to transmit information on an individual's health status, using drops inside a capillary.

# 8 Part I - Study and characterization of a cellular communication channel through an exchange of EVs

In this section, we delve into the communication between cells through the exchange of EVs. We initiated our exploration with a study which goes deeper into the nature of EVs isolated from murine Astrocytes (AS), investigating their neuroprotective effects when applied to human neuronal cells where Parkinson's disease has been induced. The results are reported in Section 8.1.

This study served as the basis for our approach to characterizing cellular communication from an engineering perspective, understood in terms of information theory. We considered individual vesicles as information packets containing bits or bytes. Consequently, our objective was to quantify the information loss when an EV is internalized through spontaneous fusion in confront of a free molecule internalized through receptor-mediated endocytosis, as detailed in Section 8.2.

Subsequently, we aimed to construct a more realistic internalization model, distinctively focusing on the two primary pathways for EV internalization: fusion and protein-dependent endocytosis by target cells. We started with real data and estimated the required parameters through an inverse engineering process, as described in Sections 8.3.1, 8.3.5, 8.3.13. We also formulated a therapeutic protocol for potential EV treatments.

Another approach to modeling EV exchange involves virtual simulations and parameters derived from existing literature, outlined in Sections 8.4.1, 8.5.1, 8.4.4.

Our goal is to support the scientific community in optimizing experimental protocols and to provide insights into how cells might respond to specific EV treatments.

# 8.1 I<sup>st</sup> Key Study: Extracellular Vesicles Communication in neuronal context

EVs have been demonstrated to play several roles in physiopathological conditions [67]. In fact, recently envisioned that natural or synthetic-engineered EVs can be engaged for the treatment of diseases to deliver drugs to target ill cells. As an example, in particular brain diseases where the success rate of existing drug delivery technologies in deep located cells is still low, EV-based therapeutic protocols may result to be a more efficient solution [107, 12].

#### 8.1.1 Small Extracellular Vesicles secreted by Nigrostriatal AS preserve vitality and mitochondrial function in Parkinson's Disease

In the context of neurodegenerative diseases, including Parkinson's disease (PD), EVs were initially identified as vehicles of misfolded proteins [108, 109], but in line with the dual role played by glial cells, EVs have been demonstrated to play also important neuroprotective functions [110, 111].

Parkinson's disease is the second most common neurodegenerative disease for which there is currently no cure to halt or reverse its progression [112, 113]. It is characterized by the selective and uncontrolled death of dopaminergic (DAergic) cell bodies located in the substantia nigra pars compacta (SNpc) in the ventral midbrain (VMB) [114, 115]. As a result, the DAergic terminals in the striatum (STR) gradually deteriorate, leading to the typical motor symptoms of PD, such as slow movements (bradykinesia), resting tremor, muscle rigidity, and postural instability. The disease's major pathological hallmarks include the chronic age-dependent degeneration of the nigrostriatal pathway [114, 116], the accumulation of abnormal intracellular aggregates of  $\alpha$ -synuclein ( $\alpha$ -syn) called Lewy bodies (LBs) and Lewy neurites (LNs), and extensive astrogliosis [116, 117]. The causes and mechanisms of DAergic neuron death in PD are still not fully understood, but current evidence suggests a complex interplay between multiple genes and environmental factors, particularly aging, inflammation, and oxidative stress, all of which strongly impact the astroglial cell population [112, 118, 111]. Mitochondrial dysfunction has been identified as a crucial final pathway in PD neurodegeneration, closely linked to the specific vulnerability of nigrostriatal neurons and the unique characteristics of the astroglial microenvironment [119, 120, 121]. AS are active contributors to both beneficial and detrimental functions during neuronal degeneration by producing various proinflammatory/anti-inflammatory molecules and neurotoxic/neuroprotective mediators [118, 111]. The delicate balance between these signaling molecules, as well as their bidirectional communication with microglial cells, ultimately determines whether the outcome will be a reparative process or neuronal failure.

Increasing evidence suggests that AS exhibit regional heterogeneity, both in terms of molecular composition and functional characteristics, which has important implications for neuronal function and vulnerability [122, 123]. Specifically, the VMB and STR regions within the central nervous system (CNS) show unique features and are highly susceptible to oxidative stress, environmental toxins, inflammation, and aging [124, 125]. Chemokines have emerged as crucial mediators of communication between glial cells and neurons during neuroinflammation and neurodegeneration [126, 127].

Within the VMB, AS play a significant role in protecting vulnerable substantia nigra pars compacta (SNpc) dopaminergic (DAergic) neurons. Reactive AS in the VMB have been identified as key contributors to neuroinflammation associated DAergic neuroprotection and repair in animal models of basal ganglia injury. In particular, reactive VMB-AS were identified as main actors linking neuroinflammation to DAergic neuroprotection and repair in the 1-methyl, 4-phenyl, 1,2,3,6 tetrahydropyridine (MPTP) mouse model of basal ganglia injury [128].

In this context, the upregulation of certain chemokines, such as CC chemokine ligand 3 (CCL3), has been implicated in promoting DAergic neurogenesis, survival, and immunomodulation [129, 130]. *In vitro* studies have demonstrated that the interaction between CCL3-activated AS and neurons is critical for neuroprotection and neurogenesis of adult neural stem cells [111, 128].

#### 8.1.2 AS from the Nigrostriatal System Secrete Small EVs in a Region-Specific Manner

To investigate potential differences in astrocyte-derived extracellular vesicles (AS-EVs) between the two main brain regions affected in PD, primary astrocyte cultures were established from the VMB and STR. Additionally, AS were grown from brains lacking these two regions, referred to as  $\Delta$ VS-AS, serving as control cells external to the nigrostriatal system. The AS were characterized under basal conditions and after 24 hours of treatment with CCL3 to examine whether the treatment conferred additional protective effects on AS-EVs [54]. All primary cultures yielded highly pure AS without any differences in proliferation rates following CCL3 treatment. Moreover, to assess the health of AS during EV isolation, cell viability and death levels were evaluated, revealing no significant differences between experimental groups. This indicates that these factors do not influence the production rate of AS-EVs from different brain regions.

Subsequently, EVs were isolated from the supernatants of astrocyte cul-

tures using differential centrifugation [54] and analyzed through a combination of techniques to evaluate their size, secretion rates, and specific markers. Nanoparticle tracking analysis (NTA) was initially performed on all EV samples, revealing an enriched population of vesicles with a peak size of approximately 100 nm, corresponding to small extracellular vesicles (sEVs) (Figure 7A). Interestingly, the origin of the brain region affected the EV secretion rate of AS. VMB-AS released 2 to 4 times more vesicles per million cells compared to STR and  $\Delta$ VS-AS, with an increased trend observed following CCL3 treatment (Figure 7B).

To further investigate this finding, transmission electron microscopy (TEM) analysis was performed on the same EV samples. The images displayed the presence of sEVs with cup-shaped structures, which is a typical result of the ultracentrifugation process (Figure 7C), with an average diameter ranging from 60 to 70 nm (Figure 7D). Once again, it was observed that VMB-AS released more vesicles compared to AS from STR and  $\Delta$ VS-AS (Figure 7D), supporting the results obtained from NTA. Interestingly, the treatment with CCL3 stimulated VMB-AS to secrete more EVs (Figure 7 1E), while the other two brain regions did not show a significant change in secretion rate following CCL3 treatment (Figure 7E).

Overall, these findings demonstrate that the secretion characteristics of AS-EVs are determined by the brain region of their origin.



Figure 7: Brain region influences the rate of secretion of AS-sEVs and responsiveness to CCL3 treatment. A) NTA analysis for size distribution displays a peak  $\simeq 100$  nm. Error bars represent SD from n = 3 independent replicates. B) EV concentration, determined by NTA, was normalized over the number of cells. The mean of particles/10<sup>6</sup> cells shows that AS from VMB region secrete more EVs than STR and  $\Delta$ VS regions. Data are presented as floating bars with line at mean from n = 3 independent replicates, indicated with different symbols. One-way ANOVA with Tukey's multiple comparison \*p < 0.05 (VMB-AS-EVs versus STR-AS-EVs; VMB-AS-EVs versus  $\Delta$ VS-AS-EVs). C) TEM ultrastructural analysis reveals the presence of sEVs secreted by AS in every condition. Scale bars: 100 nm. D) In all AS-EV samples the average diameter is  $\simeq 60/70$  nm. Raw data (diameter values) are presented as scatter dot plots with line at median  $\pm$  SD from n = 5 (for VMBand STR-AS-EVs) and n = 3 (for  $\Delta$ VS-AS-EVs) independent experiments. E)

Quantitative analysis from TEM showed that AS from VMB secrete more EVs than STR and  $\Delta$ VS regions; the treatment with CCL3 stimulates VMB-AS to release more EVs. Data are normalized considering the number of starting cells, the resuspension volume after ultracentrifugation, the volume used in the

microscope grid, and the area  $(\mu m^2)$  of each field in the grid. Data are presented as floating bars with line at mean plus individual data points based on 50 images over 5 independent replicates (for VMB- and STR-AS-EVs) and on 30 images over 3 independent replicates (for  $\Delta$ VS-AS-EVs), indicated with different symbols. One-way ANOVA with Tukey's multiple comparison: in (B) \*p < 0.05 (VMB-AS-EVs versus STR-AS-EVs and versus  $\Delta$ VS-AS-EVs; in (E) \*p < 0.05 (VMB-AS-EVs versus VMB-CCL3-AS-EVs), \*\*\*\*p < 0.001 (DVB-AS-EVs versus VMB-CCL3-AS-EVs), \*\*\*\*p < 0.001

(VMB-AS-EVs versus STR-AS-EVs and  $\Delta$ VS-AS-EVs), ns: not significant.

#### 8.1.3 Both VMB- and STR-AS-Derived Vesicles Are Enriched in sEV Markers

Subsequently, we examined the protein profiles of AS-EVs derived from the nigrostriatal system. Immunogold-labelling transmission electron microscopy (IG-TEM) was employed to assess the presence of tetraspanins CD63 and CD9, which serve as markers for sEVs. The images in Figure 8A and 8B clearly displayed well-defined 6 nm gold nanoparticles localized on the surface of EVs, indicating the presence of CD63 and CD9. To expand our analysis to other sEV markers and ensure the absence of contamination from other cellular components, we performed western blotting (WB) (Figure 8C and 8D). Consistent with the IG-TEM findings, we observed a distinct enrichment of tetraspanins CD63/CD9 and Pdcd6ip (Alix), this is an important marker to identify and discriminate the *small* vesicles from the *large* vesicles, in all EV samples compared to the donor AS. In contrast, cellular markers

such as Golga2 (for Golgi), calnexin (for endoplasmic reticulum), SDHA (for mitochondria), and actin (for cytoplasm) were predominantly retained within the cells (Figure 8C and 8D). These results provide further confirmation that our vesicular preparations are indeed enriched in sEVs.



Figure 8: AS secrete vesicles enriched in sEV markers. A,B) IG-TEM on EV samples with  $\alpha$ -CD63 (A) and  $\alpha$ -CD9 (B). Scale bars: 100 nm. C,D) WB analyses on EV lysates and corresponding AS donor cells. WBs for $\alpha$ -CD63/CD9 ((C), in non-reducing conditions) and for Pdcd6ip ((D), in reducing conditions) show an enrichment in the EV samples versus donor AS. On the contrary, the cellular markers (i.e., Golga, Calnexin, SDHA, and Actin) are mostly enriched in AS (D). All panels are representative of n = 3 independent experiments showing the same trend.

#### 8.1.4 Both VMB- and STR-AS-Derived Vesicles Are Internalized by SH-SY5Y Cells

Before examining the effects of sEVs on target neurons, we evaluated their internalization using retinoic acid (RA)-differentiated SH-SY5Y human neuroblastoma cells as a model of tyrosine hydroxylase (TH)-positive neuronal target cells. To track the AS-EVs, we used two different labeling approaches: i) both the AS of the VMB and the STR were treated with the membrane dye PKH26, followed by ultracentrifugation to isolate the labeled EVs (Figure 9 3A); and ii) PKH26 was directly applied to AS-EVs after ultracentrifugation. Both approaches produced PKH26-labelled AS-EVs, which were then administered to target cells in a 5:1 ratio (EVs derived from five AS used to treat one target cell), consistent with the local brain tissue architecture. Initially, the ability of differentiated SH-SY5Y cells to internalize AS-EVs was assessed by confocal microscopy.

As shown by the orthogonal view analyzes reported in Figure 9A, the PKH26-labelled AS-EVs were efficiently incorporated by cells and partially colocalized with TH, which has a high affinity for phospholipid membranes. A 3D volumetric reconstruction of the intracellular distribution of AS-EVs confirms the effective enrichment of vesicles in the cytoplasmic compartment. Furthermore, the combined brightfield/IF view suggests that AS-EVs are distributed throughout the cytoplasm, including neurite protrusions. Thus, AS-EVs can be efficiently transferred to neuronal target cells. Next, in order to quantify the internalization of different vesicle samples by target cells, PKH26-labelled AS-EVs were administered to differentiated SH-SY5Y cells followed by imaging flow cytometry (IFC), and the intensity of fluorescence was measured after 2, 6 and 24 hours (Figure 9B). Fluorescence increased in a time-dependent manner: i) at 2 hours there was no significant difference between untreated cells (CTRL) and treated cells; ii) at 6 hours the fluorescence intensity increases significantly by 1.4-1.7 times compared to CTRL; and iii) after 24 hours the intensity of PKH26 increased further (Figure 9C), suggesting that AS-EVs continued to enter neurons, in line with previous studies.

Although it cannot be ruled out that PKH26 may label some contaminating proteins in EV preparations, it is unlikely that autoaggregation of the dye interfered with the vesicle internalization assay, as samples containing only the dye failed to label SH cells -SY5Y at all times and with both techniques (IFC and IF) (Figure 9C).







Differentiated SH-SY5Y target cells

Figure 9: PKH26-labelled AS-EVs are internalized by differentiated,
TH-positive SH-SY5Y neuronal cells. A) Max projection and orthogonal views of representative fields show the uptake of both VMB-AS- and
STR-AS-PKH26-labelled EVs by differentiated SH-SY5Y. Each max projection is composed of a stack of 15 individual z planes, acquired every 0.4 μm along the z axis. Scale bar 10 μm. Plane a and Plane b orthogonal views represent, respectively, two selected planes located above and below the cellular nuclei (along the z axis), as represented by the cellular schematic. In all panels PKH26 is in red, TH in green, whereas nuclear DAPI counterstain is in blue. Confocal images show that PKH26 labelled EVs are present within the cellular bodies of SH-SY5Y target cells upon 6 h of incubation. B) Representative images from IFC of differentiated SH-SY5Y treated with PKH26-AS-EVs for 2, 6, and 24 h. Magnification 20×, scale bar 20 μm. C) IFC analysis of differentiated SH-SY5Y cells treated with PKH26-AS-EVs and PKH26-dye-only at different time points. Data are expressed as fold change of the mean fluorescence intensity ± SD over

CTRL set to 1 for comparison (dotted line), from n = 3 independent experiments, indicated with different symbols. One-way ANOVA with Tukey's multiple comparison versus CTRL. \*\*p < 0.01, \*\*\*\*p < 0.0001, ns: not

significant.

#### 8.1.5 EVs from CCL3-Activated AS Prevent H<sub>2</sub>O<sub>2</sub>-Induced Caspase-3 Activation in Differentiated SH-SY5Y Neurons

To assess the impact of AS-EVs on differentiated SH-SY5Y cells, which serve as a model for oxidative stress and neurodegenerative conditions, we utilized hydrogen peroxide  $(H_2O_2)$  and the neurotoxin  $MPP^+$ . These substances are well-established *in vitro* models for Parkinson's disease. Through preliminary experiments, we determined that treatment with 35  $\mu$ m  $H_2O_2$  or 1 mm  $MPP^+$  for 24 hours consistently reduced cell viability by approximately 40% and 10%, respectively, without causing acute and extensive cell death. To ensure significant internalization of vesicles, target cells were incubated with a 5:1 ratio of AS-EVs for 6 hours prior to exposure to  $H_2O_2$  or  $MPP^+$ .

First, we examined the extent of apoptosis in differentiated SH-SY5Y cells by measuring cleaved caspase-3 (c-Casp-3) levels 24 hours after  $H_2O_2$  treatment using immunofluorescence. Analysis of fluorescence intensity revealed a 3-fold increase in c-Casp-3 levels compared to untreated cells. However, the presence of VMB and STR-AS-EVs significantly reduced levels of apoptosis, with AS-EVs from CCL3-treated AS having the greatest effects on  $H_2O_2$ -induced c-Casp-3 levels, Figure 10.

To determine if the neuroprotective effect was specific to  $H_2O_2$ -injured differentiated SH-SY5Y cells, we measured caspase activity in undifferentiated SH-SY5Y cells treated with AS-EVs using the same protocols. While  $H_2O_2$  induced a 2.5-fold increase in caspase activity, pre-exposure to AS-EVs did not reduce apoptosis induced by  $H_2O_2$ . These findings further confirm that AS-EVs act as specific and effective mediators, delivering protective cargo to  $H_2O_2$ -injured differentiated SH-SY5Y cells. Additionally, these results support the potential therapeutic use of CCL3-activated AS in protecting against degeneration in the nigrostriatal system.



Figure 10: **AS-EVs significantly reduce apoptosis in differentiated SH-SY5Y neurons challenged with**  $H_2O_2$ . A) IF staining for MAP2 (in green), c-Casp-3 (in red), and DAPI (in blue), on differentiated SH-SY5Y exposed to AS-EVs and treated with 35  $\mu$ m  $H_2O_2$ . Scale bars: 50  $\mu$ m. B) Quantification of the c-Casp-3 staining in (A). The fluorescent intensities were normalized over the cell number. Data are expressed as mean  $\pm$  SD over CTRL set to 1 for comparison, from n = 3 independent replicates, indicated with different symbols. One-way ANOVA with Tukey's multiple comparison \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001 versus CTRL, ns: not significant;  $\sharp\sharp\sharpp < 0.001, \sharp\sharp\sharpp < 0.0001$  versus  $H_2O_2$ .

#### 8.1.6 Both VMB- and STR-AS-Derived EVs Preserve the Activity of Mitochondrial Complex I in Differentiated SH-SY5Y Neurons Injured by the Neurotoxin *MPP*<sup>+</sup>

Subsequently, we expanded our investigation of the neuroprotective potential of AS-EVs to the same target cells exposed to the neurotoxin  $MPP^+$ .  $MPP^+$ exerts its effects on dopaminergic (DAergic) neurons by inhibiting the activity of mitochondrial NADH-ubiquinone oxidoreductase (complex I, CI) in the electron transport chain, thereby inducing a parkinsonian-like phenotype. Additionally,  $MPP^+$  compromises the integrity of the inner mitochondrial membrane (IMM), leading to impaired ATP production through a mechanism independent of CI inhibition. We selected a dose of 1 mm  $MPP^+$ , which caused only a slight (approximately 10%) reduction in cell viability after a 24-hour incubation, thereby avoiding non-specific mitochondrial deficits due to massive cell death.

AS-EVs, in a 5:1 ratio with target cells, were applied 6 hours prior to the  $MPP^+$  challenge, and mitochondrial functionality was assessed using high-resolution respirometry (HRR) 24 hours later. The HRR analysis included the measurement of cellular oxygen consumption upon the addition of substrates and inhibitors to obtain a complete respiratory profile. Plasma membrane permeabilization and CI stimulation with specific substrates allowed for the evaluation of CI activity, Figure 11A.

 $MPP^+$  treatment did not significantly affect oxygen consumption in intact or permeabilized and fully stimulated cells but specifically impacted the contribution of CI to OXPHOS respiration. In control cells, CI accounted for approximately 73% of the overall OXPHOS, while  $MPP^+$  reduced its activity to approximately 53%. Interestingly, all AS-EV samples promoted a significant increase in CI activity in  $MPP^+$ -injured cells, ultimately achieving full rescue, Figure 11B.

The HRR measurements were also performed on undifferentiated SH-SY5Y cells exposed to 1 mm  $MPP^+$ . Although undifferentiated cells showed slight differences in the respirometry profile compared to differentiated cells,  $MPP^+$  treatment specifically affected CI activity, similar to the differentiated cells. However, the treatment with AS-EVs did not improve CI activity in undifferentiated cells, Figure 11C-D-E.

In summary, the data demonstrate the capacity of AS-EVs derived from both VMB and STR to effectively preserve CI activity in differentiated SH-SY5Y cells at concentrations significantly lower than those causing massive cytotoxicity induced by  $MPP^+$ .

#### 8.1.7 Only EVs Secreted by VMB-AS Ameliorate ATP Production in Differentiated, *MPP*<sup>+</sup>-Injured SH-SY5Y Neurons

To further investigate the impact of AS-EVs on critical aspects of  $MPP^+$ induced mitochondrial dysfunction, we evaluated their effect on features beyond CI activity. As previously demonstrated,  $MPP^+$  does not affect overall oxygen consumption in intact cells or permeabilized cells with endogenous substrates. However, it significantly reduces ATP-related fluxes, which are indicative of net fluxes. To specifically assess the effect of AS-EVs on the oxygen flux dedicated to ATP production in intact cells, HRR analysis was conducted.

The results revealed that  $MPP^+$  treatment led to a substantial reduction of the net flux by approximately 75% compared to control cells. Interestingly, treatment with VMB-AS-EVs, but not STR-AS-EVs, significantly improved ADP phosphorylation in differentiated SH-SY5Y cells injured by  $MPP^+$ . This improvement was observed regardless of the basal or CCL3 conditions. The coupling efficiency between oxidative phosphorylation and electron flows, which indicates the degree of coupling, was fully restored along with increased ATP-related flows only in the presence of VMB-AS-EVs, Figure 11F.

Control experiments using ACM/SNT or undifferentiated SH-SY5Y cells showed no significant changes in net flux or coupling efficiency when treated with EVs. These findings highlight the regional specificity of VMB-AS-EVs compared to STR-AS-EVs in rescuing the mitochondrial functional capacity of differentiated SH-SY5Y cells under  $MPP^+$  injury, Figure 11G-H.

In summary, these results demonstrate that VMB-AS-EVs have a specific ability to rescue mitochondrial functional capacity, including ATP production





Figure 11: **AS-EVs recover mitochondrial functions in differentiated SH-SY5Y neurons challenged with**  $MPP^+$ . A) Representative oxygraphic trace in untreated differentiated SH-SY5Y (control) cells alongside the specific protocol used. First, in intact cells, the physiological  $O_2$  consumption, corresponding to ROUTINE state, was measured. Second, adenylates were forced to leave the cells by a mild plasma membrane permeabilization in order to analyze the LEAK state. Third, the contribution of CI to the OXPHOS respiration was assayed in the presence of the previous addition of the appropriate substrates (pyruvate, malate, glutamate) and a saturating ADP concentration. Then, addition of succinate allowed the activation of CII (CI + CII) and the achievement of total OXPHOS respiration.

Fourth, the maximal capacity of ETS was obtained after CCCP titration. Fifth, the ROX was acquired after inhibition of ETS complexes with rotenone and

antimycin A. P, pyruvate; M, malate; G, glutamate; Dig, digitonin; S, succinate; Rot, rotenone; Ama, antimycin A. B) Schematic representation of complex I activity measurement through mitochondrial ETS complexes. C) The effects of  $MPP^+$  and EVs were tested in the same experimental conditions. The toxin reduces CI activity (CI-linked OXPHOS) of  $\simeq 30\%$  compared to CTRL while AS-EVs fully recover CI functionality of  $MPP^+$ -treated SH-SY5Y cells. The OXPHOS respiration linked to CI was expressed as flux control ratio using the total OXPHOS respiration as reference state. One-way ANOVA with Tukey's multiple comparison \*\*p < 0.01 (CTRL versus  $MPP^+$ ), and \*\*\*p < 0.001

 $(MPP^+ \text{ versus } MPP^+ + \text{VMB-AS-EVs} \pm \text{CCL3}$  and versus  $MPP^+ + \text{STR-AS-EVs} \pm \text{CCL3}$ ). D) The effects of ACM/SNT were tested as before. No significant differences were observed in  $MPP^+$ -injured cells treated with ACM or SNT samples. The OXPHOS respiration linked to CI was expressed as flux control ratio using the total OXPHOS respiration as reference state. One-way ANOVA with Tukey's multiple comparison \*\*p < 0.01 (CTRL versus  $MPP^+$ ), ns: not significant. E) The effects of AS-EVs were tested in undifferentiated SH-SY5Y cells. The toxin treatment significantly reduces CI activity versus CTRL, while no significant effect was observed in presence of AS-EV samples. The OXPHOS respiration linked to CI was expressed as flux control ratio using

the total OXPHOS respiration as reference state. One-way ANOVA with

Tukey's multiple comparison \*p < 0.05 (CTRL versus  $MPP^+$ ), ns: not significant. In panels (C–E) data are expressed as the ratio between OXPHOS driven by CI and total OXPHOS (driven by CI + CII)  $\pm$  SD. F) Schematic representation of mitochondrial ETS and ATP synthase complexes. G)  $MPP^+$ reduces  $O_2$  flux devoted to ATP production compared to CTRL in ROUTINE. Only VMBAS- EVs promote a significant recovery of the flux in differentiated SH-SY5Y cells. Net ROUTINE was expressed as flux control ratio using the maximal capacity as reference state. Data are expressed as percentage of the maximal ETS capacity  $\pm$  SD. One-way ANOVA with Tukey's multiple comparison, \*\*p < 0.01 ( $MPP^+$  versus  $MPP^+ + VMB$ -AS-EVs  $\pm$  CCL3),

\*\*\*p < 0.001 (CTRL versus  $MPP^+$ ), ns: not significant. H) Coupling efficiency in basal state (ROUTINE) of differentiated SH-SY5Y cells in the same experimental conditions. According to the ATP production data, only VMB-AS-EVs were able to significantly increase the rate of coupling between the oxidative phosphorylation and ATP production. Data are expressed as percentage of each specific state. Coupling efficiency was expressed as flux control ratio using the basal respiration (ROUTINE) as reference state. Data are expressed as means  $\pm$  SD. One-way ANOVA with Tukey's multiple comparison, \*\*p < 0.01 ( $MPP^+$  versus  $MPP^+$  + VMB-AS-EVs  $\pm$  CCL3), \*\*\*p < 0.001 (CTRL versus  $MPP^+$ ), ns: not significant.

Based on these results, we have demonstrated how cellular communication via EVs exists, and, in the neurodegenerative context, they can be useful biomarkers but also useful "weapons" to decrease the negative effects of inflammation and oxidative stress due to PD.

It is evident that in terms of information transmitted from a donor cell to a target cell through EVs, the content of the single EV can be considered useful information as well as the EV itself in its entirety, excluding its content. This dichotomy is addressed in the next Sections by dividing them into different key studies, for a better understanding by the reader.

# 8.2 *II<sup>nd</sup>* Key Study: Extracellular Vesicles as molecular packets

Cells produce thousands of molecules of different nature which provide information to other cells and influence their activity. As in the device communication, cells use different "data structure" supporting information exchange through the blood or other biofluid, as I will show you into the next paragraph.

#### 8.2.1 Extracellular Vesicles as molecular packets improve cell-tocell communication

Two cells can exchange either single molecules, or a combination of them, supporting a more complex information structure. Some examples of the first type of communication are the polar/a-polar molecules through receptors, as reported in Sections 5.1 and 6.1; examples of more complex biological data structures are EVs.

Single molecules need a ligand-receptor pathway [5]; differently structured biological information, such as EVs, may use pinocytosis, phagocytosis and fusion with the cell plasma membranes [89] that allow the encapsulated molecules to be absorbed by the cell, in most cases, without the activation of the ligand-receptor pathway.

#### 8.2.2 Key study: EVs vs packets molecules

The information, molecules, that we consider are the microRNAs  $(miRNAs)^1$ .

<sup>&</sup>lt;sup>1</sup>miRNA are small molecules of ribonucleic acid (RNA), characterized by a short sequence (22 nucleotides). Their role is to bind to mRNAs in the cytoplasm based on sequence complementarity, and downregulation of gene expression [131].



Figure 12: Comparison between ligand-receptor uptaking and EVs fusion. On the left there is a cell with 2 different receptors for AGO-miRNA-1/-1'/-2/-2'. On the right there is a cell that interact with EVs with inside miRNA 1 and miRNA 2.

EVs can concentrate, protect and carry many miRNAs simultaneously. Therefore, to compare the two signaling mechanisms, the synergistic actions of two different miRNA bound to an Argonaute protein, herein referred to as AGO-miRNA-1 and AGO-miRNA-2, will be compared with the synergistic action of miRNA-1 and miRNA-2 when transported inside the EVs.

We model the related message with a pair of random variables, X and Y, that represent the amount of miRNA-1 and miRNA-2 received at the target cell and suppose that information is encoded in the ratio between X and a Y. In the ligand-receptor communication pathway the same receptor can be occupied by a ligand different from the two miRNAs of our interest, which introduces distortion that results in information loss.

Figure 12 outlines this occurrence where the miRNA-1' and 2' represent the competing molecules and the ligand-receptor interactions will be conditioned by the probability that the receptor binds them.

The EV shields the miRNA-1 and miRNA-2 ratio it contains, enters the target cell via fusion as in Figure 12.

#### 8.2.3 Model

We model a complex information message as a K-tuple  $\mathbf{x} = (x_1, x_2, ..., x_K)$ . Due to the presence of the molecular noise, the ligands which bind with the receptors can be modeled as a random variable  $\mathbf{X} = (X_1, X_2, ..., X_K)$ . We first evaluate the probability distribution of the individual random variable



Figure 13: Numeric results. (a) PDFs of miRNA-1 and miRNA-2 ligands vs.  $N_1$  and  $N_2$  and (b) crosstalk entropy at alpha parameter variation.

 $X_k$ ; later, the *crosstalk entropy*,  $H(\mathbf{X})$ , which is a measure of the uncertainty at the target cell regarding the transmitted message. Let  $c_k^{(L)}$  be the number of molecules in the medium of the k-th signaling ligand and  $c_k^{(A-L)}$  the number of molecules in the medium of the interfering ligands concurring for binding with the k-th receptor type. The probability that a given receptor is bound to a signaling ligand can be calculated as

$$P_k^{(L)} = \frac{c_k^{(L)}}{c_k^{(L)} + c_k^{(A-L)}} = \frac{1}{1 + \alpha_k} \text{ where } \alpha_k = \frac{c_k^{(A-L)}}{c_k^{(L)}}$$
(1)

Molecular diffusion is not considered, and the concentration of molecules is much more than receptors of the cells. The parameter  $N_k$  represents the number of receptors of type k in the cell. The random variable  $X_k$  can be modeled as a Bernoulli variable. Therefore, its average and variance can be calculated as

$$\mu_k = \frac{N_k}{(1+\alpha_k)} \text{ and } \sigma_k^2 = \frac{N_k \cdot \alpha_k}{(1+\alpha_k)^2}$$
(2)

Due to the high number of receptors in a cell, the Bernoulli variable can be approximated as a Gaussian variable and since the variables  $X_1, X_2, ..., X_k$ 

are independent of each others, the crosstalk entropy of  $\mathbf{X}$  is the sum of the entropies of the individual random variables and can be calculated as:

$$H(\mathbf{X}) = 2.0471 \cdot K + \frac{1}{2} \sum_{k=1}^{K} \log_2\left[\frac{N_k \cdot \alpha_k}{(1+\alpha_k)^2}\right]$$
(3)

#### 8.2.4 Numerical results

Let  $c_1^{(L_1)}$  and  $c_2^{(L_2)}$  be the concentrations of miRNA-1 and miRNA-2 signaled to the target cell by means of ligand-receptor communication pathway. In particular we suppose to have 300 miRNA-1 molecules and 900 miRNA-2 molecules, that is  $\frac{c_1^{(L_1)}}{c_2^{(L_2)}} = \frac{1}{3}$ ; moreover, we suppose that the number of the target cell receptors specific for the two ligands are  $N_1 = 300$  and  $N_2 =$ 900. We also hypothesize that both receptors are saturated and that there are no degradation processes of the respective ligands and receptors. Figure 13a shows the PDF of the number of miRNA-1 binding receptors  $N_1$  and  $N_2$ when  $\alpha_{k_1} = \alpha_{k_2} = \alpha$  is equal to 0.4. Molecular noise dramatically reduces the probability of miRNA-1 and miRNA-2 entering the target cell, resulting in a loss of information for the target cell. Figure 13b shows the crosstalk entropy for different values of  $\alpha$ , where  $\alpha = 0$  represents the case where the EVs signaling pathway is used.

From this study, it is clear that molecular communication mediated by EVs is qualitatively better than ligand-receptor communication, since a gain is obtained in the information received by the target cell, reducing molecular noise.

Modeling and knowing the identity and concentrations of the molecules within the EVs would be very useful and interesting, so as to plan innovative therapies or specific treatments, enhancing this research work, but nowadays there are technological limits for which it is not possible to know the exact concentration of the molecules present inside the EVs and/or artificial liposomes, although it is possible to know the identity of the transported molecules. Furthermore, it is quite impossible to estimate a "threshold" of action of the molecules transported by EVs.

An approach that considers the single EV as a source of information and no longer their content is therefore more practical, which facilitates the modeling of vesicular uptake and their detection in the intra- and extracellular environment. This approach will be proposed in different strategies in the next paragraphs, adapting and evolving more and more, based on the knowledge of the parameters, the experimental data available or found in the literature and the specific experimental conditions of the system under study.

## 8.3 III<sup>rd</sup> Key Study: EVs Fusion uptake

In the previous Section I studied and modeled the cellular communication of EVs considering the content of the EVs as an element that transmits information from one cell to another.

In this Section and the next ones we will address the other side of the coin, considering the single EV, in its entirety, as a source of transmitted information and no longer its own content.

In Fig. 14 the EV communication system is depicted. The EVs, once released by donor cells, diffuse in the extracellular space. When they come in proximity of the target cells, several uptake mechanisms can take place. In particular, two of them are worth of mention: receptor-mediated endocytosis and EV fusion with the plasma membrane of the target cell [76, 77]. The general process of receptor-mediated endocytosis is well documented in literature[76]. In [132], a mathematical model of the endocytosis has been presented and in [12], it has been utilized to derive the receiver transfer function in a communication scheme focusing on endocytosis as reception mechanisms.

In this Section a particular focus on the fusion uptaking process is studied and modelled.

#### 8.3.1 Modeling Extracellular Vesicle Fusion to the Plasma Membrane of the Target Cell

The previous approach in the 8.2 Section is a study based on the content of single EVs, at the moment impossible to verify experimentally due to technological limitations. To better understand the cell-cell communication of electric vehicles, and to make the model more accessible to the scientific community, attention was focused on the integral vesicle. We have therefore studied one of the mechanisms of vesicular internalisation, fusion, and identified different phases of this process which have been described through a system of ordinary differential equations. The parameters of the equations are the rates of the chemical reactions involved in the binding and fusion of the EVs, as well as in the recycling/regeneration process of the FPs involved.

Concurrent to the endocytosis, the EVs fusion with the plasma membrane of the target cell seems to involve a great amount of EVs [77]. It is reasonable to expect that differences among various EV internalization processes performed by the target cells may be related to different functionalities, even producing different effects. Therefore, a deep investigation of the possible interactions, cooperation or competition among concurrent internalization process is of fundamental importance in large variety of applications. To this purpose, understanding the dynamics of EVs fusion with the plasma membrane of the target cell represents a first step in the direction of building a more general picture of the cell communication mechanisms.

This is crucial because poor information is available in literature about the parameters regulating the processes of our interest. In fact, the EV fusion with the plasma membrane of the target cells, in contrast with the EV fusion with membrane bound compartments and organelles inside the cell, has received attention only recently and has not been deeply investigated yet. The majority of knowledge about the external surfaces fusion between EVs and cells comes mainly from the study of either the fusion between two cells or of virus with cells, which have suggested interesting hypothesis about the identity of the proteins involved in the cell surface fusion of EVs, as well as possible new line of investigation on this topic. Nevertheless, proper meaningful values for the model parameters are still not available.

In such a context, our approach is to define a mathematical model of the EV fusion process and compare the values obtained by the model to the experimental results. This, in fact, would allow to infer reasonable values for the parameters regulating the fusion process through a reverse engineering approach.

#### 8.3.2 Fusion Model

In this section, a mathematical model of the EV fusion to the plasma membrane of the target cell is derived, with an approach similar to the one used in [132] to model endocytosis.

Let F be the concentration, in number of pairs per cell surface unit, of fusogenic protein pairs on the plasma membrane of the target cell, and let us denote as  $F_0$ ,  $F_b$  and  $F_f$  the concentrations of fusogenic protein pairs that are available for binding, or currently bound to an EV, or in a post-fusion


Figure 14: Extracellular Vesicle communication system. On the left there is the transmitter (donor cell) and on the right there is the receiver (target cell). Between them, there is the channel where EVs diffuse.

state, respectively. We assume that the EVs bind to a pairs of available FPs at a rate  $a_b$ . The binding process can be described as follows:

$$F_0 + V_e \xrightarrow{a_b} F_b + V_b \tag{4}$$

where  $V_e$  and  $V_b$  are the concentration of EVs in the extracellular space close to the plasma membrane and bound to the FPs, respectively. Near the periphery of the cell, the EVs initially exhibit a surface sliding behavior until their movements drastically decrease, as an effect of the high affinity between the FPs on the surface of both cells and EVs. The binding between those proteins is, at this point, established [77]. The duration of the surface sliding, which depends on the type of cells and EVs, determines, among other factors, the binding rate,  $a_b$ , of EVs to the plasma membrane. Soon after, the binding evolves into fusion, at a rate that we denote as  $b_f$ . The evolution of EVs from the extracellular space to the fusion EV-cell can be summarized by the following set of reactions:

$$\{extracellular medium\} \xrightarrow{k_V} V_e \xrightarrow{a_b} V_b \xrightarrow{b_f} V_f \tag{5}$$

where  $k_V$  is the delivery rate of EVs to the cell surface, i.e. the number of EV supplied to the cell by the extracellular medium in the time unit, and  $V_f$  is the concentration of EVs whose fusion to the plasma membrane is completed and whose cargos have been released into the cytosol. Note that, at this point, the fused EV does not exist anymore as a physical entity, since it has been broken down and decomposed into a piece of plasma membrane and its original content. It may be argued that all the bound EVs evolve into



Figure 15: **EV Fusion to the Plasma Membrane of the Target Cell.** This is a simple scheme that shows fusion process between EV and membrane of the target cell.

fused ones, so that  $V_e$  and  $V_f$  assume the same values. However, while this is true at the steady state, the temporal dynamics of bound and fused EVs may differ, so that the variable  $V_f$  is introduced to take into account the time needed for the fusion to take place, after the binding have been established, and how it may impact the evolution of further EVs binding.

In order to model the evolution of the fusion process, we first focus on the time-dependent concentrations of the available, bound and post-fusion FPs on the cell surface,  $F_0(t)$ ,  $F_b(t)$  and  $F_f(t)$  respectively, and consider the events that affect their temporal variation. In particular, let us note that the binding of an EV to an available fusogenic protein produces a decrease in the concentration of available FPs,  $F_0(t)$ , and a corresponding increase in the concentration of bound ones,  $F_{h}(t)$ . The contribution of this event follows by the application of the law of mass action to the reaction (4). Similarly, the fusion of a bound EV to the plasma membrane, at the rate  $b_f$ , accounts for a negative contribution in the temporal variation of the bound FPs,  $F_b(t)$  and a positive contribution to the temporal variation of the post-fusion FPs,  $F_f(t)$ . Besides the binding and fusion events, also the recycling processes of the FPs affect the temporal variation of their concentration and need to be considered. However, a deep knowledge about the FPs involved in the fusion process between EVs and target cells is not well documented vet. Nevertheless, it is reasonable to assume that a protein recycling process occurs similarly to what happens in other biological entities within the cell components and activities [133, 134]. More specifically, it is reasonably expected that after the fusion of the EVs is completed, a fraction of the post-fusion proteins becomes again available for binding new EVs, while at the same time old FPs are degraded and new available FPs are produced by the cell. Accordingly, a negative

contribution is given to the temporal variation of the post-fusion protein concentration  $F_f(t)$  by the recycled and destroyed proteins at the rates  $b_d$ and  $b_a$ , respectively. Likewise, the recycled proteins produces a positive contribution in the temporal variation of available proteins concentration  $F_0(t)$ . Additionally, let  $k_F$  be the production rate of new fusogenic protein, i.e. the concentration of new synthesized fusogenic protein pairs per unit time, which contributes positively to the concentration  $F_0(t)$ . It is worth noting that according to the dynamics implemented by the cell to regulate such a recycling process, an equilibrium between recycled, new produced and old degradated proteins may or may not be reached. Therefore to take into account the more general case, the production rate is introduces in the model as a function of time, i.e.  $k_F(t)$ . All the above considerations lead to the following equations:

$$\frac{dF_0(t)}{dt} = k_F(t) + b_d F_f(t) - a_b F_0(t) V_e(t)$$
(6)

$$\frac{dF_b(t)}{dt} = a_b F_0(t) V_e(t) - b_f F_b(t)$$
(7)

$$\frac{dF_f(t)}{dt} = b_f F_b(t) - (b_d + b_a) F_f(t)$$
(8)

With similar considerations, let us now focus on the temporal evolution of the concentrations of EVs in the extracellular space close to the target cell  $V_e(t)$ , bound to the FPs  $V_b(t)$ , and fused to the plasma membrane  $V_f(t)$ . By applying the law of mass action to reactions (5), we obtain the following equations:

$$\frac{dV_e(t)}{dt} = k_V(t) - a_b F_0(t) V_e(t)$$
(9)

$$\frac{dV_b(t)}{dt} = a_b F_0(t) V_e(t) - b_f V_b(t)$$
(10)

$$\frac{dV_f\left(t\right)}{dt} = b_f V_b\left(t\right) \tag{11}$$

Note that the concentration of EVs in the extracellular medium may not be spatially uniform. However, the fluid dynamics of EVs delivery to the cell surface [12] is out the scope of this work, since we are here interested on the dynamics of binding and fusion of the EVs to the plasma membrane. For this reason, we assume that there is a layer of fluid close to the plasma membrane of the target cell, where the concentration of the EVs can be considered spatially uniform and we define  $V_e$  as the concentration of EVs in this portion of extracellular fluid. Therefore, we can consider here the solely dependance of  $V_e$  from time. This concentration of EVs, close to the plasma membrane of the target cell, is supplied to the system from an external source at the delivery rate  $k_V(t)$ .

#### 8.3.3 Model solution

The set of equations (6)-(11) constitute, in its general form, the system of Ordinary Differential Equations (ODEs) that models the binding and fusion process of EVs to the plasma membrane of a target cell. However, note that equations (7) and (10) have the same expression. In fact, since a pair of FPs bind with a single EV, the number of bound fusogenic protein pairs is equal to the number of bound EVs, i.e.:

$$V_b\left(t\right) = F_b\left(t\right) \tag{12}$$

Therefore we can substitute (10) with (12).

Furthermore, the variable  $V_f(t)$  appears only in (11), and can be calculated by integration from  $V_b(t)$ , or equivalently  $F_b(t)$ , that is:

$$V_f(t) = c + b_f \int F_b(t) dt$$
(13)

where c is the constant of integration. Thus, let us focus on the system of ODEs (6)-(9). These equations are non-linear, due to the term with the product between  $F_0(t)$  and  $V_e(t)$ . However, since this term appears in three of the four above equations, we can easily derive the following identities:

$$a_b F_0 V_e = \frac{dF_b}{dt} + b_f F_b = -\frac{dF_0}{dt} + k_F + b_d F_f = -\frac{dV_e}{dt} + k_V$$
(14)

where the dependency from time have been omitted for the sake of simplicity. Now from (8) let derive the expression of  $F_b$  as a function of  $F_f$ , i.e.:

$$F_b = \frac{1}{b_f} \frac{dF_f}{dt} + \frac{b_d + b_a}{b_f} F_f \tag{15}$$

By substituting (15) in (14) and integrating each side of (14), after some manipulation the following relations are obtained:

Table 2: Coefficients of Equation (19)

$$\mathcal{A} = -\frac{a_b}{b_f}$$

$$\mathcal{B} = -2\frac{a_b}{b_f} (b_d + b_a + b_f)$$

$$\mathcal{C} = -a_b (b_d + 2b_a)$$

$$\mathcal{D}(t) = b_d + b_a + b_f + a_b \int (k_V (t) + k_F (t)) dt$$

$$\mathcal{E} = -\frac{a_b}{b_f} (b_d + b_a + b_f)^2$$

$$\mathcal{F} = -a_b (b_d + 2b_a) (b_d + b_a + b_f)$$

$$\mathcal{G}(t) = b_f (b_d + b_a) + a_b (b_d + b_a + b_f) \int (k_V (t) + k_F (t)) dt$$

$$\mathcal{H} = -a_b b_f b_a (b_d + b_a)$$

$$\mathcal{I}(t) = a_b b_f (b_d + b_a) \int k_F (t) dt + a_b b_f b_a \int k_V (t) dt$$

$$\mathcal{J}(t) = -a_b b_f \int k_F (t) dt \int k_V (t) dt$$

$$F_{0} = \int k_{F}dt - \frac{1}{b_{f}}\frac{dF_{f}}{dt} - \frac{b_{d} + b_{a} + b_{f}}{b_{f}}F_{f} - b_{a}\int F_{f}dt$$
(16)

$$V_e = \int k_V dt - \frac{1}{b_f} \frac{dF_f}{dt} - \frac{b_d + b_a + b_f}{b_f} F_f - (b_d + ba) \int F_f dt \qquad (17)$$

In order to simplify the notation, let apply the following substitutions:

$$\int F_f dt = z, \quad F_f = z'_t, \quad \frac{dF_f}{dt} = z''_t, \quad \frac{d^2 F_f}{dt^2} = z'''_t$$
(18)

where the notations  $z'_t = \frac{dz}{dt}$ ,  $z''_t = \frac{d^2z}{dt^2}$ ,  $z''_t = \frac{d^3z}{dt^3}$ ,... are used for the total derivatives. Let now substitute (18) in (16), (17) and (15), and the resulting equations in (7). After some manipulation, the following ODE is obtained:

$$z_{t}^{\prime\prime\prime} + \mathcal{A} (z_{t}^{\prime\prime})^{2} + \mathcal{B} z_{t}^{\prime} z_{t}^{\prime\prime} + \mathcal{C} z z_{t}^{\prime\prime} + \mathcal{D} z_{t}^{\prime\prime} + + \mathcal{E} (z_{t}^{\prime})^{2} + \mathcal{F} z z_{t}^{\prime} + \mathcal{G} z_{t}^{\prime} + \mathcal{H} z^{2} + \mathcal{I} z + \mathcal{J} = 0$$
(19)

whose coefficients are calculated as in

Table 2.

So far, the system of ODE (6)-(9) has been reduced to the single nonlinear ODE of the third order (19), for which, unfortunately, from the best of our knowledge, a solution method is not known. Therefore, in the following, several substitutions are applied to transform the equation into a more easy to manage form. We will see, that (19) can be transformed in an Abel ODE of the second kind, in normal form, whose solution has been proposed in [135, 136].

The first substitution to be applied is:

$$z''_{t} = r(z)$$

$$z''_{t} = \frac{dr(z)}{dt} = \frac{dr}{dz}\frac{dz}{dt} = r'_{z}z'_{t} = r'_{z}r$$

$$z'''_{t} = \frac{d(rr'_{z})}{dt} = \frac{d(rr'_{z})}{dz}\frac{dz}{dt} = r^{2}r''_{z} + r(r'_{z})^{2}$$
(20)

which reduces the third order ODE in (19) into the following second order ODE:

$$r_{z}'' = f_{1}(r)(r_{z}')^{2} + f_{2}(r, z, [t])r_{z}' + f_{3}(r, z, [t])$$
(21)

where:

$$f_{1}(r) = -\mathcal{A} - \frac{1}{r}$$

$$f_{2}(r, z, t) = -\mathcal{B} - \frac{\mathcal{C}z + \mathcal{D}(t)}{r}$$

$$f_{3}(r, z, t) = -\mathcal{E} - \frac{\mathcal{F}z + \mathcal{G}(t)}{r} - \frac{\mathcal{H}z^{2} + \mathcal{I}(t)z + \mathcal{J}(t)}{r^{2}}$$
(22)

The coefficients f2 and f3 in (22) are functions of the variables r, z, and t. However, through the substitution (20), the independent variable in (21) is now z, whereas t serves as a parameter. Therefore the notation adopted in (21) and in the following, to clearly distinguish whether in a given equation the arguments of a function have to be handled as independent variables or parameters, consist of embracing the list of parameters in square bracket. Note that, for the sake of simplicity and without loosing generality, the parameters can be sometimes omitted.

The second order ODE in (21), through the following substitution:

$$r'_{z} = u(r)$$

$$r''_{z} = \frac{du(r)}{dz} = \frac{du}{dr}\frac{dr}{dz} = u'_{r}r'_{z} = u'_{r}u$$
(23)

can be further reduced to the following first order ODE in the form of an Abel differential equation of the second kind, where the independent variable

is r, while z and t act as parameters:

$$uu'_{r} = f_{1}u^{2} + f_{2}\left(r, [z, t]\right)u + f_{3}\left(r, [z, t]\right)$$
(24)

The following functional transformation [137]:

$$w(r) = u(r) E(r),$$
  

$$E(r) = e^{-\int f_1(r)dr} = \alpha r e^{\mathcal{A}r}$$
(25)

where  $\alpha$  is a constant of integration, allows to rewrite (24) as:

$$ww'_{r} = g_{1}(r, [z, t]) w + g_{0}(r, [z, t])$$
(26)

where:

$$g_{1}(r, z, t) = f_{2}(r, z, t) E(r)$$
  

$$g_{0}(r, z, t) = f_{3}(r, z, t) E^{2}(r)$$
(27)

Finally, by the following substitution [137]:

$$x(r) = \int g_1(r, z, t) dr = R(r) \quad \Leftrightarrow \quad r = R^{-1}(x)$$
  
$$y(x) = w(R^{-1}(x)) \qquad \Leftrightarrow \quad w(r) = y(R(r))$$
(28)

the Abel equation of second kind (26) can be written in the normal form:

$$y(x) y'_{x}(x) - y(x) = Q(x)$$
 (29)

where:

$$Q(x) = \frac{g_0(x)}{g_1(x)} = \frac{g_0(R^{-1}(x))}{g_1(R^{-1}(x))}$$
(30)

More specifically, after some manipulation, (27) and (28) can be calculated, respectively as follows:

$$g_{1}(r, z, t) = -\alpha \left(\mathcal{B}r + \mathcal{C}z + \mathcal{D}\right) e^{\mathcal{A}r}$$
  

$$g_{0}(r, z, t) = -\alpha^{2} \left(\mathcal{E}r^{2} + \left(\mathcal{F}z + \mathcal{G}\right)r + \mathcal{H}z^{2} + \mathcal{I}z + \mathcal{J}\right) e^{2\mathcal{A}r}$$
(31)

and

$$x(r) = -\alpha \left(\frac{\mathcal{B}}{\mathcal{A}}r - \frac{\mathcal{B}}{\mathcal{A}^{2}} + \frac{\mathcal{C}z + \mathcal{D}}{\mathcal{A}}\right)e^{\mathcal{A}r} = R(r)$$
  
$$r(x) = \frac{1}{\mathcal{A}}\left(W\left(-\frac{\mathcal{A}^{2}}{\alpha\mathcal{B}}e^{-1 + \frac{\mathcal{A}}{\mathcal{B}}(\mathcal{C}z + \mathcal{D})}x\right) + 1 - \frac{\mathcal{A}}{\mathcal{B}}(\mathcal{C}z + \mathcal{D})\right)$$
(32)

where W is the Lambert W function.

A methodology to solve (29) has been presented in [135, 136] and is provided in (Appendix 8.3.4) at the end of this paper.

Now, following the inverse procedure, from (37) and (28), the solution of (26) is:

$$w(r, [z, t]) = y(R(r), [z, t])$$
(33)

which through (25) gives the solution of (24) as:

$$u(r, [z, t]) = \frac{w(r, [z, t])}{E(r)}$$
(34)

Now, by solving the differential equation in the first of (23) after substituting (34) in it, r(z, [t]) has to be derived from the following implicit equation:

$$z = \int \frac{dr}{u(r, [z, t])} = S(r, z, [t])$$
(35)

Similarly, by substituting the solution of (35) in the first of (20), z(t) has to be derived from the following implicit equation:

$$t = \int \frac{dz}{r(z, [t])} = T(z, t)$$
(36)

Now, substituting the solution of (36) in (18), the expression of  $F_f(t)$  is provided, which in turn allows to calculate  $V_e(t)$ ,  $F_0(t)$  and  $F_b(t)$  from (17), (16) and (15), respectively. Finally, once the expression of  $F_b(t)$  is known, also  $V_f(t)$  and  $V_b(t)$  can be calculated from (13) and (12), respectively.

# 8.3.4 Solution of the normal Abel ODE of the second kind.

In this section, is provided the methodology to solve the normal Abel differential equation of the second kind (29), presented in [135, 136].

The solution of (29) is given by [135, 136]:

$$\prod_{k=1}^{n_{A}} |y(x) - y_{k}(x)|^{m_{k}(x)} = C_{A}$$
(37)

where  $C_A$  is a constant of integration,  $y_k(x)$  are particular independent solutions of (29), constructed as follows:

$$y_k(x) = \frac{1}{2}x \left[ N_k(x) + \frac{1}{3} \right], \quad k = 1, ..., n_A$$
 (38)

on the roots  $N_k(x)$  of the cubic equation:

$$N^3 + pN + q = 0 (39)$$

in which:

$$p = -\frac{a^2}{3} + b, \quad q = \frac{2}{27}a^3 - \frac{1}{3}ab + c$$

$$a = -4, \quad b = 3 + \frac{4(G(x) + Q(x))}{x}, \quad c = -\frac{4(G(x) + 2Q(x))}{x}$$

$$G\left(\xi\right) = \frac{\left((\xi\sin\xi + \cos\xi)Ci(\xi) + \cos\xi^2\right)(4\xi Ci(\xi) + \cos\xi)}{16(\xi Ci(\xi))^3}e^{-\xi} - 2Q\left(\xi\right)$$

$$Ci\left(\xi\right) = \gamma + \ln\xi - \int_0^{\xi} \frac{1 - \cos\vartheta}{\vartheta}d\vartheta \quad \text{for arg } |\xi| < \pi$$

$$\gamma \approx 0.57721566 \quad (\text{Euler-Mascheroni constant})$$

$$\xi = \ln|x|$$

$$(40)$$

and where  $n_A$  and  $m_k(x)$  depend on the discriminant  $\Delta_3$  of the cubic equation (39), that is  $\Delta_3 = \left(\frac{p}{3}\right)^3 + \left(\frac{q}{2}\right)^2$ . More specifically, if:

•  $\Delta_3 = 0$ : the cubic equation (39) admits two real roots, one of them with multiplicity 2, which implies that only  $n_A = 2$  independent particular solutions have to be considered in (37), and the two corresponding exponents  $m_1(x)$  and  $m_2(x)$  are:

$$m_1(x) = \frac{x + 12Q(x)}{x + 18Q(x)} M(x), \quad m_2(x) = \frac{6Q(x)}{x + 18Q(x)} M(x)$$
(41)

where

$$M(x) = \frac{\ln |C_A|}{\int \frac{4(x+15Q(x))}{3(x+12Q(x))(x+18Q(x))} \ln \left|4 + \frac{x}{3Q(x)}\right| dx}$$
(42)

The solutions of the cubic equation (39) are in this case:

$$N_1 = -2\sqrt[3]{\frac{q}{2}}, \quad N_2 = \sqrt[3]{\frac{q}{2}}$$
 (43)

•  $\Delta_3 \neq 0$ : the cubic equation (39) admits three roots, so that  $n_A = 3$  independent particular solutions can be considered in (37), and the three corresponding exponents  $m_k(x)$  for k = 1, 2, 3 are:

$$m_{1}(x) = \frac{y_{1}(x)}{(y_{1}(x) - y_{2}(x))(y_{1}(x) - y_{3}(x))} M(x)$$

$$m_{2}(x) = \frac{y_{2}(x)}{(y_{2}(x) - y_{1}(x))(y_{2}(x) - y_{3}(x))} M(x)$$

$$m_{3}(x) = -(m_{1}(x) + m_{2}(x))$$
(44)

where

$$M(x) = \left(C_A - \frac{y_1(x)(y_2(x) - y_3(x))}{2y_1(x)y_2(x) + (y_1(x) + y_2(x))y_3(x)}\right)^{(y_1(x) - y_2(x))}$$
(45)

The three solutions of the cubic equation (39) are, in this case, when  $\Delta_3 < 0$ :

$$N_{1} = 2\sqrt{-\frac{p}{3}}\cos\frac{\beta}{3}, \quad N_{2} = -2\sqrt{-\frac{p}{3}}\cos\frac{\beta-\pi}{3}, \\ N_{3} = -2\sqrt{-\frac{p}{3}}\cos\frac{\beta+\pi}{3}, \quad \cos\beta = -\frac{q}{2\sqrt{-(\frac{p}{3})^{3}}} \quad 0 < \beta < \pi$$
(46)

or, when  $\Delta_3 > 0$ .:

$$N_{1} = \sqrt[3]{-\frac{q}{2} + \sqrt{D}} + \sqrt[3]{-\frac{q}{2} - \sqrt{D}}$$

$$N_{2} = -\frac{1}{2} \left( \sqrt[3]{-\frac{q}{2} + \sqrt{D}} + \sqrt[3]{-\frac{q}{2} - \sqrt{D}} \right) + \frac{1}{2i\frac{\sqrt{3}}{2}} \left( \sqrt[3]{-\frac{q}{2} + \sqrt{D}} - \sqrt[3]{-\frac{q}{2} - \sqrt{D}} \right)$$
(47)

In this work, some parameters involved in the vesicular fusion process have been defined based on the known literature. The model we created appears to be accurate and will be verified and implemented in the next paragraph.

# 8.3.5 Evaluation of EV Fusion Process Parameters at the Receiving Cell

The lack of knowledge of all the molecular processes and all the parameters involved, combined with the continuous progress of scientific research, add completeness and new ideas to improve and make previous models more realistic. Therefore, a thorough investigation of possible interactions between competing internalization processes is crucial for biomedical purposes. For this reason, an evolution of the previous model is necessary with a real estimate of the parameters involved in the fusion process and a possible application in a very specific context, which could be relevant for successful therapeutic strategies. However, it has only recently received attention and has not yet been thoroughly studied.

For all these reasons, in [138] we have presented a mathematical model of the internalization of EVs through their fusion with the plasma membrane of the target cells, based on the model reported in the previous paragraphs. There were several phases of the fusion process described through a system of ordinary differential equations whose parameters are the speeds of the chemical reactions involved in the bonding and fusion of the electric vehicles, as well as in the recycling/regeneration process of the proteins involved in the process, from hereinafter generically referred to as FP, for convenience.

Unfortunately, to the best of our knowledge, little information is available in the literature on the parameters that regulate the processes of interest to us.

To overcome this drawback, in this work we initially use the mathematical model to study the influence of relevant parameters on the evolution of EV fusion and provide a framework for future comparisons between numerical solutions and biological experimental measurements. Next, we use the model as a tool to infer the range of most probable values for these biological parameters, given some common biological laboratory measurements, such as the rate of internalization of EVs into the recipient cell.

#### 8.3.6 Modeling of the fusion process

In this section, a mathematical model of the EV fusion to the plasma membrane of the target cell is derived, with an approach similar to the one used in [132] to model endocytosis.

Let F be the concentration of FPs on the plasma membrane of the target cell, and let  $F_a$ ,  $F_b$  and  $F_f$  denote the concentrations of FPs that are available for binding, currently bound to an EV, and in a post-fusion state, respectively. As already mentioned in Section 6.1.2, two FPs on the plasma membrane of the target cell are necessary to bind an EV [77], (see also Fig. 15). Therefore, for the sake of simplicity, let us measure F in number of FPs pairs<sup>2</sup> per unit of volume. Further, let V be the concentration of EVs, and let  $V_e$ ,  $V_b$  and  $V_f$  denote the concentration of EVs that are in the extracellular space close to the target cell (for brevity, in the following, also called external EVs), currently bound to pairs of FPs, and in a post-fusion state. Near the periphery of the cell, the external EVs initially exhibit a surface sliding behavior until their movements drastically decrease, as an effect of the high affinity between the proteins on the surface of both EVs and cells. The bond between EVs and FPs is, at this point, established [77], which implies

<sup>&</sup>lt;sup>2</sup>In the following we may generically refer to FPs meaning always FPs pairs.

that the available FPs,  $F_a$ , and the external EVs,  $V_e$ , change their state to bound FPs,  $F_b$ , and bound EVs,  $V_b$ , respectively. This happens at a binding rate constant,  $a_b$ , determined, among other factors, by the duration of the surface sliding described above, which depends on the type of FPs and EVs. The binding process described so far, with an approach similar to the one in [132], can be schematized as follows:

$$F_a + V_e \xrightarrow{a_b} F_b + V_b \tag{48}$$

Soon after, the binding between EVs and FPs evolves into fusion, with a rate constant that we denote as  $b_f$ . The evolution of EVs from the extracellular space to the EV-cell fusion can be schematized by the following set of reactions, similarly to (48):

$$\{extracellular medium\} \xrightarrow{k_V} V_e \xleftarrow{a_b}{\underset{b_u}{\longleftrightarrow}} V_b \xrightarrow{b_f} V_f$$
(49)

where  $k_V$  is the delivery rate of EVs to the cell surface, i.e. the number of EV supplied to the cell by the extracellular medium in the time unit.

Note that, a fraction of the EV-FP bonds may disassociate, with a rate constant  $b_u$ , before the fusion activation. Therefore, not all the bound EVs evolve into fused ones and the variable  $V_f$  counts the EVs whose fusion is successfully completed.

A simple scheme of the EV fusion is illustrated in Fig. 15.

In order to model the evolution of the fusion process, we first focus on the time-dependent concentrations of the available, bound and post-fusion FPs on the cell surface,  $F_a(t)$ ,  $F_b(t)$  and  $F_f(t)$ , respectively, and consider the events that affect their temporal variation. In particular, let us note that the binding of an EV to an available FPs produces a decrease in the concentration of available FPs,  $F_a(t)$ , and a corresponding increase in the concentration of bound ones,  $F_b(t)$ . The contribution of this event follows by the application of the law of mass action to the reaction (48). Similarly, the fusion of a negative contribution in the temporal variation of the bound FPs,  $F_b(t)$ , and a positive contribution to the temporal variation of the post-fusion FPs,  $F_f(t)$ .

Besides the binding and fusion events, also the recycling processes of the FPs affect the temporal variation of their concentration and need to be considered [133, 134]. More specifically, it is expected that after the fusion of the EVs is completed, a fraction of the post-fusion proteins becomes again available for binding new EVs, while at the same time old FPs are degraded and new available FPs are produced by the cell. Accordingly, a negative contribution is given to the temporal variation of the post-fusion protein concentration  $F_f(t)$  by the recycled proteins with the rate constants  $b_d$ . Likewise, the recycled proteins produces a positive contribution in the temporal variation of available proteins concentration  $F_a(t)$ , whereas the destroyed proteins produce a negative contribution to  $F_a(t)$  with a constant rate  $b_x$ . Additionally, let  $k_F(t)$  be the production rate of new FPs, i.e. the concentration of new synthesized FPs pairs per time unit, which contributes positively to the concentration  $F_a(t)$ .

Finally, the possibility that a fraction of the EV-FP bonds may disassociate before the fusion process is activated, produces, with the rate constant  $b_u$ , an increase of the concentration of available FPs,  $F_a(t)$ , and a decrease of the bound ones,  $F_b(t)$ .

All the above considerations lead to the following equations:

$$\frac{dF_a(t)}{dt} = k_F(t) - b_x F_a(t) + b_d F_f(t) + b_u F_b(t) - a_b F_a(t) V_e(t)$$
(50)

$$\frac{dF_b(t)}{dt} = a_b F_a(t) V_e(t) - (b_f + b_u) F_b(t)$$
(51)

$$\frac{dF_f(t)}{dt} = b_f F_b(t) - b_d F_f(t)$$
(52)

With similar considerations, let us now focus on the temporal evolution of the concentrations of EVs in the extracellular space close to the target cell  $V_e(t)$ , bound to the FPs  $V_b(t)$ , and fused to the plasma membrane  $V_f(t)$ . By applying the law of mass action to reactions (49), we obtain the following equations:

$$\frac{dV_{e}(t)}{dt} = k_{V}(t) + b_{u}V_{b}(t) - a_{b}F_{a}(t)V_{e}(t)$$
(53)

$$\frac{dV_b(t)}{dt} = a_b F_a(t) V_e(t) - (b_f + b_u) V_b(t)$$
(54)

$$\frac{dV_f\left(t\right)}{dt} = b_f V_b\left(t\right) \tag{55}$$

The set of equations (50)-(55) constitute the system of ordinary differential equations (ODEs) that models the protein-mediated fusion process of EVs to the plasma membrane of a target cell.

## 8.3.7 Initial conditions

In the model presented so far, the variables are the EV and FP concentrations in different phases of the process, and the coefficients are the parameters regulating the processes, i.e. the rates of the chemical reactions involved in EV binding and fusion, as well as in the recycling/regeneration process of the FPs. However, as already said, poor information is available in literature about those parameters. On the contrary, through specific biological experiments, the concentration of the external, bound and fused EVs can be measured over the time, i.e.  $V_e(t)$ ,  $V_b(t)$  and  $V_f(t)$  can be known. Then, a change of perspective can be applied to the model, so that the Vs are no more unknown variables, and instead the model parameters, together with the concentration of FPs, are the unknowns in the equations (50)-(55). Therefore, we may exploit the model to infer, through a mathematical reverse process, some reasonable values for the model parameters.

Unfortunaltely, to the current state of the art, the experimental measures needed for the above approach are challenging to achieve. In fact, to selectively measure the concentration of the EVs internalized through fusion, the *in vitro* selection or inhibition of specific uptake mechanisms is required. However, technical limitations currently hinder the possibility to efficiently achieve this target. More specifically, the EV-cell interactions involve mechanisms which are physiologically used by cells also for the internalization of other molecules. Therefore the inhibition of some uptake mechanism interferes with the other cellular functions, affecting the normal physiology of the cells, even compromising the cell survival. For these reasons, in this work, we provide a frame of reference for future comparisons between numerical solutions and biological experimental measures, by considering the conditions of common feasible biological experiments, as described in [62], to numerically solve the ODE system (50)-(55).

To this purpose, let us consider the case of a usual *in vitro* experiment where a single dose of EVs is supplied to the target cell at the beginning of the experiments and no other EVs are supplied successively, in the time interval under examination, i.e.:

$$k_V(t) = 0 \tag{56}$$

Moreover we can assume that, in the time interval under examination, the cell dynamics regulating the regeneration of proteins do not introduce, over time, great variations of the concentration of proteins, that is, an equilibrium between old degraded and new produced proteins is maintained. Therefore, in the following we will consider numerical solutions of the ODE system in the case:

$$b_x = 0$$
  

$$k_F(t) = 0$$
(57)

Typical initial conditions, at the time instant  $t_0$  (i.e. at the beginning of the observation of the bio-lab experiment), correspond to a situation where the cells have not yet received, i.e. not bound nor internalized, EVs. Therefore, the initial concentration of bound EVs and proteins, as well as fused EVs and post-fused FPs are equal to zero, while the initial concentration of the external EVs and available FPs on the plasma membrane of the target cell is indicated as  $V_0$  and  $F_0$ , respectively. This situation is summarized as follows:

$$\begin{cases} V_e(t_0) = V_0 \\ V_b(t_0) = 0 \\ V_f(t_0) = 0 \\ F_a(t_0) = F_0 \\ F_b(t_0) = 0 \\ F_f(t_0) = 0 \end{cases}$$
(58)

where the initial concentrations  $V_0$  of external EVs is assigned during the biological experiment planning, whereas the initial concentration  $F_0$  of FPs is unknown. Therefore, in order to make the numerical results independent from the initial concentration of proteins, let us normalize the concentration of all Fs and Vs to the initial concentration of available proteins  $F_0$ . So doing the solutions of the ODE system in (50)-(55), can be analyzed in terms of the ratio  $\frac{V_0}{F_0}$ .

To the purpose, let us denote as

$$S(t) = (F_a(t), F_b(t), F_f(t), V_e(t), V_b(t), V_f(t))$$
(59)

the generic solution of the ODE system in (50)-(55), and with calligraphic letters the normalization of (59) with respect to the concentration of proteins  $F_0$ , i.e.:

$$\mathcal{S}(t) = (\mathcal{F}_a(t), \mathcal{F}_b(t), \mathcal{F}_f(t), \mathcal{V}_e(t), \mathcal{V}_b(t), \mathcal{V}_f(t)) = = \left(\frac{F_a(t)}{F_0}, \frac{F_b(t)}{F_0}, \frac{F_f(t)}{F_0}, \frac{V_e(t)}{F_0}, \frac{V_b(t)}{F_0}, \frac{V_f(t)}{F_0}\right)$$
(60)

Note that, due to the presence in the system (50)-(55) of the non-linear term  $F_a V_e$ , the normalization  $\mathcal{S}(t)$  of the initial condition (58) is solution of the ODE system as long as we redefine, under the considered assumptions,  $a_b$  as  $\alpha_b$ , as follows:

$$\alpha_b = a_b F_0 \tag{61}$$

The equivalent initial conditions, in this case are:

$$\begin{aligned}
\mathcal{V}_e(t_0) &= \mathcal{V}_0 \\
\mathcal{V}_b(t_0) &= 0 \\
\mathcal{V}_f(t_0) &= 0 \\
\mathcal{F}_a(t_0) &= \mathcal{F}_0 \\
\mathcal{F}_b(t_0) &= 0 \\
\mathcal{F}_f(t_0) &= 0
\end{aligned}$$
(62)

where:

$$\mathcal{V}_0 = \frac{V_0}{F_0}$$

$$\mathcal{F}_0 = \frac{F_0}{F_0} = 1$$
(63)

#### 8.3.8 Analysis of the ODE solutions

In this section, we will discuss the numerical results of the ODE system, in a time interval of 72 hours, for different orders of magnitude of the model parameters. More specifically, in Section 8.3.9 we will investigate the effects of the model parameters on the time evolution of the fusion process. In particular, we will focus on the *basic* fusion process, without considering possible EV-FP bond disassociation, i.e. for  $b_u = 0$ . The impact of  $b_u \neq$ 0 will be examined in Section 8.3.10. In Section 8.3.11, we will formally define and evaluate the internalization rate for a wide range of values of relevant parameters. Finally in Section 8.3.12, we will provide a discussion regarding what are most likely parameter values associated to the measured internalization rate.

For the sake of simplicity, in the following, we will refer to the parameter values without specifying the measurement units, which are meant to be as specified in Table 3.



Table 3: Unit of measurement of model parameters

Description	Parameter	Unit of measurement
Binding rate constant	$a_b$	$\mathrm{ml} \cdot \mathrm{mol}^{-1} \cdot \mathrm{h}^{-1}$
Fusion rate constant	$b_f$	$h^{-1}$
Bond dissociation rate constant	$b_u$	$h^{-1}$
FPs recycling rate constant	$b_d$	$h^{-1}$
FPs destroyed rate constant (post bond disassociation)	$b_x$	$h^{-1}$



Figure 17: Concentration of FPs and EVs for different values of parameters. In those graphs the parameter  $b_u$  remain 0, while others parameters change from 0.1 to 1000.

# 8.3.9 Temporal evolution of FP and EV concentrations

First, let us consider the effects of the parameters  $\alpha_b$ ,  $b_f$  and  $b_d$ , regulating the *basic* fusion process, without considering possible EV-FP bond disassociation, i.e.  $b_u = 0$ . In Fig. 17, the time evolution of the concentration of FPs and EVs is shown, in a time interval of 72 hours, for different values of the above mentioned model parameters<sup>3</sup>.

Let us initially focus on Fig. 17c, where the temporal evolution of the concentrations  $\mathcal{F}_a(t)$ ,  $\mathcal{F}_b(t)$  and  $\mathcal{F}_f(t)$ , for different values of the binding rate constant  $\alpha_b$ , are shown, in a time interval of 72 hours, when the fusion rate constant  $b_f$  is equal to 10 and FPs recycling rate constant  $b_d$  is 1000. In the small box inside the figure, the 18 minutes after the time instant t = 0 is analyzed more in details. As shown in these figures, the concentration  $\mathcal{F}_a$  of available FPs is, at the time instant t = 0, equal to 1, which represents the total concentration of proteins (see (62) and (63)). Soon after, it rapidly decreases, while the concentration of bound FPs  $\mathcal{F}_b$  increases complementary

 $<sup>^{3}</sup>$ In Fig. 17, some curves stop earlier than 72 hours. This happens because, for computational reasons, the calculation of the ODE system solutions automatically stops once the concentration of external EV becomes zero.



Figure 18: Concentration of  $\mathcal{V}_e(t)$  for different values of parameters.

to  $\mathcal{F}_a$ , meaning that the bond between FPs and EVs is taking place. As expected, for lower values of the binding rate constant  $\alpha_b$  (e.g.  $\alpha_b = 0.1$ , solid lines in Fig. 17c), the concentration  $\mathcal{F}_a$  decreases until it reaches about half of the initial concentration of proteins, which is a relatively large value in comparison to the concentration obtained for greater values of  $\alpha_b$ , (e.g.  $\alpha_b = 100$ , dotted lines in Fig. 17c), which instead is close to zero. In fact, low binding rate constants imply (for equal concentrations of reactants) large time intervals where the FPs remain available, waiting for a successful EV-FP bond. On the contrary, the larger the binding rate constant  $\alpha_b$ , the shorter the time period the FPs remain available. Therefore, for low binding rate constants, the average over time of the concentration  $\mathcal{F}_a$  of available proteins is larger, with respect to the case of high binding rate constants.

After this first rapid decrease, the concentration  $\mathcal{F}_a$  increases. The slope of such an increment is sensibly dependent on the value of the binding rate constant  $\alpha_b$ . More specifically, for high values of  $\alpha_b$ , the concentration  $\mathcal{F}_a$  increases slowly, for about the first 10 hours, to steeply increase and stabilize to the initial concentration of FPs. This time interval before the concentration of  $\mathcal{F}_a$  stabilizes to the initial concentration of FPs, corresponds to the internalization period, i.e., the duration of the whole internalization process, as shown also in Fig. 17d, where the concentrations  $\mathcal{V}_e$  and  $\mathcal{V}_f$  of external and fused EVs, respectively, are drawn. Indeed, when  $\mathcal{F}_a$  stabilizes around 1 (e.g., about 10 hours for high values of  $\alpha_b$ ), the concentration  $\mathcal{V}_e$  of external EVs to be internalized is close to zero, while the concentration  $\mathcal{V}_f$  of fused EVs is close to the initial concentration of EVs.

Let us now compare Fig. 17e to Fig. 17c, where only the recycling rate constant  $b_d$  changes, i.e., it decreases by one order of magnitude. As shown in the figures, the internalization period does not change appreciably. In this time interval, the concentration of  $\mathcal{F}_f$  appears to be greater when  $b_d = 100$ than when  $b_d = 1000$ . This happens because a slower recycling rate constant, introduces a recycling delay, which means that the proteins, that completed successfully the EV fusion, need more time to become again available for binding new EVs. Again, a longer permanence in the fused state, corresponds to a larger average over time of concentration of fused proteins. As a counter part, since the total concentration of proteins is assumed to be constant in our analysis, such an increase of fused proteins should correspond to a decrease of available and bound proteins. However, as shown in the figures, the concentration  $\mathcal{F}_a$  does not show appreciable variations with respect to the previous case, especially for high values of the binding rate constant  $\alpha_b$ , because the new available proteins are soon involved in new EV-FP bonds, so maintaining  $\mathcal{F}_a$  low. Therefore, the increase of  $\mathcal{F}_f$  mainly produces a decrease of the average concentration  $\mathcal{F}_b$  of bound FPs. A comparison between Fig. 17d and Fig. 17f shows that the concentrations  $\mathcal{V}_e$  and  $\mathcal{V}_f$  of external and fused EVs, respectively, are not affected by  $b_d$  variations significantly.

When  $b_d$  is further reduced to 10, as in Fig. 17g and Fig. 17h, the recycling delay, introduced before the fused proteins become again available, has a higher impact on both  $\mathcal{F}_a$  and  $\mathcal{F}_b$ . More specifically, the concentration of  $\mathcal{F}_b$  decreases significantly as  $\mathcal{F}_f$  increases, whereas the concentration of  $\mathcal{F}_a$  remains quite as low as in the previous considered cases. However, the internalization time is almost doubled, with respect to previous analyzed cases.

Let us now maintain  $b_d$  constant and consider an increment of  $b_f$ , corresponding to a faster fusion process. By comparing Fig. 17g and Fig. 17h to

Fig. 17i and Fig. 17j, the concentration  $\mathcal{F}_b$  reduces while the concentration  $\mathcal{F}_f$  increases, as  $b_f$  grows. The concentration of  $\mathcal{F}_a$  is not noticeably affected by the variation of  $b_f$  during the internalization time, but the internalization time decreases and the slopes of  $\mathcal{V}_e$  and  $\mathcal{V}_f$  increase, accordingly. The same trend appears by further increasing  $b_f$ , as shown in Fig. 17k and Fig. 17l. Note that increasing  $b_f$  and reducing  $b_d$ , symmetrically with respect to the case  $b_d = b_f$ , produces analogous results as of  $\mathcal{F}_a$ ,  $\mathcal{V}_e$  and  $\mathcal{V}_f$ , while swapping the trends of  $\mathcal{F}_b$  and  $\mathcal{F}_f$ , that is, the distribution among bound and fused protein  $\mathcal{F}_b$  and  $\mathcal{F}_f$  changes in favor of the first or the second, depending on whether the ratio  $\frac{b_f}{b_d}$  decreases or increases.

# 8.3.10 Impact of model parameters on the concentration of internalized EVs

In this section we focus on the concentration  $\mathcal{V}_e$ . Let us explain the reason for this choice. The variable that is usually most accessible to measure in bio-labs experiments is the sum of the EVs internalized (i.e. fused EVs  $\mathcal{V}_f$ ) and still attached to the cell membrane (i.e. bounds EVs  $\mathcal{V}_b$ ). Moreover, an interesting metric for the future applications of this study is the internalization rate, which may be inferred, again, by the analysis of  $\mathcal{V}_b + \mathcal{V}_f$ . Since we are considering the case where no additional EVs are supplied to the cells in the time interval under examination, the total concentration of EVs (i.e. the sum of external, bound, and fused EVs,  $\mathcal{V}_e(t)$ ,  $\mathcal{V}_b(t)$ , and  $\mathcal{V}_f(t)$ respectively) is constant and equal to the initial concentration  $\mathcal{V}_0$  of external EVs. Threfore, the sum of bound and fused EVs,  $\mathcal{V}_b + \mathcal{V}_f$  is complementary to the concentration  $\mathcal{V}_e$  of external EVs, with respect to  $\mathcal{V}_0$ . So, for the sake of simplicity, we will focus on a single variable  $\mathcal{V}_e$ , instead of the sum  $\mathcal{V}_b + \mathcal{V}_f$ , keeping in mind that the results apply to them equivalently.

In Section 8.3.9 we have already glimpsed how the model parameters  $\alpha_b$ ,  $b_f$  and  $b_d$  influence the evolution of  $\mathcal{V}_e$ . More specifically, it is possible to infer that increasing values of the binding rate constant  $\alpha_b$ , produce an increase of the slope of  $\mathcal{V}_e$  and reduce the internalization period. The same trend appears by increasing  $b_f$  and/or  $b_d$ . Let us now, analyze the impact of the EV-FP bond disassociation rate constant  $b_u$ . This parameter measures the possibility that the bonds between EVs and FPs disassociate before the fusion takes place. When this event occurs, the FPs involved in the bond move from the state *bound* to the state *available*, and the same occurs for the EVs. Therefore, intuitively we expect that increasing the values of  $b_u$  for given

binding rate constants  $\alpha_b$  produces the same effects of lower binding rate constants with  $b_u = 0$ . This intuition is confirmed by the analysis of Fig. 18, where it can be seen how the slope of  $\mathcal{V}_e$  reduces and the internalization time increases, as  $b_u$  grows.

### 8.3.11 Internalization Rate

In this section, let us focus on the internalization rate, which may be defined as the slope of  $\mathcal{V}_e$ . Since  $\mathcal{V}_e$  shows in general a non linear decrease, its slope changes according to the time instant when we measure it. Therefore, let us define the *average internalization rate at 95%*,  $r_{95}$ , as follows:

$$r_{95\%} = \frac{0.95\mathcal{V}_0}{t_{95\%}} \tag{64}$$

where  $t_{95}$  is the time instant where the 95% of the initial external EVs have been internalized.

Figs. 19 shows the internalization rate  $r_{95}$  for different values of the fusion parameters  $\alpha_b$ ,  $b_f$ ,  $b_d$ ,  $b_u$  and defined as in (63).

As expected, the figures show that the internalization rate increases when the binding rate  $\alpha_b$  increases, and decreases for increasing values of the bond disassociation rate,  $b_u$ . The impact of  $b_u$  is however lighter. As far as the fusion rate constant,  $b_f$ , and the recycling rate constant,  $b_d$ , are concerned, the internalization rate increases as both parameters assume higher values. The analysis of the cases where the initial concentration ratio is  $\mathcal{V}_0 = \{100, 1000, 10000\}$ , has confirmed the same results.

# 8.3.12 Parameter inference

In this section we elaborate on the most likely EV fusion process parameter values associated to an internalization rate measured in a bio-lab experiment, in the assumption of uniform distribution of the parameter values.

In particular Fig. 20 shows, for each parameter, the likelihood distribution of the parameter values for given order of magnitude of the measured internalization rate. In Fig. 20a we can infer that if the measured internalization rates is in the order of  $10^{-1}$ , than the values of  $\alpha_b$  is in the order of  $10^{-1}$  with high likelihood. As increasing values of the internalization rate are measured in a bio-lab experiment, the highest likelihood of the  $\alpha_b$  parameter



moves toward greater values. However, in these cases, the likelihood distribution of the  $\alpha_b$  parameter values tends to flatten out. Thus, the inference of the parameter values given the internalization becomes more uncertain.

From Fig. 20b and Fig. 20c we can infer with high likelihood the values of the parameters  $b_f$  or  $b_d$  for each given order of magnitude of the measured internalization rate. More specifically, increasing values of the internalization rate correspond to increasing values of  $b_f$  or  $b_d$  parameters.

Fig. 20d shows that the parameter  $b_u$  has a lower impact on the internalization rate, since the likelihood distribution appears to be uniform for each value of the internalization rate. Therefore, the uncertainty about the value of such parameter remains high.



(a) Distribution of the model parameter  $\alpha_b$  given the Internalization Rate





(b) Distribution of the model parameter  $b_f$  given the Internalization Rate (d) Distribution of the model parameter  $b_u$  given the Internalization Rate



(c) Distribution of the model parameter  $b_d$  given the (e) Distribution of the model parameter  $\mathcal{V}_0$  given the Internalization Rate

Figure 20: Numerical results. Likelihood of the model parameter values given the Internalization Rate.

Finally, Fig. 20e shows the likelihood distribution of the ratio  $\mathcal{V}_0$  for each given order of magnitude of the measured internalization rate. This information is useful to infer information about the initial concentration  $F_0$ of FPs. In fact, as mentioned in 8.3.7, the initial concentration  $V_0$  of EVs is assigned during biological experiment planning. Therefore, through (63), the most likely values of the ratio  $\mathcal{V}_0$  can be easily translated in terms of the initial concentration  $F_0$  of proteins. The results in Fig. 20e show that the measured internalization rates in the order of  $10^{-1}$  are with high likelihood achieved for  $\mathcal{V}_0$  in the order of 10, as well as internalization rates in the order of  $10^3$  are with high likelihood achieved for  $\mathcal{V}_0$  in the order of  $10^5$ . However, for intermediate values of the internalization rates, the inference of  $\mathcal{V}_0$  values is performed with great uncertainty.

A practical application of this approach will be shown in the next paragraph.

### 8.3.13 An Analytical Model for the generic internalization of EV

Molecular mechanisms involved in the uptake of EVs are not fully understood. Ideally, for understanding the whole process, it would be necessary to work at a single EV (even single molecule) resolution, over a dense set of time points [139]. However, this remains a technical challenge for the field, considering that it is possible to visualize and to estimate the internalization, but without any knowledge of the number or concentration of internalized EVs and their relationship with the effects observed after the treatment. Furthermore, the experiments carried out at different time points, require multiple replicates, since for each measurement the experiment need to be stopped for the analysis. For example, in the set of experiments carried out to generate the model describing the dynamics of EV internalization, target cells were seeded in several wells of 12-well plates, with 3 wells representing a single condition. Therefore, to produce different conditions at different time points, multiple wells were provided. At the end of the treatment, to perform the measurement of EV-derived fluorescence by the imaging flow cytometry, the cells were detached from the well, washed of their medium to eliminate both cellular debris and residual EVs, and then resuspended in a specific buffer to be finally analyzed at single cell level. All passages are repeated for every time point. As expected, this kind of experiments is, in general, very expensive (in particular in terms of EV production, other than for instruments' costs) and time-consuming, and it is hardly affordable to repeat

them for a dense set of time points. For these reasons, a good design of the experiments, based on the estimation of the expected results, is crucial to avoid waste of resources.

Several models of EV uptake are present in literature [138]. The uptake models developed so far are specific of some particular mechanisms and would be useful on the design of selective uptake experiments. Unfortunately, considering that the internalization mechanisms are used by cells, not only for the EV uptake, but also for internalization of other molecules and for the physiological recycle of membranes, the inhibition or selection of specific uptake pathways may be toxic for the cells and the development of efficient pharmacological treatments is challenging.

Now, we introduce an analytical model of a generic uptake process of EVs by a target cell, the model parameters, which correspond to the rate of chemical reactions involved in the absorption process, are not known. For this reason, based on laboratory data, an estimation of the unknown parameters is performed and used to design future experiments so reducing costs and time. More specifically, the model solutions describing the evolution of the process are compared with experimental data collected *in vitro* from a neuroblastoma cell line (SH-SY5Y) treated with EVs isolated from primary murine AS. From these experiments the values of the parameters associated with the uptake of EVs by SH-SY5Y are deduced. Then, the use of the model as a tool for the design of new biological experiments is assessed by comparing the EV internalization forecasted by the model with experimental data obtained in the same conditions.

# 8.3.14 Uptake Model

In this work we focus on a generic EV uptake process, which develops on two phases: *i*)the EVs in the extracellular medium are supposed to bind with some generic receptors, *ii*) they are internalized by the target cell. Let  $V_e$ indicate the number of available EVs in the extracellular space close to the plasma membrane of the target cell, and let  $V_b$  and  $V_i$  indicate the bound and internalized EVs, respectively. The dynamics of the binding and internalization processes are assumed to be regulated by the binding and internalization rate constants, denoted as  $r_b$  and  $r_i$ , respectively. Note that, a fraction of the EV-cell bonds may disassociate before the internalization is activated and the involved EVs come back to the extracellular medium. To take into account this occurrence, the bond disassociation rate constant  $r_d$  has been introduced. The evolution of the EV uptake can be summarized by the following simplified reaction scheme:  $r_{b_{i-1}} r_{i_i}$ 

$$\{extracellular medium\} \xrightarrow{g_V} V_e \xleftarrow{r_d}{r_d} V_i$$

$$(65)$$

where  $g_V$  is the supply rate of EVs, i.e. the number of EV supplied to the cell by external sources in the time unit, and  $r_r$  indicates the recycling rate constant, i.e. the rate at which the target cell may recycle or release a fraction of the internalized EVs.

In order to model the evolution of the EV uptake process, we first focus on the time-dependent number of the available, bound and internalized EVs,  $V_e(t), V_b(t)$  and  $V_i(t)$ , respectively, and consider the events that affect their temporal variation. In particular, let us note that the binding of an EV to the target cell produces a decrease in the number of available EVs,  $V_e(t)$ , and a corresponding increase in the number of bound ones,  $V_b(t)$ . Similarly, the internalization of a bound EV, at the rate  $r_i$ , accounts for a negative contribution in the temporal variation of the bound EVs,  $V_b(t)$ , and a positive contribution to the temporal variation of the internalized ones,  $V_i(t)$ . Besides the binding and internalization events, also the recycling processes of EVs, as well as the possibility that a fraction of the EV-cell bonds disassociates before the fusion process is activated, affect the EV amount in the three considered states. More specifically, both events produce, at the rate  $r_r$  and  $r_i$ , respectively, an increase of the number of available EVs,  $V_e(t)$ , and a decrease of the internalized and bound ones,  $V_i(t)$  and  $V_b(t)$ , respectively. All the above considerations lead to the following equations:

$$\begin{cases} \frac{dV_{e}(t)}{dt} = -r_{b}V_{e}(t) + r_{d}V_{b}(t) + r_{r}V_{i}(t) + g_{V}(t) \\ \frac{dV_{b}(t)}{dt} = r_{b}V_{e}(t) - (r_{d} + r_{i})V_{b}(t) \\ \frac{dV_{i}(t)}{dt} = r_{i}V_{b}(t) - r_{r}V_{i}(t) \end{cases}$$
(66)

Note that the number of EVs in the extracellular medium may not be spatially uniform. However, the fluid dynamics of EVs delivery to the cell surface [12] is out the scope of this work, since we are here interested on the dynamics of binding and internalization of the EVs to the plasma membrane. For this reason, we assume that there is a layer of fluid close to the plasma membrane of the target cell, where the number of the EVs can be considered spatially uniform and we define  $V_e$  as the number of EVs in this portion of extracellular fluid. Therefore, we can consider here the solely dependance of  $V_e$  from time. The number of EVs, close to the plasma membrane of the target cell, is supplied to the system from an external source at the delivery rate  $g_V(t)$ , which in general is a function of time.

#### 8.3.15 Model solution

The set of equations ordinary differential equations (66) constitutes the model of the binding and internalization process of EVs by a target cell. In this section we provide the solution of this system, which is linear and solvable with standard methods.

As usual, we need to find three linearly independent solution of the homogeneous system associated to (66), i.e. for  $g_V(t) = 0$ . So let us look for solutions in the form:

$$\mathbf{V} = e^{\lambda t} \mathbf{v} \tag{67}$$

where  $\lambda$  and  $\mathbf{v}$  are the eigenvalues and eigenvectors, respectively, of the coefficient matrix  $\mathbf{A}$  of (66), and  $\mathbf{V}$  is the vectorial notation for the number of external, bound and internalized EVs, i.e.:

$$\mathbf{V} = \begin{bmatrix} V_e \\ V_b \\ V_i \end{bmatrix} \quad \mathbf{A} = \begin{bmatrix} -r_b & r_d & r_r \\ r_b & -(r_d + r_i) & 0 \\ 0 & r_i & -r_r \end{bmatrix}$$
(68)

As known, the eigenvalues  $\lambda$  are the roots of the characteristic equation of the matrix **A**, i.e.  $|\mathbf{A} - \lambda \mathbf{I}| = 0$  (where  $|\cdot|$ , when applied to a matrix, denotes its determinant, and **I** is the identity matrix), which is easy to verify that can be written as follows:

$$\lambda \left( \lambda^{2} + (r_{b} + r_{i} + r_{d} + r_{r}) \lambda + r_{r} (r_{d} + r_{i}) + r_{b} (r_{r} + r_{i}) \right) = 0$$
 (69)

The roots of (69) are:

$$\lambda_1 = 0 \lambda_{2,3} = -\frac{1}{2} \left( r_b + r_i + r_d + r_r \right) \pm \frac{1}{2} \sqrt{\Delta}$$
(70)

where

$$\Delta = (r_b + r_i + r_d + r_r)^2 - 4 \left( r_r \left( r_d + r_i \right) + r_b \left( r_r + r_i \right) \right)$$
(71)

The eigenvector  $\mathbf{v}_i$  associated to the *i*-th eigenvalue  $\lambda_i$  must satisfy the following equation:

$$\mathbf{A}\mathbf{v}_i = \lambda_i \mathbf{v}_i \tag{72}$$

However, when  $\Delta = 0$ , the characteristic equation (69) presents a double root, i.e.  $\lambda_2 = \lambda_3$ , which means the eigenvalue is incomplete or defective, and a third linearly independent solution can be found in the form:

$$\mathbf{V} = e^{\lambda_2 t} \left( t \mathbf{v}_2 + \mathbf{v}_3 \right) \tag{73}$$

where  $\mathbf{v}_2$  is found from (72), and  $\mathbf{v}_3$  is found from:

$$\left(\mathbf{A} - \lambda_2 \mathbf{I}\right) \mathbf{v}_3 = \mathbf{v}_2 \tag{74}$$

After some calculation, the eigenvectors associated to the eigenvalues in (70) are:

$$\mathbf{v}_{1} = \begin{bmatrix} r_{r} (r_{d} + ri) \\ r_{b}r_{r} \\ r_{i}r_{b} \end{bmatrix} \mathbf{v}_{2} = \begin{bmatrix} (\lambda_{2} + r_{r}) (\lambda_{2} + r_{d} + r_{i}) \\ r_{b} (\lambda_{2} + r_{r}) \\ r_{i}r_{b} \end{bmatrix} \\ \mathbf{v}_{3} = \begin{cases} \begin{bmatrix} (\lambda_{3} + r_{r}) (\lambda_{3} + r_{d} + r_{i}) \\ r_{b} (\lambda_{3} + r_{r}) \\ r_{i}r_{b} \end{bmatrix} & \text{if } \Delta \neq 0 \\ \begin{bmatrix} 2\lambda_{2} + r_{d} + r_{i} + r_{r} \\ r_{b} \\ 0 \end{bmatrix} & \text{if } \Delta = 0 \end{cases}$$
(75)

The general solution of the system (66) is the linear combination of the solution (67) and (73) with the eigenvalues and eigenvectors found so far, and can be summarized, according to the sign of  $\Delta$ , as follows:

$$\mathbf{V} = \begin{cases} k_1 \mathbf{v}_1 + k_2 e^{\lambda_2 t} \mathbf{v}_2 + k_3 e^{\lambda_3 t} \mathbf{v}_3 & \text{if } \Delta > 0\\ k_1 \mathbf{v}_1 + e^{\lambda_2 t} \left( k_2 \mathbf{v}_2 + k_3 \mathbf{v}_3 + k_3 \mathbf{v}_2 t \right) & \text{if } \Delta = 0\\ k_1 \mathbf{v}_1 + e^{\mathcal{R}[\lambda_2]t} \left( \cos\left(\mathcal{I}[\lambda_2]t\right) \left( k_2 \mathcal{R}[\mathbf{v}_2] + k_3 \mathcal{I}[\mathbf{v}_2] \right) + \\ + \sin\left(\mathcal{I}[\lambda_2]t\right) \left( k_2 \mathcal{I}[\mathbf{v}_2] + k_3 \mathcal{R}[\mathbf{v}_2] \right) \right) \\ & \text{if } \Delta < 0 \end{cases}$$
(76)

where  $\mathcal{R}[\cdot]$  and  $\mathcal{I}[\cdot]$  indicate real and imaginary part of a complex number, respectively, and  $k_1$ ,  $k_2$ , and  $k_3$  are constants to be find according to the initial conditions.

Note that the solution found so far is the solution of the homogenous system associated to (66). To find the complete solution of the system, the term  $g_V(t)$  has to be considered. However, the model solution will be compared with experimental results obtained in the case  $g_V(t) = 0$ ,  $\forall t$ . For this reason, the computation of the general solution of (66), with  $g_V(t) \neq 0$ , is out the scope of this work.

### 8.3.16 Experimental case study

In this section, we describe the biological experiments for the measurement of the EV uptake evolution.

The number of EVs obtained were  $2.73 \cdot 10^9$  per million of AS (the donor cells), as measured by Nanoparticle Tracking Analysis [140]. EVs were labelled with the very stable (about 100 days) CellVue Claret Fluorescent Cell Linker Dye (Merck), using the protocol described in [140]. This dye is excited by a laser at a wavelength of 655 nm and emits at a wavelength of 675 nm corresponding to the far-red spectrum [141].

The target cells are SH-SY5Y, differentiated into dopaminergic-like neurons with retinoic acid and serum deprivation [140]. SH-SY5Y cells were seeded in a 12 well plate, in an amount of  $1 \cdot 10^6$  cells per well. 24 hours after seeding, target cells were treated with EVs using two ratios 5:1 and 0.5:1, that correspond to the EVs produced by 5 or 0.5 donor AS, respectively, and given to 1 target cell. In total,  $1.37 \cdot 10^{10}$  EVs were used for the 5:1 ratio, while for the 0.5:1 ratio  $1.37 \cdot 10^9$  EVs. In the following we refer to this two experiment conditions as Exp1 and Exp2 for the ratio 5:1 and 0.5:1, respectively. The time of EV treatment represent the time  $t_0$ . Then cells were left with EVs for 6, 24, 48 and 72h, to evaluate EV uptake over time. Target cells at each time points were detached by trypsinization, washed of their medium and cellular debris, and resuspended in 1 mm EDTA + 1% BSA [140].

The EV uptake by the target cells was detected with the imaging flow cytometer (IFC, Amnis FlowSight), using the IDEAS [142] software [143]. This approach allows to evaluate the amount of EV internalization at single cell level. The first 2000 single cells, in order of acquisition, were analyzed, and the data were expressed in terms of mean fluorescence intensity. In order to convert such a measure to number of internalized EVs, the fluorescence of a single EVs should be known. However, the Amnis FlowSight magnification does not allow the detection of single EV fluorescence, neither within the cells, nor as a sample to be directly measured by the instrument before the treatment on target cells. Therefore, a parallel set of experiments was carried out seeding cells in 96-well plates, in an amount of 85.000 cells per well. The treatment with EVs was performed following the same strategy as for the IFC analysis ( $1.16 * \cdot 10^9$  EVs for the 5:1 ratio, and  $1.16 \cdot 10^8$  EVs for the 0.5:1 ratio). The EV-derived fluorescence was measured by using a plate reader (PR, Varioskan, Thermo Fisher Scientific), with use of Skanlt<sup>TM</sup>

software [144]. To derive the fluorescence of the single EVs, the same amount of labelled vesicles has been measured with the PR in the 96-well plate, in the absence of cells. Note that the amount of EVs and cells in the 96 well plate differs from the ones in the 12 well plate (although a normalization per area/well has been used) for the analysis through the flow cytometer, while the ratio between EVs and cells is kept constant. Notably, the exclusive use of PR for the measurement of EV derived fluorescence in target cells is not possible since this instrument does not allow the analysis at single cell level. On the contrary, only the fluorescence of the whole well (including both cells and the medium) can be measured. For this reason, we used two different strategies to obtain the required unknown variables. Indeed, the measurements obtained through IFC and PR have been compared, and the fluorescence of a single EV (in the unit of measurement of the IFC) has been deduced through a simple proportion, in the assumption of linearity, as follows:

$$F(EV)_{IFC} = \frac{F(EV)_{PR} \cdot F(Cell + EVs)_{IFC}}{F(Cell + EVs)_{PR}}$$
(77)

where  $F(EV)_{IFC}$  and  $F(EV)_{PR}$  indicate the fluorescence intensity of a single EV for flow cytometer and plate reader, respectively, and  $F(Cell + EVs)_{IFC}$  and  $F(Cell + EVs)_{PR}$  indicate the fluorescence intensity of a single cell after EVs internalization for flow cytometer and plate reader, respectively.

By applying the results of (77) to the flow cytometer results, we obtain the temporal evolution of the number of EVs internalized by the target cells for two different initial conditions (namely the initial number of EVs the cells are treated with), above referred to as Exp1 and Exp2. The initial number of EVs per cell, in the following indicated as  $V_0$ , has been calculated according to the percentage of cells detected as positive by the imaging flow cytometer (i.e. the cells that present a non negligible level of internalization). The values obtained are  $V_0 = \{16000, 3400\}$ , for the case Exp1 and Exp2, respectively.

# 8.3.17 Model application

In 8.3.14 we have derived an analytical model describing the uptake process of EVs from a target cell. In this model, the variables are the EV in different phases of the process, and the coefficients are the parameters regulating the processes, i.e. the rates of the chemical reactions involved in EV binding and internalization, as well as in the disassociation/recycling process of the EVs. However, as already said, poor information is available in literature about those parameters. On the contrary, through the biological experiments, as described in 8.3.16, the concentration of the external, bound and internalized EVs, i.e. the model variables  $V_e(t)$ ,  $V_b(t)$  and  $V_i(t)$ , can be measured over the time. Then, a change of perspective can be applied to the model, so that the Vs are no more unknown variables, and instead the model parameters, are the unknowns in the equations (66). Therefore, we will exploit the model to infer, through a mathematical inverse process, the values for the model parameters that apply to the case under study, i.e. the administration of EVs from murine AS to the target cells SH-SY5Y.

With this in mind, in this section we will first consider, in 8.3.18, which model assumption are needed, to make the model representative of the experimental conditions. Then in 8.3.19, we will compare the analytical solution of the model with experimental results, to infer the model parameter that apply for the case under study. Finally, in 8.3.20 we will use the model with the parameter values found in 8.3.19, to provide a forecasting of the results of a second experiment, and to demonstrate that the model could also be used for the design of new experiment, based on desired results.

#### 8.3.18 From the model to lab experiments

In this section, we will consider the model assumptions that makes the model representative of the experiments described in 8.3.16.

Let us first focus on the initial condition of the experiment. In particular, let us note that the labelled EVs are placed all at once in the well at the beginning of the experiment, while the cells have not yet either bound or internalized EVs. In terms of the variable of the model, this means that at the initial time  $t_0 = 0$ , we can write:

$$V_e(0) = V_0, \quad V_b(0) = 0, \quad V_i(0) = 0$$
(78)

where  $V_0$  indicates the initial amount of EVs per cell.

Moreover, no additional EVs are supplied during the duration of the experiment to the system under study. Therefore we can consider:

$$g_V(t) = 0 \quad \forall t \tag{79}$$

According to (78), the constants  $k_1$ ,  $k_2$  and  $k_3$  in (76), can now be calculated. Let us note, that we do not know yet which case we need to consider, according to the sign of  $\Delta$ . Nevertheless, as we will see later in Fig. 21b, the measurements of  $V_i$  do not show oscillating behaviour, which allows us to exclude, in our study, the case of  $\Delta < 0$ . Moreover, the  $\Delta = 0$  is a singular case, for which the parameters need to be fine-tuned, and this occurrence can be reasonably considered unlikely in real-world experiments and meaningless from the biological/physical point of view. Therefore, in the following, if not otherwise specified, we will consider only the case  $\Delta > 0$ .

From (76), together with (75) and (78), we obtain the following expressions for the constants  $k_1$ ,  $k_2$  and  $k_3$ :

$$k_1 = \frac{V_0}{\lambda_2 \lambda_3}, \quad k_2 = \frac{V_0}{\lambda_2 (\lambda_2 - \lambda_3)}, \quad k_3 = \frac{V_0}{\lambda_3 (\lambda_3 - \lambda_2)}$$
(80)

By replacing (80), in (76), we obtain the general solution of the model for the experiments described in 8.3.16.

### 8.3.19 Model parameter inference

In this section, we will exploit the model to infer, through a mathematical inverse process, the values for the model parameters that apply to the case under study, i.e. the administration of EVs from murine AS to the target cells SH-SY5Y.

To this purpose, let us consider the measurement of  $V_i$  from the experiment denoted as Exp2 in 8.3.16, and let us look for the best fitting of this curve. According to (76), the best fitting function has to be in the following form:

$$V_i = a + be^{\lambda_2 t} + ce^{\lambda_3 t} \tag{81}$$

with:

$$a = k_1 v_{31}, \quad b = k_2 v_{32}, \quad c = k_3 v_{33}$$
 (82)

where  $v_{ij}$  indicates the *i*-th component of the vector  $\mathbf{v}_j$  of (75). It is easy to verify that from (75), together with (80), the following constraints between the coefficients a, b, and c of (81) hold:

$$b = a \frac{\lambda_3}{\lambda_2 - \lambda_3}, \quad c = a \frac{\lambda_2}{\lambda_3 - \lambda_2}$$
 (83)

Therefore, according to the coefficient constraints in (83), the best fitting function for  $V_i$  has to be in the form:

$$V_i = a \left( 1 + \frac{\lambda_3}{\lambda_2 - \lambda_3} e^{\lambda_2 t} + \frac{\lambda_2}{\lambda_3 - \lambda_2} e^{\lambda_3 t} \right)$$
(84)

where a,  $\lambda_2$  and  $\lambda_3$  are to be determined by the fitting. Let us indicate as  $a_f$ ,  $\lambda_{f2}$  and  $\lambda_{f3}$ , the values obtained in this way. By replacing the above values

in (82), and combining them with (70), (71), (75) and (80), we obtain the following system of equations:

$$\begin{cases} V_0 \frac{r_b r_i}{\lambda_{f^2} \lambda_{f^3}} = a_f \\ \sqrt{\Delta} = |\lambda_{f3} - \lambda_{f2}| \\ -\frac{1}{2} \left( r_b + r_i + r_d + r_r \right) = \frac{1}{2} (\lambda_{f2} + \lambda_{f3}) \end{cases}$$
(85)

where  $|\cdot|$ , when applied to a number, denotes its absolute value.

Let us recall that the unknowns in this problem are the model parameters  $r_b$ ,  $r_d$ ,  $r_i$ , and  $r_r$ , that regulates the internalization process. Therefore, we need to solve a system of four independent equations in those unknowns. The system (85), provides three of the four needed equations. The last equation is obtained considering the measurement of the number of EVs in the extracellular medium at a given time-point. In terms of the variables of the model, the measurement of the EVs in the extracellular medium correspond to the variable  $V_e$ . By equating the measured value of  $V_e(t_p)$ , at the time point indicated here as  $t_p$ , to the analytical expression obtained from (76), together with (80), and solving the obtained equation together with (85), after some mathematical manipulation, we obtain the following formulas for the computation of the model parameters:

$$\begin{cases}
P = \frac{a_{f}\lambda_{f2}\lambda_{f3}}{V_{0}} \\
r_{r} = -\frac{1}{2}(\lambda_{f2} + \lambda_{f3}) \pm \frac{1}{2}\sqrt{4P + (\lambda_{f2} - \lambda_{f3})^{2}} \\
S = -(\lambda_{f2} + \lambda_{f3} + r_{r}) \\
T = \frac{V_{e}(t_{p}) - k_{2}\lambda_{f2}(\lambda_{f2} + r_{r})e^{\lambda_{f2}t} - k_{3}\lambda_{f3}(\lambda_{f3} + r_{r})e^{\lambda_{f3}t}}{k_{1}r_{r} - k_{2}(\lambda_{f2} + r_{r})e^{\lambda_{f2}t} - k_{3}(\lambda_{f3} + r_{r})e^{\lambda_{f3}t}} \\
r_{b} = S - T \\
r_{i} = \frac{P}{r_{b}} \\
r_{d} = T - r_{i}
\end{cases}$$
(86)

where, for the sake of simplicity, we have introduced the quantities P, S and T, defined as:

$$P = r_b r_i, \quad S = r_b + r_d + r_i, \quad T = r_d + r_i$$
 (87)

In Fig. 21a, the measurement of  $V_i$  from the experiment Exp2 in 8.3.16 is shown together with the best fitting functions in the form of (84). The corresponding values of  $a_f$ ,  $\lambda_{f2}$ , and  $\lambda_{f3}$  are resumed in Table 4a. The fit has been performed with the curve-fitting toolbox in the statistical analysis package of Matlab (MathWorks), with the nonlinear least squares method, by using the default algorithm "Trust Region". The starting points have been empirically found to help the fit to be found.



Figure 21: Numerical results.(a) Comparison between the experimental data, curve fitting and model solution, for the case *Exp2*.(b) Comparison between the experimental data and the EVs internalization forecasted by the model.

According to the values in Table 4a, together with the measured value of the EVs in the extracellular medium for the experiment Exp2, which is  $V_e(t_p) = 1.3534 \cdot 10^3$ , for  $t_p = 6$ h, we proceed to calculate the model parameters from (86). Note that, as it is easy to verify, the expression of  $r_r$ in (86) comes from a second order equation, which provides in general two solutions. Accordingly, two theoretically possible combinations of parameters are found. Nevertheless, negative values of the model parameters do not have physical meaning. For this reason, if some negative theoretical value is found, the corresponding combination of parameters is discarded. With all this in mind, the computation of the parameter according to (86), for the values in Table 4a, provides one feasible combination of parameter values, which are provided in Table 4b.

The analytical solution, obtained according to (76), by replacing the values from Table 4b, is drawn in Fig. 21a. As expected, the figure shows that the analytical solution of the model describes with good approximation the experimental data.

parameter variable according to (co) (b)					
	(a)		Name	Value	
Name	Value		Traine	1 00005 10 1	
	$2.0694 \pm 10^3$		$r_b$	$1.68335 \cdot 10^{-1}$	
$u_f$	2.0024 • 10		$r_{J}$	$4.8915 \cdot 10^{-2}$	
$\lambda_{f2}$	$-2.395 \cdot 10^{-1}$			1 1 4 475 10-1	
\	$1.998 \ 10^{-1}$		$r_i$	$1.14475 \cdot 10^{-1}$	
$\wedge f3$	$-1.226 \cdot 10$		$r_{-}$	$3.0550 \cdot 10^{-2}$	
			'r	0.0000 10	

Table 4: (a) Parameters for the best fitting curve in Fig. 21a - (b) Modelparameter values according to (86)(b)

# 8.3.20 Forecasting and Design

In this section we will exploit the model with the parameter values found in 8.3.19, to forecast the result of another experiment session. In particular, let us consider the case study, denoted as Exp1 in 8.3.16, where the amount of initial EVs changes with respect to the experiment Exp2, used to infer the model parameter. Let us consider the solution of the model according to the new initial condition. Fig. 21b shows the comparison between the experimental results and the model solutions. As we can see the model solution is close to the experimental results.

To show the potentiality of the model let us consider an example. Let us suppose we would like to know the initial amount  $V_0$  of EVs to provide at the time instant  $t_0 = 0$ , such that, at the time instant  $t^*$ , the amount of EVs inside the cell is  $V^*$ . In term of the model variables, this condition can be written as:

$$V_i(t^*) = V^* \tag{88}$$

where the expression of  $V_i$  is given by the third component of (76). The change of  $V_0$  affects the values of the coefficients  $k_1$ ,  $k_2$ , and  $k_3$ , that have to be calculated again. Although this time  $V_0$  is the unknown, the initial condition is again expressed by (78). Therefore,  $k_1$ ,  $k_2$ , and  $k_3$  can be calculated through (80), as function of  $V_0$ .

Substituting these values into (88), together with the expression from (76) and (75) and solving the equation in  $V_0$ , we find that the initial number of EV to be administered to the target cell is  $V_0 = 1.6745 \cdot 10^4$ , which in fact corresponds with a good approximation to the initial number of  $1.6 \cdot 10^4$  EV considered for experiment Exp1.
Our model allows us to faithfully reconstruct the internalization of EVs in this very specific context. This can be useful in designing experiments by reducing material waste.

# 8.4 IV<sup>th</sup> Key Study: EVs Endocytosis Uptake

In this Section molecular communication through EVs is studied focusing on endocytosis key study, mediated by receptors at the level of the target cell.

The propagation of EVs in the extracellular matrix is another important point to study this type of molecular communication. An important issue to define is the degradation of EVs due to their interaction with hindering cells or half-life. This would be characterized by a rate of channel degradation in the communication link. Furthermore, the conditions that govern the boundaries of the medium, e.g. in intracellular communication are determined by EV internalization and cell membrane binding rates [145]. These rates also specify the uptake mechanism at the receiver. The EVs that are bound to the cell might be recycled into the environment through a backward reaction as well [146]. Furthermore, there are some *in vitro* and *in vivo* evidences [147, 148] that shown EVs are taken up by the same cells that are released from, which should be taken into the account for the release rate estimation.

# 8.4.1 Evaluation of reaction rates for the endocytosis phenomena in Extracellular Vesicles cell-to-cell Communications

All these issues necessitate estimating chemical reaction rates involved in EVs reception, as a prior step in EV-based Molecular Communication. This also has applications in designing drug delivery systems for therapeutic reasons or optimizing experimental test-beds by providing initial results.

Data acquisition for the parameter estimation is usually based on "in vivo", "in vitro" or "in silico" (i.e. computer simulations) [134, 149] experiments. There are also various strategies for the data analysis including Nano-Tracking Analysis (NTA) [150], Confocal Microscopy Imaging (CMI) [151], Scansion Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) [152]. NTA is just aimed at overall uptake estimation while the internalization and binding mechanism could be distinguished by CMI, SEM and TEM at the expense of performing several experiments and using more resources.



Figure 22: Experimental set-up of EVs treatment. Isolated EVs are added into the well in which the target cells are cultured.

For the parameter estimation, Cock et al. proposed a stochastic model for a first-order chemical reaction using the maximum likelihood method [153]. The number of biomolecules involved in the chemical reaction is approximated by a Gaussian distribution. However, this work lacks the backward reaction due to the first-order assumption. Furthermore, the estimation of reaction rates is given by [154] upon an ill-posed inverse problem. This approach hardly satisfies the uniqueness and stability of the results specially for noisy acquired experimental data.

This model encompasses all the processes of EVs binding, internalization and recycling. It is expressed in terms of ODEs whose coefficients are estimated through a complex fitting function that we derive in the frequency domain. This model corresponds to an comparably simple experimental setup shown in Figure 22 that we discuss later in this work.

Since all the reaction rates are incorporated in this model, there is no need to exploit advanced techniques such as confocal microscopy imaging to evaluate internalization and binding process. All reaction rates can be estimated based on a single experiment, which eliminates the need for several more complex experiments that may be affected by changing conditions.

## 8.4.2 System Model

As we discussed before, being aware of the chemical reaction rates on the cell membrane can help us to assess the channel response and receiver performance in a EV-mediated communication link. Here, we exploit a simplified reaction mechanism given by

$$\varnothing \xleftarrow{k_{\perp}h} A \xleftarrow{-[k_b][k_r]B} \xrightarrow{k_{\perp}i} \varnothing$$
 (89)

where 'A' and 'B' indicate the stimulating and bound EVs, respectively. In this model  $k_{\rm b}$ ,  $k_{\rm i}$ ,  $k_{\rm r}$ ,  $k_{\rm h}$  denote binding, internalization, recycling and halflife rate of EVs, respectively. Some of these parameters may get zero value regarding to the type of endocytic mechanism. Endocytosis is influenced by the number of endocytosis sites on the cell surface. Here, we ignore the receptor saturation on the target cell for which all the binding spaces on the cell membrane are occupied. This is because the number of endocytosis sites is about 3000 in average per cell [155], while in this study we consider the number of initial EVs in the well by 1000. However, the model can be easily extended by taking receptor saturation into account. Please note that this model is just to apply to some of endocytosis mechanisms, which are already studied in the literature [155, 156, 157, 158].

In order to estimate the reaction rates, we suggest a scenario of data acquisition as shown in Figure 22. In this setup, targeted cells which are to be evaluated for the uptake mechanism are cultivated at the bottom of a well which is filled in by  $q_0$  number of stimulating EVs. This process can be done in parallel at several wells to get the remained number of EVs at different time samples. At first, EVs should be isolated from the wells through the stages centrifugation. Then, we need to characterize EVs by using NTA which can count the number of particles with varied sizes. NTA calculate the particles' hydrodynamic diameters based on Stokes Einstein equation and gives the size distribution of EVs. EVs are injected in, specifies the EVs half-life rate so  $k_{\rm h}$  is assumed to be known (or obtained through another experiment).

Please note that we ignore the release of same EVs as those taken up by the target cells and exclude the release rate function in the formulation. However, EVs release could be incorporated through a separate experiment in which the cells are left in the EV-free wells and the number of generated EVs is specified over the time. This uptake experiment is represented through a set of ODEs given by

$$\frac{\partial q_{\rm B}(t)}{\partial t} = k_{\rm b} q_{\rm A}(t) - k_{\rm r} q_{\rm B}(t) - k_{\rm i} q_{\rm B}(t), \qquad (90)$$

$$\frac{\partial q_{\rm A}(t)}{\partial t} = -k_{\rm b}q_{\rm A}(t) + k_{\rm r}q_{\rm B}(t) - k_{\rm h}q_{\rm A}(t), \qquad (91)$$

$$q_{\rm A}(0) = q_0, \quad q_{\rm B}(0) = 0,$$
 (92)

where  $q_{\rm A}(t)$  and  $q_{\rm B}(t)$  stand for the number of EVs in the environment and the EVs bound to the cells, respectively.

Reaction rates are estimated by measured  $q_A(t)$ , through the model function derived from (90)-(92). In the cell-line experiment, EVs would be isolated from the wells at different time points and counted by NTA device to specify  $q_A(t)$  function. For now, we exploit the particle based simulation (PBS) to generate  $q_A(t)$  artificially. We remove the initial conditions given by (92) and insert the impulse function  $q_0\delta(t)$  to the right-hand side of (91) and derive  $q_A(t)$  by taking Fourier transform of (90) and (91) and solving the set of equations in frequency domain. After some simple manipulations, we have

$$\frac{q_0 - (j\omega + k_{\rm h})\tilde{q}_{\rm A}(j\omega)}{\tilde{q}_{\rm A}(j\omega)} = \frac{k_{\rm b}k_{\rm i} + k_{\rm b}j\omega}{k_{\rm r} + k_{\rm i} + j\omega},\tag{93}$$

where  $\tilde{q}_{\rm A}(j\omega)$  is the Fourier transform of  $q_{\rm A}(t)$  at different angular frequency samples  $\omega$ . It is observed in (93) that  $k_{\rm b}$  is mainly dependent on the high frequency components of  $\tilde{q}_{\rm A}(j\omega)$ .

In order to estimate the rates coefficients  $k_{\rm b}$ ,  $k_{\rm r}$ , and  $k_{\rm i}$ , the number of environmental EVs over time,  $q_{\rm A}(t)$ , is transformed into the Fourier domain and the left-hand side of (93) over which the model function (i.e. the righthand side) is fitted, would be obtained, where  $k_{\rm b}$ ,  $k_{\rm r}$ , and  $k_{\rm i}$  represent the non-negative fitting coefficients. The curve fitting can be solved, for example, by a nonlinear least-squares algorithm. It should be noted again that  $k_{\rm h}$  is assumed to be known in this work, since half-life of EVs is a well-studied and easy to investigate issue. Nevertheless,  $k_{\rm h}$  can also be determined by a reformulation of (93), although this will involve an overall rate estimate error increase.

Eqs. (90)-(92) can be exploited for various types and sizes of EVs for which different reaction rates would be obtained.

Parameters	Symbol	Value	Ref.
Binding rate	$k_{ m b}$	$2.3{ m s}^{-1}$	[149]
Recycling rate	$k_{ m r}$	$0.11{ m s}^{-1}$	[160]
Internalization rate	$k_{ m i}$	$0.005{ m s}^{-1}$	[161]
Half-life rate	$k_{ m h}$	$3.85 \times 10^{-4}  \mathrm{s}^{-1}$	[162]
Initial EV quantity	$q_0$	1000	
Time step in PBS	$\Delta t$	$0.01\mathrm{s}$	
Maximum time in PBS	$T_{\rm max}$	$1800\mathrm{s}$	
PBS Monte Carlo runs	N	$10^{4}$	

 Table 5: Default simulation parameters, which are applied throughout the numerical results, if not stated otherwise.

### 8.4.3 Simulation Results

In this section we investigate the validity of the suggested model and the estimation error through numerical particle-based simulation. PBS results resemble the experimental data from the microscopic point of view. The curve fitting for parameter estimation is performed by the *MATLAB Optimization Toolbox*<sup>TM</sup>, which uses a trust-region-reflective method [159]. The simulation parameters and reference rate parameters under consideration are given in Table 5. Figure 23 shows  $q_A(t)$  and  $q_B(t)$  for a single realization of the PBS. As a comparison, the ODEs (90) and (91) are numerically solved as well, taking into account the reference rate parameters k, and the rate parameters  $\hat{k}$  estimated by the PBS. It can be observed that the noisy PBS simulation follows the ODE solution. Furthermore, the ODE solution for  $\hat{k}$  differs only slightly from the solution with k. From this, it can already be concluded that (93) leads to a good estimation, at least for the parameters under investigation. To have a precise assessment of the estimation results, the normalized mean squared estimation error (NMSEE) is defined as

$$\overline{\text{NMSEE}} = \frac{1}{N} \sum_{n=0}^{N-1} \left| \frac{k - \hat{k}}{k} \right|^2.$$
(94)

Figure 24 shows the influence of the initial number of EVs  $q_0$  in the environment on the NMSEE when estimating  $k_{\rm b}$ ,  $k_{\rm r}$  and  $k_{\rm i}$ . For all rates, the estimation improves with increasing  $q_0$ . This is to be expected, since



Figure 23: Numerical results. Obtained number of EVs in environment and bound to the cell with respect to time for a single PBS realization and ODE solution of (90) and (91). The considered parameters are given in Table 5.

the deviation from the expected value decreases with increasing number of EVs in PBS. In other words, the PBS result approaches the solution given in (90) and (91) as  $q_0$  increases. Thus, Figure 24 also validates the proposed estimation approach according to (93). The estimation errors of  $k_{\rm b}$ ,  $k_{\rm r}$ , and  $k_{\rm i}$  are of the same order of magnitude in the considered scenario.

Number of initial EVs,  $q_0$  might be overestimated or (as indicated in Figure 25 by  $\hat{q}_0$ ) due to the limited accuracy of the NTA device. Figure 25 illustrates  $\overline{\text{NMSEE}}$  for the rate parameters versus the relative estimation error of  $q_0$  in percent. As shown, for  $q_0 = 10^3$ ,  $\overline{\text{NMSEE}}$  mainly changes for  $k_b$ and increases at most by 0.05 and 0.08 respectively, for 20% overestimation and underestimation of the initial EVs in the well. This demonstrates that the model is robust enough against  $q_0$  variation.

Figure 26 shows the effect of time step size  $\Delta t$  in PBS on rate estimation. While  $\Delta t$  for PBS can be chosen arbitrarily, the choice in laboratory measurements is limited by the available equipment and the total time required. Therefore it is interesting to investigate the influence of  $\Delta t$  on the



Figure 24: Error estimation graph respect EVs number. Normalized mean squared estimation error for the reaction rates with respect to initial number of EVs in the environment. The considered parameters are given in Table 5.

parameter estimation, especially if  $\Delta t$  increases. As  $\Delta t$  increases, two effects cause the rate estimate to degrade. The first effect is that fewer samples are available for estimation according to (93), which worsens the fitting. A suitable interpolation between the measured values can reduce this effect. The second effect is that PBS results become imprecise with increasing step size due to very high reaction probabilities. It should be noted that this is only a problem of implementation of PBS and not of practical measurements. For the considered scenario, a significant degradation of the estimate starts from  $\Delta t > 0.01$  s. A significant improvement of the estimate is not observed for  $\Delta t < 0.01$  s.

Figure 27 shows the effect on the NMSEE of  $k_{\rm b}$ ,  $k_{\rm r}$ , or  $k_{\rm i}$  when these are varied, respectively. For this purpose, the rates were varied by a factor of 10 from their default value. The NMSEE is consistently low over the considered range. However, it can be seen that the rate value has an influence on the estimation error and the simulation parameters should be adjusted



Figure 25: Error estimation graph about estimation of EVs number. Normalized mean squared estimation error for the overestimation (negative relative estimation error) and underestimation (positive relative estimation error) of the number of initial EVs in the well.

carefully. Larger reaction rates will, like an increase of  $\Delta t$ , lead to high reaction probabilities, which limits the accuracy of the PBS. Consequently,  $\Delta t$  should be decreased. For lower reaction rates,  $q_0$  and the observation window given by  $T_{\text{max}}$  should be increased to acquire the entire reaction dynamics.

In this work the topic of endocytosis is therefore addressed, we have created a model based on the known literature that simulates the absorption conditions of EVs. In the next paragraph, we will apply this model in a specific tumor context and validate our model.

#### 8.4.4 Modeling EVs interactions into a tumor microenvironment

As a particular case, EVs in the tumor microenvironment (TME) are involved in different processes like cascade metastasis, cells motility and/or adhesion [163]. TME as a complex biological structure is characterized by a chronic inflammation and composed of various immune cells, fibroblasts, cancer cells



Figure 26: Error estimation graph about reaction rate estimation. Normalized mean squared estimation error for the reaction rates with respect to time step size in PBS. The considered parameters are given in Table 5.

and vascular and lymphatic cells [164]. Tumor-derived EVs (TDEVs) have a crucial role in cell physiology for the homeostasis, cell division, migration and differentiation. EVs are used in the oncology for diagnosis [165] and in tumor pathology to investigate disease stages [166]. Cell interactions mediated by TDEVs can modulate the TME causing immunosuppression and cancer development. Tumor cells mainly interact with the immune cells; in the TME, EVs can inhibit the immune cells, stimulating inflammation and cancer dissemination [167]. There is some evidence that TDEVs can regulate the immune system by their specific content, after a therapeutic intervention or through the changes they make in TME [166]. This process can reduce the invasive action of immune cells, e.g. T lymphocyte cells (T cells), i.e. transform the healthy cell into a cancer cell [168]. The release of immune cell-derived EVs (IDEV) could also be affected by the tumor activities [169], Fig. 28. For example, a miRNA, carried by the TDEVs toward the T-cells [170, 171], can contribute in the TDEVs binding to the immune cell membrane through the receptor-ligand pathways, can adjust the  $Ca^{++}$  influx, and can cause reprogramming of the cells [172, 173]. In contrast, the immune cells



Figure 27: Error estimation graph about reaction rates estimation and time. Normalized mean squared estimation error for the reaction rates with respect to reaction rate value. The considered parameters are given in Table 5.

can also release pro-apoptotic EVs to kill and/or inhibit the tumor cells in different ways [174]. IDEVs must be internalized and release their miRNA cargo inside the intracellular environment to induce apoptosis in the tumor cells [175]. This might be done through an artificial vesicle called liposome, inserted in a drug or a biological molecule and involved in the recovery of tumor-killing pathways [176]. Natural EVs (e.g. macrophage EVs) can also mediate the disease treatment without any side effect that comes with the chemical drug [177]. Also, like in the communication systems, some EVs are able to interact with a specific target cell more efficiently which is referred to as *homing process* [178]. For example, tumor-homing EVs derived from the natural killer (NK) cells can bind to the tumor cells with a high value of binding rate which could be incorporated into the uptake model [179].

This biological evidence reveals the necessity of a comprehensive model of EV-mediated interactions to encompass the release by a donor cell, diffusion in the extracellular matrix (ECM), and various uptake mechanisms. A mass of tumor cells is an inherent biological transmitter of TDEVs which gets en-

larged continuously due to cell proliferation. These donor cells also could be impressed by the received signal from another cell (e.g. an anti-proliferative signal from a NK cell). In fact, in the tumor microenvironment, especially between immune and tumor cells, we encounter a biological closed-loop system in the area of cell to cell communications. This interaction can modulate the particle release and uptake rate of each cell. The feedback effect is caused by the particles taken up by the receiver which leads releasing a number of particles of the same type. These re-emitted particles are received by the transmitter and change the release rate. This phenomenon is mostly observed in the short range of the interactive cells and significantly manipulates the overall release rate of the particles [180]. This paper is aimed at modeling this feedback-based system in the framework of molecular communication. This model is of interest to investigate the T-cell immunity suppression due to the cancer progression. On the other hand, it could be exploited in the cancer immunotherapy to assess the efficacy of anti-tumor drugs in re-activating the dysfunctional immune cells.

Many of the TME cells can be assumed like a spheroid with the boundary conditions assigned by the binding and internalization process over the cell membrane. We also need to consider TDEVs diffusion in the cells interstitial space defined by ECM. ECM as a sub-diffusive porous space (see Fig. 28), mediates the cell-cell or cell-matrix communications in TME which conducts different cellular processes including proliferation, differentiation, tumor cell metastasis and immunity suppression [181]. There are several proteins in ECM that define some diagnostic bio-markers. Cancer-associated fibroblasts release some kind of ECM macromolecules like collagens, glycoproteins, proteoglycans and many others, which leads to ECM stiffening and increased tumor cell migration. The interaction of propagating EVs as heterogeneous particles with ECM depends on the type and size of those EVs and the ECM specifications described by the diffusion and degradation coefficients in the diffusion-reaction model. The space occupied by ECM with respect to the whole TME, defined by the volume fraction, has a main influence on the ECM binding of the diffusing EVs which leads to an effective degradation coefficient. Modeling the transmitter, channel and receiver has been well investigated in the paradigm of molecular communication [182]. Some papers deal with the unbounded structures with the transparent receiver which are not relevant in the current application. Noel et. al in [183] compare the performance of a passive or transparent receiver with an absorbing one through the closed-form expressions obtained for the received number of EVs. The

model could not be applied for the partially absorbing receivers (RXs) as a common feature of the cells in TME. In [184], an analytical model is derived for the reversible adsorption receiver in which the error probability at the receiver is assessed in terms of the adsorption and desorption rates. The model in this paper lacks the distinction between binding and internalization of the particles in the uptake process. An improved model which incorporates the reversible binding at the receiver is proposed by Ahmadzadeh et. al [185], where a detector based on maximum likelihood estimation of ISI-causing signal is developed. Also, a general homogeneous model for the interaction of particles with a spherical boundary is proposed in [186], which could be applied to the cell membrane in intracellular communication.

Here, we develop a reaction-diffusion model for the propagation of EVs in the intercellular space and their uptake by the TME cells. Since the model comprises the interactions of EVs on the cell membrane, it could be exploited as a simulation platform for developing engineered EVs. The internalization frequency response of a cell is derived in a single-cell network as well as a feedback-based communication link. Number of internalized EVs with antitumor cargoes which could be excited by immune cells, can be adapted as a criteria for drug delivery investigations. We insert the relay effect of the target and donor cells in the closed-form solution that we derive for the cells interactions. The release function of a donor cell is expressed in terms of a natural release and an induced release triggered by the other cells. The induced release could be affected by the inhibitory activity of the other cells which yields a release reduction for some specific types of EVs. The model also encompasses the cells feedback in a closed-loop system that simulates the ping-pong communication for example between tumor and immune cells in TME, which has not been observed before.

# 8.4.5 System Model

Modeling cell to cell interaction in TME is discussed here. As we explained in the previous section, the release of EVs from a donor cell (e.g. a tumor cell) could be activated partially by the EVs received from the target cell (e.g. an immune cell) where the donor cell acts as a relay. There is a similar behavior for the target cell in response to a stimulating signal from the donor cell. Here, this is referred to as the induced release in contrast to the natural release which occurs regardless to the cells interactions. Various cell types in TME, e.g. tumor cell, fibroblast, regulated T-cell, helper T-cell, and B-cells,



Figure 28: Relationship between T-cells, Tumor cells and EVs into the ECM environment. The tumor microenvironment is made of an acellular part based on proteins and gel, called extracellular matrix (ECM), and a cellular part based on immune cells (T-cells) and tumor cells. These cells release different types of molecules, including extracellular vesicles (EVs), into the extracellular space (ES) that are involved in inflammatory processes. The same processes of release and uptake of EVs happen in both cells type.

might play the role of transmitter (TX) and receiver (RX) in the prescribed molecular communication system. We denote the EVs which derive from the donor cell by  $EV^D$  and those derive from the target cell by  $EV^T$ . Diffusing  $EV^D$ s inside the ECM, are degraded by the rate  $k_d^D$  due to the ECM binding and undergo the uptake process on the target consisting of binding to the plasma membrane, recycling to the ECM and internalizing into the cell by the rates  $k_b^T$ ,  $k_r^T$  and  $k_i^T$ , respectively. On the one hand, internalized  $EV_i^D$ s relay the target cell-derived  $EV^T$ s by the release function  $f^T(i^D(t))$ . On the other hand, internalized  $EV_i^T$ s relay the donor cell-derived  $EV^D$ s by the release function  $f^T(i^T(t))$ . Here,  $i^D(t)$  and  $i^T(t)$  indicate the internalization rate function (IRF) of  $EV^D$  and  $EV^T$  in target and donor cells, respectively. The diffusion-reaction model from the donor cell to the target cell is given by

$$\varnothing \xleftarrow{k^{\mathrm{D}}_{-\mathrm{d}}} \mathrm{EV}^{\mathrm{D}}_{\mathrm{i}} - \xrightarrow{k_{-\mathrm{b}}^{\mathrm{T}}} \mathrm{EV}_{\mathrm{b}}^{\mathrm{D}} \xrightarrow{k_{-\mathrm{i}}^{\mathrm{T}}} \mathrm{EV}_{\mathrm{i}}^{\mathrm{D}} \longrightarrow f^{T}(i^{D}(t)) \mathrm{EV}^{\mathrm{T}}.$$
(95)

The same model exists from the target cell to the donor cell in terms of corresponding degradation rate  $k_{\rm d}^{\rm T}$  and reaction rates  $k_{\rm b}^D$ ,  $k_{\rm r}^D$  and  $k_{\rm i}^D$  on the donor cell given by

$$\varnothing \xleftarrow{k^{\mathrm{T}}_{\mathrm{-}}\mathrm{d}} \mathrm{EV}^{\mathrm{T}}_{\mathrm{i}} \xrightarrow{k_{\mathrm{-}}\mathrm{b}^{\mathrm{D}}} \mathrm{EV}_{\mathrm{b}}^{\mathrm{T}} \xrightarrow{k_{\mathrm{-}}\mathrm{i}^{\mathrm{D}}} \mathrm{EV}_{\mathrm{i}}^{\mathrm{T}} \xrightarrow{f^{D}} f^{D}(i^{T}(t)) \mathrm{EV}^{\mathrm{D}}.$$
(96)

As an initial step, we consider the linear approximations of  $f^{T}(i^{T}(t))$  and  $f^{D}(i^{D}(t))$  as

$$f^T(i^T(t)) \approx k_{\rm re}^T i^T(t), \tag{97}$$

and

$$f^D(i^D(t)) \approx k^D_{\rm re} i^D(t), \tag{98}$$

where  $k_{\rm re}^T$  and  $k_{\rm re}^D$  are the induced release coefficients (IRC) on the target and donor cells, respectively. Based on the aforementioned model, the following differential equations are obtained for the concentration of  $EV^T$  and  $EV^D$ in the ECM denoted by  $c^T(r,t)$  and  $c^D(r,t)$ , respectively:

$$\frac{\partial}{\partial t}c^{T}(r,t) = D^{T}\nabla^{2}c^{T}(r,t) + k_{d}^{T}c^{T}(r,t) + S^{T}(r,r^{T}) \times \qquad (99)$$

$$\left(k_{\rm re}^{T}i^{D}(t) + re_{0}^{T}(t)\right),$$

$$\frac{\partial}{\partial t}c^{D}(r,t) = D^{D}\nabla^{2}c^{D}(r,t) + k_{d}^{D}c^{D}(r,t) + S^{D}(r,r^{D}) \times \\
\left(k_{\rm re}^{D}i^{T}(t) + re_{0}^{D}(t)\right),$$
(100)

where  $S^T(r, r^T)$  and  $S^D(r, r^D)$  characterize the source spatial function of the target and donor cells in terms of their locations  $r^T$  and  $r^D$ , respectively.

In (99) and (100),  $re_0^T(t)$  and  $re_0^D(t)$  stand for natural release functions. These functions could also be exploited for a controlled release that results from an external factor such as drug injection. Here, we consider two different diffusion coefficients  $D^T$  and  $D^D$ , which are determined based on the size and the type of  $EV^T$  and  $EV^D$ , respectively. For the special case, where the EVbased induced interaction of the cells is not significant, we can assume a unilateral process in which  $k_{\rm re}^T = k_{\rm re}^D = 0$ .

 $c^{T}(r,t)$  and  $c^{D}(r,t)$  in (99) and (100) satisfy the following boundary conditions on the donor cell membrane (DCM) and the target cell membrane (TCM), respectively [186]:

$$\begin{aligned}
D^{T}(\frac{\partial}{\partial t} + k_{r}^{D} + k_{i}^{D}) \nabla c^{T}(\bar{r}, t) \cdot \hat{n} \Big|_{\bar{r} \in DCM} &= \\
k_{b}^{D}(\frac{\partial}{\partial t} + k_{i}^{D}) c^{T}(\bar{r}, t) \Big|_{\bar{r} \in DCM}, \\
D^{D}(\frac{\partial}{\partial t} + k_{r}^{T} + k_{i}^{T}) c^{D}(\bar{r}, t) \cdot \hat{n} \Big|_{\bar{r} \in TCM} &= \\
k_{b}^{T}(\frac{\partial}{\partial t} + k_{i}^{T}) c^{D}(\bar{r}, t) \Big|_{\bar{r} \in TCM}.
\end{aligned}$$
(101)
$$(102)$$

In the following we clarify how to obtain the internalization rate function in terms of the frequency responses of  $c^T(r,t)$  in (99) and  $c^D(r,t)$  in (100), when the induced and natural release terms are replaced by an impulse function.

## 8.4.6 Analytical Solution for EV-based Cell Interactions

Here, we solve equations (99) and (100) regarding to the boundary conditions given by (101) and (102) in the frequency domain, respectively. According to the information acquired from the cell morphology, a realistic assumption could be a spheroid shape with radius  $a^T$  and  $a^D$  for the target and donor cells, respectively. We have already developed a diffusion-based molecular communication link inside a spheroid [186]. Here, we extend it to derive a solution for the diffusion-reaction model of EVs in the intercellular environment. In fact, we obtain the channel impulse response of propagating EVs, originally released from a point source transmitter at a distance d from the receiver center, undergoing a reversible uptake process on the TCM. We apply this impulse response in the frequency domain inside a closed-loop system to get the overall release rate of a cell (i.e. combination of natural and induced release) as well as the overall IRF represented by a closed-form expression. This is of interest particularly to diagnose the stage of cancer development or evaluate the anti-tumor drug in terms of TDEVs.

#### 8.4.7 Frequency Response of EVs Internalization

Due to the spherical symmetry, the number of internalized EVs is a function of  $r^{D(T)}$  regardless the angular location of target (donor) cell. If we replace the last two terms at the right hand-side of (100) ((99)) with the impulse point source  $\frac{1}{4\pi r^{D(T)^2}}\delta(r-r^{D(T)})\delta(t)$  and take the Fourier transform from two



Figure 29: Block diagram of a biological closed-loop system for the cell to cell EV-based interaction. The natural release function of the donor and the target cells is given by  $R_0^{\rm D}(j\omega)$  and  $R_0^{\rm T}(j\omega)$ , respectively. Overall release functions as the combination of the natural release and the induced release are also denoted by  $R^{\rm D}(j\omega)$  and  $R^{\rm T}(j\omega)$ . The induced releases are related to the overall internalization functions  $I^{\rm D}(j\omega)$  and  $I_0^{\rm T}(j\omega)$  through the release factors  $k_{\rm re}^{\rm T}(j\omega)$  and  $k_{\rm re}^{\rm D}(j\omega)$  in the frequency domain. Internalization functions are also

expressed in terms of internalization frequency responses  $H_{na}^{(.)}$  and  $H_{ind}^{(.)}$ , respectively, due to the natural and induced release, given by (124)-(127).

sides of the equation,  $H^{D(T)}(r, j\omega)$  as the concentration of  $EV^{D(T)}$ s in the frequency domain is given by

$$j\omega H^{D(T)}(r,j\omega) = \frac{1}{r^2} D^{D(T)} \frac{\partial^2}{\partial r^2} H^{D(T)}(r,j\omega) + k_d^{D(T)} H^{D(T)}(r,j\omega) + \frac{1}{4\pi r^{D(T)^2}} \delta(r-r^{D(T)}),$$
(103)

satisfying

$$D^{D(T)}M^{T(D)}\nabla H^{D(T)}(\bar{r},t)\cdot \hat{n} = N^{T(D)}H^{D(T)}(\bar{r},t),$$
(104)

where

$$M^{T(D)}(j\omega) = (j\omega + k_r^{T(D)} + k_i^{T(D)}),$$
(105)

and

$$N^{T(D)}(j\omega) = -k_b^{T(D)}(j\omega + k_i^{T(D)}).$$
(106)

 $M^{T(D)}(j\omega)$  and  $N^{T(D)}(j\omega)$  are dependent on the reaction rates at the target (donor) cell and are obtained by Fourier transforming both sides of (102) ((101)). We impose two extra boundary conditions by removing the source term  $\frac{1}{4\pi r^{D(T)^2}}\delta(r-r^{D(T)})$  in the right hand-side of (103), and considering continuity and derivative discontinuity of  $H^{D(T)}(r, j\omega)$  at  $r = r^{D(T)}$  which results in [186]

$$H^{D(T)}(r,\omega)\Big|_{r=r_{+}^{D(T)}} = H^{D(T)}(r,\omega)\Big|_{r=r_{-}^{D(T)}},$$
(107)

and

$$r^{2} \frac{\partial H^{D(T)}(r,\omega)}{\partial r} \bigg|_{r=r_{+}^{D(T)}} - r^{2} \frac{\partial H^{D(T)}(r,\omega)}{\partial r} \bigg|_{r=r_{-}^{D(T)}} = 1, \quad (108)$$

respectively.

The general solution of (103) in the homogeneous form is given by

$$H^{D(T)}(r,\omega) = \begin{cases} A^{D(T)} j_0(k^{D(T)}r) + B^{D(T)} n_0(k^{D(T)}r) \\ a^{T(D)} < r < r^{D(T)} \\ C^{D(T)} h_0^{(2)}(k^{D(T)}r) & r \ge r^{D(T)}, \end{cases}$$
(109)

where  $k^{D(T)} = \sqrt{\frac{-k_d^{D(T)} - j\omega}{D^{D(T)}}}$  and  $j_0(.)$ ,  $n_0(.)$  and  $h_0^{(2)}(.)$  are 0th order of the first and second type of spherical Bessel function and second kind of spherical Hankel function, respectively. We fulfill the boundary conditions of (104)-(108) to derive a closed-form expression for  $A^{D(T)}, B^{D(T)}, C^{D(T)}$  in (109) as follows:

4

$$A^{D(T)} = \frac{-\alpha_0^{T(D)} B^{D(T)}}{\alpha_1^{T(D)}},$$
(110)

$$C^{D(T)} = -\frac{(-\alpha_2^{D(T)}\alpha_0^{T(D)} / \alpha_1^{T(D)} + \alpha_3)^{D(T)}B^{D(T)}}{\alpha_4^{D(T)}},$$
(111)

$$B^{D(T)} = B^{D(T)} = \frac{1/D_0}{(-\alpha_5^{D(T)}\alpha_0^{T(D)}/\alpha_1^{T(D)} + \alpha_6^{D(T)}) - \frac{(-\alpha_2^{D(T)}\alpha_0^{T(D)}/\alpha_1^{T(D)} + \alpha_3^{D(T)})}{\alpha_4^{D(T)}}},$$
(112)

and

$$\alpha_0^{T(D)} = M^{T(D)}(j\omega)\sqrt{k^{D(T)}}j_0(k^{D(T)}a^{T(D)}) - N^{T(D)}(j\omega)j_0'(k^{D(T)}a^{T(D)}),$$
(113)

$$\alpha_1^{T(D)} = M^{T(D)}(j\omega)\sqrt{k^{D(T)}}n_0(k^{D(T)}a^{T(D)}) - N^{T(D)}(j\omega)n_0'(k^{D(T)}a^{T(D)}),$$
(114)

$$\alpha_2^{D(T)} = j_0(k^{D(T)}r^{D(T)}), \tag{115}$$

$$\alpha_3^{D(T)} = n_0(k^{D(T)}r^{D(T)}), \tag{116}$$

$$\alpha_4^{D(T)} = -h_0^{(2)}(k^{D(T)}r^{D(T)}), \qquad (117)$$

$$\alpha_5^{D(T)} = r^{D(T)^2} \sqrt{k^{D(T)}} j_0'(k^{D(T)} r^{D(T)}), \qquad (118)$$

$$\alpha_6^{D(T)} = r^{D(T)^2} \sqrt{k^{D(T)}} n_0'(k^{D(T)} r^{D(T)}), \qquad (119)$$

$$\alpha_7^{D(T)} = -r^{D(T)^2} \sqrt{k^{D(T)}} h_0^{(2)'} (k^{D(T)} r^{D(T)}).$$
(120)

Obviously, (115)-(120) are independent of the target (donor) cell parameters (i.e. reaction rates and radius of the target (donor) cell), and just specified by the distance between the donor (target) cell and the target (donor) cell, as well as the diffusion coefficient and degradation rate of donor (target) derived EVs. Finally, the number of internalized  $EV^{D(T)}$  into the receiver is given by

$$H_i^{T(D)}(j\omega) = \frac{k_i^{T(D)} k_b^{T(D)}}{j\omega(j\omega + k_r^{T(D)} k_i^{T(D)})} a^{T(D)^2} \times \left(A^{D(T)} j_0(k^{D(T)} a^{T(D)}) + B^{D(T)} n_0(k^{D(T)} a^{T(D)})\right).$$
(121)

 $H_i^{T(D)}(j\omega)$  in (121) is the unilateral frequency response of the internalization function for  $EV^{D(T)}$ , that is characterized by the distance between donor and target cells, size of target cell, and the reaction rate of EVs on the target cell. Subsequently, we apply this function to develop the frequency response of a bilateral cell to cell communication link.

# 8.4.8 Release and Internalization Functions for a Closed-loop Cell to Cell Interaction

In this sub-section, we derive the release rate of the donor cell,  $re^{D}(t)$  as the aggregate of natural release given by  $re_{0}^{D}(t)$  and the induced release originated from target cell as a feedback.  $re^{D}(t)$  in the frequency domain, i.e.  $R^{D}(j\omega)$ , can be written as

$$R^{D}(j\omega) = R^{D}_{0}(j\omega) + k^{D}_{\rm re}I^{D}(j\omega), \qquad (122)$$

where  $I^{D}(j\omega)$  denotes the internalization function at donor cell (due to the EVs released from target cell) in the frequency domain that is obtained by taking the Fourier transform of  $i^{D}(t)$  in (98). A similar equation exists for  $R^{T}(j\omega)$  in terms of  $I^{T}(j\omega)$  (i.e. the Fourier transform of internalization function  $i^{T}(t)$  at target cell in (97)) given by

$$R^{T}(j\omega) = R_{0}^{T}(j\omega) + k_{\rm re}^{T}I^{T}(j\omega).$$
(123)

 $R_0^D(j\omega)$  and  $R_0^T(j\omega)$  denote the Fourier transform of  $re_0^D(t)$  and  $re_0^T(t)$ , respectively, and the second terms in (122) and (123) indicate the stimulated or induced release from the other cell.

We need to specify  $I^{D}(j\omega)$  and  $I^{T}(j\omega)$  to extract the overall release of a cell through a feedback controlled closed-loop system. These functions are obtained in terms of the internalization frequency response given by (121). We place the TX at the middle of donor cell (see point 'A' in Fig. 28) to get the frequency response of natural release since there is no preference for the EVs discharge toward a specific direction. On the other hand, the channel frequency response for the internalized EVs due to the induced release is derived assuming TX is located at a point on the donor cell membrane with the least distance to the target cell center (see point 'B' in Fig. 28). This assumption is consistent with the cell's intention to ensure maximum EVs concentration at the cell from which a stimulating signal is received. Now, we can define

$$H_{na}^{\mathrm{D}}(j\omega) = H_{i}^{\mathrm{D}}(j\omega) \Big|_{r^{D}=a^{D}+d+a^{T}}$$
(124)

and

$$H_{na}^{\mathrm{T}}(j\omega) = H_i^{\mathrm{T}}(j\omega) \bigg|_{r^T = a^D + d + a^T},$$
(125)

where d denotes the membrane to membrane distance of the donor and target cells. Here,  $H_{na}^{\rm D}(j\omega)$  and  $H_{na}^{\rm T}(j\omega)$  stand for the internalization frequency response for the natural release of the target and donor cells, respectively. The corresponding frequency responses for the induced release are given by

$$H_{ind}^{\rm D}(j\omega) = H_i^{\rm D}(j\omega) \bigg|_{r^D = d + a^T}$$
(126)

and

$$H_{ind}^{\mathrm{T}}(j\omega) = H_i^{\mathrm{T}}(j\omega) \bigg|_{r^T = d + a^T}.$$
(127)

Thus, we express the overall internalization function in terms of the aforementioned frequency responses as

$$I^{T}(j\omega) = H^{T}_{na}(j\omega)R^{D}_{0}(j\omega) + H^{T}_{ind}(j\omega)k^{D}_{re}I^{D}(j\omega)$$
(128)

for the target cell and

$$I^{D}(j\omega) = H^{D}_{na}(j\omega)R^{T}_{0}(j\omega) + H^{T}_{ind}(j\omega)k^{T}_{re}I^{T}(j\omega)$$
(129)

for the donor cell.  $I^{T}(j\omega)$  and  $I^{D}(j\omega)$  deduced from (128) and (129) are given by

$$I^{T}(j\omega) = \frac{H_{na}^{T}(j\omega)R_{0}^{D}(j\omega) + k_{\rm re}^{D}H_{ind}^{T}(j\omega)H_{na}^{D}(j\omega)R_{0}^{T}(j\omega)}{1 - k_{\rm re}^{D}H_{ind}^{D}(j\omega)k_{\rm re}^{T}H_{ind}^{T}(j\omega)}$$
(130)

and

$$I^{D}(j\omega) = \frac{H^{D}_{na}(j\omega)R^{T}_{0}(j\omega) + k^{T}_{re}H^{D}_{ind}(j\omega)H^{T}_{na}(j\omega)R^{D}_{0}(j\omega)}{1 - k^{D}_{re}H^{D}_{ind}(j\omega)k^{T}_{re}H^{T}_{ind}(j\omega)},$$
(131)

respectively. It is clear that (130) and (131) reduce to the internalization function due to the natural release upon a unilateral communication link when  $k_{\rm re}^T = k_{\rm re}^D = 0$ . The block diagram of the closed-loop system for the cell to cell interaction is shown in Fig 29. The overall internalization as well as the release functions as the outputs of the system are denoted in the figure.

## 8.4.9 Simulation Results

In this section we present the numerical results for two scenarios inspired from the exchange of EVs between tumor and immune cells in TME. In the first scenario, we consider a unilateral communication between the cells in which the feedback effect is not observed. In contrast, the second scenario addresses the reciprocal interactions of the cells through the model derived in this paper. We also applied particle based simulations (PBS) to verify the results of the closed-form solution. The parameters that we used in these simulations are indicated in Fig. 30 and their reference values are reported in Table ??. In each study, it is stated if other values are selected.



Figure 30: Schematic representation of the system model for immune-tumor interaction.

# 8.4.10 Tumor-immune Unilateral Cell Communication

The natural release of EVs from a tumor (immune) cell and their uptake by an immune (tumor) cell in the TME are discussed here. The EVs uptake is generic, so includes all the processes (endocytosis, fusion, juxtacrine, etc.) without any specification [195, 196]. For now, we ignore the feedback effect of the cells. In addition, the process of EV transfer between cells is supposed to be unilateral. Thanks to the cell imaging modalities, we have accurate information about the size of the cells. Here, we choose the average values of  $a^{tumor} = 6.5 \,\mu\text{m}$  and  $a^{immune} = 4 \,\mu\text{m}$  [193] for the radius of tumor and immune cells, respectively. Also, we assume  $d = 2 \,\mu\text{m}$  for the membrane-to-membrane distance between the cells. Regarding the size of the EVs and the specifications of the ECM, the diffusion coefficients for the IDEVs and TDEVs are set to  $D^{IDEV} = 3.3 \,\mu\text{m}^2/\text{s}$  and  $D^{TDEV} = 6.6 \,\mu\text{m}^2/\text{s}$ , respectively. The uptake parameters of EVs on the target cells (i.e. the binding, internalization, and recycling rates) are quantified in Table II for the tumor and immune cells. Table II also contains the degradation rates

Parameters	Symbol	Value	Unit	Ref.
Internalization rate of TDEV on immune cell	$k_{\rm i}^{\rm immune}$	0.005	$s^{-1}$	[187]
Internalization rate of IDEVs on the tumor cell	$k_{i}^{tumor}$	0.02	$s^{-1}$	[188]
Binding coefficient of TDEV on immune cell	$k_{ m b}^{ m immune}$	60	$\mu m  s^{-1}$	[189]
Binding coefficient of IDEV on tumor cell	$k_{\rm b}^{\rm tumor}$	110	$\mu m  s^{-1}$	[190]
Re-cycling rate of TDEV on immune cell	$k_{ m r}^{ m immune}$	0.1	$s^{-1}$	[187]
Re-cycling rate of IDEVs on the tumor cell	$k_{ m r}^{ m tumor}$	0.2	$s^{-1}$	[191]
Diffusion coefficient of IDEVs	$D^{\text{IDEV}}$	3.3	$\mu m^2  s^{-1}$	[192]
Diffusion coefficient of TDEVs	$D^{\text{TDEV}}$	6.6	$\mu m^2  s^{-1}$	[192]
Degradation rate of IDEVs	$k_d^{\text{IDEV}}$	0.1	$s^{-1}$	[192]
Degradation rate of TDEVs	$k_d^{\tilde{T}DEV}$	0.2	$s^{-1}$	[192]
Tumor cell radius	$a^{\mathrm{tumor}}$	6.5	$\mu m$	[193]
Immune (lymphocyte) cell radius	$a^{\text{immune}}$	4	$\mu m$	[193, 194]

Table 6: Parameter values of the system model in Fig. 30.

of TDEVs and IDEVs due to the ECM binding and hindrances. All the parameters reported in Table II were evaluated from real experimental data in the literature [193, 192, 187, 189, 188, 190, 191, 194].

Fig. 31 depicts the internalization impulse response (IIR) given by taking inverse Fourier transform of (124)-(127). It also includes the PBS results (1000 EVs per impulse, time steps of 50 µs,  $10^5$  repetitions) which are quite matched to the results of the proposed analytic solution. As shown in Fig. 31, the impulse responses associated with the induced release are more dominant, since in these cases the point source is located on the membrane of the donor cell and thus closer to the target cell (in contrast to natural release, where the point source is located at the cell center). In contrast, the higher internalization rate on the tumor cell leads to an IIR with a longer tail and a larger number of internalized EVs.

Now, we proceed with considering a natural release having a specific release function for the tumor and immune cells. TDEVs and IDEVs releases can follow the cells' survival functions that are modeled here through an exponential rule given by

$$re_0^{\text{tumor}}(t) = 100(e^{-3 \times 10^{-5}t})$$
 (132)

for the TDEVs, and

$$re_0^{\text{immune}}(t) = 50(e^{-1.5 \times 10^{-5}t})$$
 (133)

for the IDEVs. Fig. 32 depicts the IRF of TDEV and IDEV at immune and tumor cells, respectively, for different cell sizes. As it is shown, IRF of



Figure 31: Numerical results. Internalization impulse response for tumor and immune cells when they are stimulated by a point source located at the other cell's center (for natural release) or membrane (for induced release).

IDEVs at tumor cells is higher than IRF of TDEVs at immune cells despite the more powerful release from tumor toward immune cell (see (132) and (133)). If these EVs are responsible for cell apoptosis, this process is performed more quickly in tumor cells. Furthermore, cells with a larger surface area may provide more influx of EVs. Also, Fig. 33 demonstrates the IRF with respect to the diffusion coefficient of the EVs, which is dependent on the size of the EVs. This dependence is described by the Einstein formula. Accordingly, smaller EVs (e.g. exosomes) are more internalized into the cell in comparison to larger EVs (e.g. microvesicles) and could therefore be of greater importance in cell-to-cell communication. It is also indicated by the figure that the sifting property of the cell based on the EV sizes is more significant at tumor cells, which are more sensitive to the variations in EV diffusion. This illustrates a filtering behavior of tumor cells on EVs' size



Figure 32: Numerical results. IRF at tumor and immune cells for different cell sizes.

distribution function. Fig. 34 depicts the IRF for various reaction rates of the immune and tumor cells. As shown, a higher internalization rate value leads to a larger number of internalized EVs. As expected, the recycling rate conversely influences the internalization of EVs since a larger  $k_r$  causes more EVs to get dissociated from the cell membrane. It is also perceived from the figure that the IRF is slightly sensitive to the binding rate.

# 8.4.11 Tumor-immune Bilateral Cell Communication

TDEVs and/or IDEVs are able to stimulate or inhibit the respective release at immune and/or tumor cells, respectively, which is exploited to reduce chronic inflammation and tumor spread as well as reciprocal apoptosis through a feedback pathway [197, 198]. In fact, cell release stimulation (inhibition) amplifies (degrades) the budding release pathway for the *Large* 



Figure 33: Numerical results. IRF at the tumor and immune cells for different diffusion coefficients of TDEVs and IDEVs.

Vesicles and the endocytosis release pathway for the Small Vesicles [199]. In this subsection, we incorporate the feedback effect between cells by adopting non-zero values for  $k_{\rm re}^D$  and  $k_{\rm re}^T$ . According to this, promotion or inhibition of the natural release at each cell as a transceiver, is characterized by the positive or negative  $k_{\rm re}$ . Fig. 35 illustrates the IRF curves for  $k_{\rm re}^{\rm tumor} = 0.5$ and  $k_{\rm re}^{\rm immune} = 0.25$  regarding the pathway activation and  $k_{\rm re}^{\rm tumor} = -0.5$  and  $k_{\rm re}^{\rm immune} = -0.25$  regarding the pathway inhibition at tumor and immune cells. The figure also includes IRF results for  $k_{\rm re}^{\rm tummor} = k_{\rm re}^{\rm immune} = 0$  i.e. the unilateral case. As shown, the induced release significantly changes the IIR specially at the immune cell. Also, it is clear from the figure that the impact of the inhibition process on IRF degradation is less than that of the activation process on IRF amplification.

The maximum overall release rate of the tumor and immune cell in the closed-loop system, given by (122) and (123), respectively, is depicted in Figs.



Figure 34: Numerical results. IRF at the tumor and immune cells with different internalization, binding, and recycling rates.

36 and 37 for various induced release coefficients and distances between the membranes of the cells. In both figures we assume  $k_{re}^{\text{immune}} = 0.5 k_{re}^{\text{tumor}}$ . As shown, the maximum release rate gets enhanced for the positive values of IRC and degraded for the negative values of IRC. Also, the overall release is substantially distinguished from the natural release for the short distances between the cells. For example, at the tumor cell, the release rate of TDEVs grows up at least 30% for the cells distances below 0.5 µm and IRC more than 1.25. On the other hand, the release rate is degraded by at least 10% for the distances below 0.5 µm and IRC less than -1.25 due to the prohibitory process of the cells.

Induced release of EVs relies on a feedback mechanism in which the rate of release by donor cells is influenced by the EVs returned by the target cell. A limited number of secreted EVs may encounter the target cells and get internalized to contribute to the induced release. This back-and-forth



Figure 35: Numerical results. IRF at the tumor and immune cells for different induced release coefficients of cell activation and inhibition.

shuffling significantly deteriorates induced release, especially for cells with a large distance between them. The distance between cells with poor bilateral effect is mainly dependent on the diffusion and degradation coefficients of the particles in the environment, as well as the binding, internalization, and induction rates. For the current scenario as shown in Figs. 36 and 37, when the cells move away more than  $3 \,\mu m$  from each other, the impact of the induced release is not significant and the overall release is closely equivalent to the natural release. We remark that the unilateral communication between the cells exists even for more than  $3 \,\mu m$  distances [200, 201]. As expected, for smaller values of CRI, cells should be closer together to be more affected by the feedback effect.

In this work we have applied and implemented the model presented in paragraph 8.4.1, in a very specific tumor context which allows us to have an idea of what could actually happen and plan possible future therapies aimed at stimulating the release of EVs by the immune system, against cancer cells.

In the next Section, a model similar to this but *in silico* will be presented, further analyzing the aspects of secretion, diffusion and interaction of EVs with target cells for possible implementations and future applications.

# 8.5 V<sup>th</sup> Key Study: Evaluation of EVs uptaking rates in silico approach

The processes of EV release and uptake, Figure 38, are characterized by chemical reaction rates which are studied in chemical kinetics [202, 203], as before discussed.

Values of chemical reactions involved in EVs transport yield information about the reaction's mechanism and transition states, and are critical for the creation of mathematical models that are used as a tool to describe the characteristics of EVs transport. Moreover, such values are essential for prototyping EV-based therapy [204, 205], for example, anticancer therapy [206], giving insights into how fast the biomolecule-carrying EVs absorption occurs or what is the survival rate of cancer cells that is closely dependent on the rate and amount of the received biomolecules. Furthermore, values of chemical reactions are important for effectively establishing a communication link between bio-transceivers in an EV-based communication network [207, 208].

However, this strategy is not only technically challenging because it is hard to differentiate between the bound and internalized EVs when assaying acquired data [77], but also sensitive to potential mechanical deformations of the cell membrane incurred by its interaction with EVs [209].

# 8.5.1 Estimation of Chemical Reaction Rates in Extracellular Vesicle Signaling through *in silico* approach

Mathematical modeling is often used in combination with *in vitro* experiments to alleviate such limitations when deeply examining cell-to-cell signaling and identifying critical components involved in the signaling cascade [210]. Some examples where transport of (nano)particles were studied through mathematical models based on Fick's second law of diffusion and a convection-diffusion-reaction equation, in combination with experimental models, include the stent-based drug release and delivery [211], the permeation of drugs through different layers of the skin [212], and the transport of



Figure 36: Numerical results. Maximum overall release (natural release + induced release) of TDEVs in terms of distance between the cells membrane (d) and induced release coefficient for (a) pathway activation process and (b) pathway inhibition process.



Figure 37: Numerical results. Maximum overall release (natural release + induced release) of IDEVs in terms of distance between the cells membrane (d) and induced release coefficient for (a) pathway activation process and (b) pathway inhibition process.



Figure 38: EV uptake by the recipient cell can be driven by many different pathways: endocytosis, fusion and juxtacrine. This illustration is created using BioRender.com.

a water-soluble drugs from cylindrical tablets [213]. Other examples include the models for tumor cell apoptosis upon drug reception [214] and nutrient uptake [215], as well as the models of the growth and invasion of Glioblastoma spheroids [216, 217].

Different wells are used for different time-points of data acquisition where only the EVs present in the medium were quantified by NTA; in this regard, the time-course of the environmental EVs could be characterized. The suggested computational approach is based on a closed-form fitting function derived from ODEs that correspond to the kinetic model of EVs uptake [204]. The suggested model, however, does not consider the number of available binding sites on the cell membrane which is a critical factor in the receptormediated endocytotic pathway of EVs uptake.

Here we extend our previous model, in Section 8.4.1 by exploiting a nonlinear system model in which the impact of limited receptor sites on the recipient cell membrane is incorporated. The reaction rates are then computed through a suggested iterative approach by which the rate parameters are obtained after several updates to satisfy the predefined relative error requirements. In the literature, several *time domain approaches* for parameter estimation have been well studied [218]. These techniques use a numerical method to iteratively solve the ODEs and estimate the parameters such that the norm of the object function is minimized. However, optimization algorithms often encounter convergence issues. For example, deterministic optimization methods are highly dependent on the initial estimation to avoid local minima, while stochastic optimization methods are time-consuming. Classical statistical estimators, such as the maximum likelihood method and the least square method, require large temporal data sets collected under different conditions, which are computationally intensive. Bayesian approaches require prior knowledge of parameters, which is usually obtained from preliminary experiments that increase the time and cost of the estimation process. To overcome these issues, we propose a frequency domain approach that results in closed-form expressions for the reaction rates. The closed-form expressions are used together with an iterative approach to simultaneously estimate the nonlinear part of the model and the rate parameters. This approach is also advantageous over existing methods, for example, an ill-posed inversion method [154] which is applicable for linear models and hardly satisfies the result uniqueness and stability.

In addition, we extend our previous linear system model based solution in [204] by deriving closed-form expressions for rate estimation which avoids computationally complex curve fitting. Moreover, we suggest computing the reaction rates by applying Michaelis-Menten kinetics directly in the frequency domain under the pseudo-steady-state assumption. We compare the validity region of these three approaches with respect to the ratio between free receptor sites and environmental EVs, as well as the absolute values of the rate parameters. This is done by numerical particle-based simulations (PBS) which are set to resemble experimental data from the microscopic point of view, thus avoiding the time and resource-consuming experiments for the purpose of the presented results. Of note, our proposed methodology is not restricted to the estimation of the chemical reaction rates involved in EV signaling, and could be applied to analyses of reaction mechanisms and transition states involved in other types of molecular communication.

# 8.5.2 System Model

The Fick's law equation is exploited when constructing mathematical models of the spatio-temporal concentrations of particles which are governed by diffusion (including sub-diffusion in an anomalous media) and advection [213, 216, 217, 219]. Such models are typically referred to as partialdifferential equation (PDE)-based models. On the other hand, ODE-based models are used to investigate the release and uptake rates of particles, as well as the dynamics of cell growth and death. In ODE-based models, the system model is characterized by a series of ODEs in which the unknowns are functions of time; in such models, coefficients characterizing diffusion and/or advection are excluded. Some examples where ODE-based models are utilized include studying the interactions between microglia and neural stem cells [220], the functionality of the immune system and normal cells in tumor growth [221], and the uptake of nutrients by a tumor cell with the presence of biomolecular fuel, such as glucose and lactate [215].

Here, we exploit an ODE-based system model to describe various mechanisms including fusion, receptor-mediated endocytosis, macropinocytosis and phagocytosis associated with EV uptake by cells [76] (Figure 38). EV fusion is accomplished by merging EVs with the cell membrane and releasing their content into the EV recipient cell cytoplasm. On the other hand, receptormediated pathways such as clathrin-mediated- and caveolae-mediated endocytosis require a ligand on the EV membrane surface to employ specific receptors on the cell plasma membrane and leverage binding and internalization. We assume that the EV can be degraded in the environment via a first-order chemical reaction mechanism of the form

$$A \xrightarrow{k_{\rm h}} \varnothing, \qquad (134)$$

where 'A' indicates the unbound EVs and  $k_h$  is the degradation (half-life) chemical reaction rate. We also assume that the EV may reversibly react with a receptor 'R' to form an activated EV-receptor complex 'AR', via a second-order chemical reaction mechanism of the form

$$A + R_{i} \xrightarrow{k_{b}} AR \xrightarrow{k_{i}} P + R, \qquad (135)$$

where  $k_{\rm b}$ ,  $k_{\rm r}$ , and  $k_{\rm i}$  are the binding-, recycling-, and internalization chemical reaction rate, respectively. Furthermore, 'P' denotes an EV finally internalized into the cell. Here we assume the number of the recipient cells is constant during the simulations and the chemical reaction rates are time-independent. Also, we assume that the uptake mechanism is not influenced by the number of initial EVs in the medium where the recipient cells are cultured [222].

Following (134) and (135), the dynamics of bound and environmental

EVs are given by the following ODEs:

$$\frac{\mathrm{d}q_{\mathrm{AR}}(t)}{\mathrm{d}t} = k_{\mathrm{b}}f(t) - k_{\mathrm{r}}q_{\mathrm{AR}}(t) - k_{\mathrm{i}}q_{\mathrm{AR}}(t), \qquad (136)$$

and

$$\frac{dq_{\rm A}(t)}{dt} = -k_{\rm b}f(t) + k_{\rm r}q_{\rm AR}(t) - k_{\rm h}q_{\rm A}(t), \qquad (137)$$

respectively, satisfying the following initial conditions:

$$q_{\rm A}(0) = Q_0, \quad q_{\rm AR}(0) = 0.$$
 (138)

The term  $q_A(t)$  is the number of EVs in the environment (initially set to  $Q_0$ ) and  $q_{AR}(t)$  is the number of EVs bound to the cells.

We explore how limited receptor sites affect the dynamics of EVs using a function defined as

$$f(t) = [N - q_{\rm AR}(t)] q_{\rm A}(t), \qquad (139)$$

where N is the total number of receptor sites per cell. When EVs are already bound to the receptors on the recipient cell membrane, the binding probability of the remaining EVs decreases. To account for this effect, we introduce a modification factor, given by  $[N - q_{AR}(t)]$  in (139), which modifies the binding rate. This generates nonlinear terms in the equations for the binding of EVs to the cell membrane, namely (136) and (137). We discuss how to determine f(t) using an iterative approach in Section 8.5.4.

## 8.5.3 Computational Approaches

We exploit a frequency domain approach to derive the closed-form expressions for the chemical rates estimation. This approach imposes low computational cost and provides a platform to characterize the binding, recycling and internalization rates simultaneously. The input data are supposed to be provided for the environmental EVs which are quantified by PBS when combining the approach with *in silico* modeling or NTA when combining the approach with *in vitro* modeling. Taking the Fourier transform of (136) and (137) yields

$$j\omega\tilde{Q}_{\rm AR}(j\omega) = k_{\rm b}\tilde{F}(j\omega) - k_{\rm r}\tilde{Q}_{\rm AR}(j\omega) - k_{\rm i}\tilde{Q}_{\rm AR}(j\omega), \qquad (140)$$

and

$$j\omega\tilde{Q}_{\rm A}(j\omega) = -k_{\rm b}\tilde{F}(j\omega) + k_{\rm r}\tilde{Q}_{\rm AR}(j\omega) - k_{\rm h}\tilde{Q}_{\rm A}(j\omega) + Q_0, \qquad (141)$$

respectively, where  $\tilde{Q}_{AR}(j\omega)$ ,  $\tilde{F}(j\omega)$  and  $\tilde{Q}_A(j\omega)$  denote the Fourier transform of  $q_{AR}(t)$ , f(t) and  $q_A(t)$ , respectively. A closed-form expression for  $\tilde{Q}_{AR}(j\omega)$  is obtained from (140) in terms of  $\tilde{F}(j\omega)$  as

$$\tilde{Q}_{\rm AR}(j\omega) = \frac{k_{\rm b}\tilde{F}(j\omega)}{j\omega + k_{\rm r} + k_{\rm i}},\tag{142}$$

and a useful equation for the chemical rates estimation is derived from (141) as

$$H(j\omega) = \frac{F(j\omega)}{(j\omega + k_{\rm h})\tilde{Q}_{\rm A}(j\omega) - Q_0} = -\frac{j\omega + k_{\rm r} + k_{\rm i}}{j\omega k_{\rm b} + k_{\rm b}k_{\rm i}}.$$
 (143)

We assume that  $k_{\rm h}$  is known, as the half-life of EVs is a well-studied and easily investigated issue. In principle,  $k_{\rm h}$  can also be estimated by reformulating (143), although this will increase the overall rate estimation error. Eq. (143) provides a way to extract closed-form expressions for the reaction rates, as we explain below. This advantage significantly reduces the estimation time, unlike numerical methods in the time domain and statistical approaches.  $H(j\omega)$  is a complex function that can be expressed in the following form

$$H(j\omega) = R(\omega) + jI(\omega), \qquad (144)$$

where  $R(\omega)$  and  $I(\omega)$  denote the real and imaginary parts of  $H(j\omega)$  given by

$$R(\omega) = -\frac{\omega^2 + k_{\rm r}k_{\rm i} + k_{\rm i}^2}{k_{\rm b}(\omega^2 + k_{\rm i}^2)},\tag{145}$$

and

$$I(\omega) = \frac{\omega k_{\rm r}}{k_{\rm b}(\omega^2 + k_{\rm i}^2)},\tag{146}$$

respectively.  $I(\omega)$  has the peak value  $I(\omega_{\rm p}) = I_{\rm p}$  at

$$\omega = \omega_{\rm p} = k_{\rm i}.\tag{147}$$

Two more expressions could be simply derived for  $k_{\rm b}$  and  $k_{\rm r}$  in terms of  $I_{\rm p}$  given by

$$k_{\rm b} = -\frac{k_{\rm r} + k_{\rm i}}{k_{\rm i}R(0)},$$
 (148)

and

$$k_{\rm r} = -\frac{2k_{\rm i}I_{\rm p}}{R(0) + 2I_{\rm p}},\tag{149}$$

respectively. Thus, we have closed-form solutions for the chemical reaction rates, which depend on only two points of  $H(j\omega)$ , the DC component and the maximum of the imaginary component. According to (143), we need to specify  $\tilde{F}(j\omega)$  before estimating the rate parameters. This is discussed in the following subsection through an introduced iterative approach.

### 8.5.4 Iterative Approach

Here we propose an iterative approach to evaluate  $\tilde{F}(j\omega)$ . Given  $\tilde{F}^{(i)}(j\omega)$  as the estimated function at the *i*<sup>th</sup> iteration,  $H^{(i)}(j\omega)$  can be expressed as

$$H^{(i)}(j\omega) = \frac{\tilde{F}^{(i)}(j\omega)}{(j\omega + k_{\rm h})\,\tilde{Q}_{\rm A}(j\omega) - Q_0},\tag{150}$$

where  $\tilde{Q}_{\rm A}(j\omega)$  and  $Q_0$  are either measured by PBS or NTA, and  $\tilde{F}^{(i)}(j\omega)$  is assumed to be known from the  $(i-1)^{\rm th}$  iteration.  $H^{(i)}(j\omega)$  is updated at each iteration leading to the new estimated parameters i.e.,  $k_{\rm b}^{(i)}$ ,  $k_{\rm r}^{(i)}$ ,  $k_{\rm i}^{(i)}$  given by (147)-(149). This approach is here referred to as the iterative closed-form (Iter. CF) approach, where the rate parameters are calculated by the closedform expressions. The closed-form solution depends only on two points in the spectral signal and is thus quite sensitive to variations of them. Therefore, we propose a second method, termed iterative fitting (Iter. FIT), where we estimate the reaction rates by applying curve fitting to (150) instead of using the closed-form solution. Since the curve fitting is based on several samples, Iter. FIT is more robust against variations in the spectral signal. However, it encounters more computational costs and needs the initial guess of the reaction rates.

In both iterative approaches,  $k_{\rm b}^{(i)}$ ,  $k_{\rm r}^{(i)}$ , and  $k_{\rm i}^{(i)}$  are used to calculate a new estimate  $\tilde{F}^{(i+1)}(j\omega)$ . In order to determine  $\tilde{F}^{(i+1)}(j\omega)$ , we combine (139) and (142) to derive an operator equation given by

$$q_{\rm A}(t) = \left[\frac{\mathcal{I}}{N} + \mathcal{A}^{(i)}\right] \left\{ f^{(i+1)}(t) \right\},\tag{151}$$

where  $\mathcal{I}\{\cdot\}$  is the identity operator and  $\mathcal{A}^{(i)}\{\cdot\}$  is given by

$$\mathcal{A}^{(i)}\{\cdot\} = \frac{q_{\rm A}(t)}{N} \mathcal{F}^{-1}\left\{\frac{k_{\rm b}^{(i)}}{j\omega + k_{\rm r}^{(i)} + k_{\rm i}^{(i)}} \mathcal{F}\{\cdot\}\right\}.$$
(152)


Figure 39: Flowchart of the iterative approach.

The operators  $\mathcal{F}$  and  $\mathcal{F}^{-1}$  indicate the Fourier transform and the inverse Fourier transform, respectively.<sup>4</sup> Also,  $f^{(i+1)}(t)$  denotes the estimated f(t) at the  $(i+1)^{th}$  iteration. We apply the estimated rate factors  $k_{\rm b}^{(i)}, k_{\rm r}^{(i)}$ , and  $k_{\rm i}^{(i)}$ in (152) and solve (151) using the gradient descent method (GDM) to find the updated value  $f^{(i+1)}(t)$ . At each iteration, we compare the relative errors of estimated rates with a predefined tolerance  $\epsilon$  and make a decision about continuing or ending the iterative process. We assume the initial value  $f^{(0)}(t)$ to be equal to  $q_{\rm A}(t)$ . The flowchart for the proposed approach is illustrated in Figure 39.

However, the iterative process suffer from an accumulated estimation error by error propagation, especially for small values of N. Smaller values of N result in stronger nonlinearity and lower power of f(t), as given by (139). Consequently, imprecise rate estimates will lead to a larger estimation error of  $f^{(i+1)}(t)$ . This yields inaccurate  $H^{(i+1)}(j\omega)$  given by (150), which once again gets back to estimation of  $f^{(i+2)}(t)$  through (151). We will compare the performance of Iter. CF and Iter. FIT in terms of N in Section 8.5.8.

 $<sup>^{4}\</sup>mathrm{Here},$  we use a calligraphic notation to indicate an operation on a function rather than a variable.

#### 8.5.5 Michaelis-Menten Approach

As discussed in Section 8.5.4, convergence of the iterative approach towards precise rate estimates is challenging when the number of free sites on the target cells, N, is small compared to the environmental EVs. Thereby, we exploit an alternative approach based on *Michaelis-Menten* (MM) kinetics, which is one of the most commonly used models of enzyme kinetics, and discuss its performance through error analysis simulations. Based on the MM kinetics, we derive an equation for the rates estimation which exclude  $F(j\omega)$ and is not significantly influenced by the noise. The determination of reaction rates based on MM kinetics typically requires a couple of measurements at different initial environmental EV concentrations. From the observation of the internalized EVs, the initial reaction rate is derived as a function of initial environmental EV concentrations. Finally, the reaction rates are obtained using a nonlinear regression of the results to MM equation. By using approximate Bayesian computation, the number of measurements can be reduced to the measurement at a single initial environmental EV concentration [223]. In contrast, the approach presented in the following exploits MM kinetics in the frequency domain and is based only on the temporal observation of environmental EVs at a single given initial concentration. Consequently, experimental determination of internalized EVs is not necessary in our approach.

The MM model is obtained under the quasi-steady-state assumption in which the concentration of bound EVs does not change in the time-scale of the internalization process. This condition is well-satisfied when the number of binding sites is small compared to the number of initial EVs. Mathematically, this means  $\frac{\partial q_{AR}(t)}{\partial t} = 0$  and (136) is then transformed to

$$0 = k_{\rm b} \left( N - q_{\rm AR}(t) \right) q_{\rm A}(t) - k_{\rm r} q_{\rm AR}(t) - k_{\rm i} q_{\rm AR}(t) \,. \tag{153}$$

Replacing  $q_{AR}(t)$  in (137) using (153) and solving the equation in the frequency domain, after some simple manipulations, yields

$$\left(\left(j\omega+k_{\rm h}\right)k_{\rm r}+\left(j\omega+k_{\rm b}N+k_{\rm h}\right)k_{\rm i}\right)\tilde{Q}_{\rm A}(j\omega)+\left(\frac{j\omega k_{\rm b}}{2}+k_{\rm b}k_{\rm h}\right)\tilde{Q}_{\rm s}(j\omega)=\left(\frac{k_{\rm b}Q_{\rm 0}}{2}+k_{\rm r}+k_{\rm i}\right)Q_{\rm 0},\quad(154)$$

where,

$$\tilde{Q}_{\rm s}(j\omega) = \mathcal{F}\left\{q_{\rm A}(t)\,q_{\rm A}(t)\right\}.\tag{155}$$

Eqs. (154) and (155) provide the new fitting equations for the rate estimations. Because this approach does not require approximating the nonlinear part of the model (i.e. f(t)) iteratively, it is computationally faster than the iterative approach. The performance of all presented approaches is compared in terms of N in Section 8.5.8.

#### 8.5.6 Linear Model

When the number of receptor sites, N, is sufficiently large compared to the number of bound EVs,  $q_{AR}(t)$  can be ignored in f(t) given by (139), and the dynamics of  $q_{AR}(t)$  and  $q_A(t)$  are approximated by the following linear ODEs:

$$\frac{\mathrm{d}q_{\mathrm{AR}}(t)}{\mathrm{d}t} \approx k_{\mathrm{b}} N q_{\mathrm{A}}(t) - k_{\mathrm{r}} q_{\mathrm{AR}}(t) - k_{\mathrm{i}} q_{\mathrm{AR}}(t) , \qquad (156)$$

$$\frac{\mathrm{d}q_{\mathrm{A}}(t)}{\mathrm{d}t} \approx -k_{\mathrm{b}}Nq_{\mathrm{A}}(t) + k_{\mathrm{r}}q_{\mathrm{AR}}(t) - k_{\mathrm{h}}q_{\mathrm{A}}(t) \,. \tag{157}$$

In this way,  $H(j\omega)$  in (143) is rewritten as

$$H(j\omega) = \frac{N\tilde{Q}_{\rm A}(j\omega)}{Q_0 - (j\omega + k_{\rm h})\tilde{Q}_{\rm A}(j\omega)}.$$
(158)

and the rate parameters are obtained using (147)-(149) accordingly. Please note that the iterative approach that addresses the estimation of  $\tilde{F}(j\omega)$  is not relevant for the linear model.

#### 8.5.7 Particle-based Simulation (PBS)

To analyze the performance of the rate estimation methods presented in Section 8.5.3, we use numerical particle-based simulations, where a particle here is equivalent to an EV. PBS is a cost-effective and a more precise/highresolution alternative to experiments, which are often expensive in terms of time and resources. PBS results resemble the experimental data from the microscopic point of view, in our case the interaction between EVs and the cell's receptors and their internalization. Due to the full control of the relevant parameters, PBS is well suited for parameter studies such as the one performed in Section 8.5.8. Besides the time and resource consuming effort, the direct control of all parameters as well as the determination of reference parameters in experiments is crucial. In contrast to the ODE solution according to (136) and (137) which describes the mean expected behavior, in PBS the reaction statistics are applied to each particle. Thus, randomness is introduced and an individual result is obtained for each realization, which deviates from the ODE solution. We denote the deviation of the PBS results from the ODE solution as noise.

We focus our PBS on the degradation and uptake mechanism given in (134) and (135). In this context, we assume that a total of N receptor sites are available in the environment to interact with EVs. These receptor sites can be either from a single cell or from multiple cells. Furthermore, we assume that all environmental EVs are subject to the same reaction rates and are present within the range of the receptor sites all the time and thus are available for interaction. By this assumption we can neglect the spatial component and focus on the reactions according to (134) and (135). Depending on whether an EV is in the environment or bound to a receptor site, it can react via two (pseudo) first-order pathways. Based on the reaction rates, we can determine probabilities for the reaction of an EV within a time interval  $\Delta t$  [224]:

$$p_{\rm h} = \frac{k_{\rm h}}{k_{\rm h} + k_{\rm b^*}} \left( 1 - e^{-\Delta t (k_{\rm h} + k_{\rm b^*})} \right), \tag{159}$$

$$p_{\rm b} = \frac{k_{\rm b^*}}{k_{\rm h} + k_{\rm b^*}} \left( 1 - e^{-\Delta t (k_{\rm h} + k_{\rm b^*})} \right), \tag{160}$$

$$p_{\rm r} = \frac{k_{\rm r}}{k_{\rm r} + k_{\rm i}} \left( 1 - e^{-\Delta t (k_{\rm r} + k_{\rm i})} \right), \qquad (161)$$

$$p_{\rm i} = \frac{k_{\rm i}}{k_{\rm r} + k_{\rm i}} \left( 1 - e^{-\Delta t (k_{\rm r} + k_{\rm i})} \right), \qquad (162)$$

$$k_{\rm b^*} = k_{\rm b} \left( N - q_{\rm AR}(t) \right),$$
 (163)

where  $p_{\rm h}$ ,  $p_{\rm b}$ ,  $p_{\rm r}$ ,  $p_{\rm i}$  are the degradation (half-life), effective binding, recycling, and internalization probabilities of an EV, respectively. Furthermore,  $k_{\rm b^*}$ denotes the effective binding rate, which depends on the binding rate and on the number of free receptor sites. It allows us to interpret the binding reaction of an EV as a pseudo first order reaction. In PBS, for discrete time steps of length  $\Delta t$  for each EV, the probability of the possible chemical reactions is evaluated by a Bernoulli random variable where the probability of success is equal to the probability of reaction. If a reaction occurs, the number of environmental and/or bound EVs, as well as the number of free receptor sites, is adjusted for the next discrete time step.

#### 8.5.8 Numerical Results

In this section, we investigate the validity of the approaches presented in Section 8.5.3 and their estimation errors by numerical particle-based simulations

colorblackdiscussed in Section 8.5.7. The curve fitting for non-negative parameter estimation in the Iter. FIT and MM approaches is performed by the MATLAB Optimization  $Toolbox^{TM}$ , which uses a trust-region-reflective method [159]. For our considered parameters discussed in Section 8.5.9, the initial guesses of the curve fitting algorithm applied in the Iter. FIT and MM approaches are chosen to be  $10^{\circ}$  for  $k_i$  and  $10^{-1}$  for  $k_b$  and  $k_r$ . These are not to be confused with the initialized rates  $k_b^{(-1)} = k_r^{(-1)} = k_i^{(-1)}$  for the initial tolerance check in Figure 39. All rates in the curve fitting algorithm are upper bounded by 10. When performing curve fitting, we do not consider the entire spectral signal, but only the region in which 97% of the spectral energy of  $\hat{Q}_{\rm A}(j\omega)$  in MM or of the denominator of  $H(j\omega)$  in Iter. FIT lies (typically at low frequencies). The Iter. CF and linear approach depend only on the DC part and the maximum of the imaginary part of  $H(j\omega)$  whose angular frequency is equal to the internalization rate (see (147)). By observing  $q_A(t)$ , we can roughly estimate the order of magnitude of  $k_i$ . Therefore, we generously restrict the considered frequency range for these two approaches in our scenario to  $|\omega| < 10$ . By this procedures we prevent a large influence of low energy signal components on the curve fitting and numerical edge effects.

In order to evaluate the performance of the different proposed approaches, we utilize the normalized mean squared estimation error (NMSEE) which is defined as

$$\overline{\text{NMSEE}} = \frac{1}{N_{\text{MC}}} \sum_{n=0}^{N_{\text{MC}}-1} \left| \frac{k - \hat{k}}{k} \right|^2, \qquad (164)$$

where  $N_{\rm MC}$  is the number of PBS realizations and  $\hat{k}$  is the estimated rate.

#### 8.5.9 Parameter Selection

The simulation parameters and rate parameters under consideration are given in Table 7. We use the listed parameters as reference parameters, which are assumed for the numerical simulations if the parameter is not varied. Inspired by [225], we consider a dimensionless scenario which is more general and compact since units are omitted. To achieve this, all concentrations

Parameters	Symbol	Value	Ref.
Binding rate	$k_{\rm b}$	0.13	[225]
Recycling rate	$k_{ m r}$	0.11	[226]
Internalization rate	$rac{k_{ m i}}{ar{k}_{ m i}}$	$\frac{1}{0.0046{\rm s}^{-1}}$	[225]
Half-life rate	$k_{ m h}$	0	[225]
Binding sites	Ν	0.1 (MM) 1 (Iter. FIT) 10 (Iter. CF) 10 (Lin.)	
Initial EV quantity	$egin{array}{c} Q_0 \ ar Q_0 \end{array}$	$1 \\ 10^5$	[225]
Samples in PBS	$N_{\rm s}$	$10^{5}$	
PBS Monte Carlo runs	$N_{ m MC}$	$10^{3}$	
Tolerance	$\epsilon$	$10^{-3}$	

Table 7: Default simulation parameters, which are applied throughout the numerical results, if not stated otherwise. Parameters with a bar denote dimensional parameters.

are given relative to the initial environmental EV concentration  $\bar{Q}_0$  and all temporal parameters are given relative to  $\bar{k}_i$ . Accordingly, the dimensionless parameters can be determined as

$$Q_{0} = \frac{\bar{Q}_{0}}{\bar{Q}_{0}}, \quad N = \frac{\bar{N}}{\bar{Q}_{0}}, \quad k_{\rm b} = \frac{\bar{k}_{\rm b}\bar{Q}_{0}}{\bar{k}_{\rm i}}, \quad k_{\rm h,r,i} = \frac{\bar{k}_{\rm h,r,i}}{\bar{k}_{\rm i}}, \quad t = \bar{k}_{\rm i}\bar{t}.$$
(165)

In [225],  $\bar{k}_b$  is given in units of mL/molecule/s and the reactant in molecule/mL. In PBS, we work with the absolute number of EVs and receptor sites, which needs to be taken into account in the calculation of the dimensionless binding rate  $k_b$ . However, this is not important for the evaluation of our estimation methods and we therefore omit the calculation and directly adopt the dimensionless parameter from [225] for the sake of simplicity. The variation of individual parameters can influence the total time until all environmental EVs are internalized and thus the temporal dynamics. To counteract this effect and capture the full dynamics of the signal, we consider in the simulations the period adaptive between zero and ten times the duration that is



N = 0.1.

theoretically needed to internalize 90% of all EVs based on (136) and (137). We divide this range into  $N_{\rm s}$  equally distributed samples. As an example, for the scenario in Figure 40 this results in a total time of  $10 \times 163 = 1630$  with a step size of  $\Delta t = 0.0163$ . It should be noted that in PBS  $N_{\rm s}$  is limited only by hardware, whereas in laboratory measurements the sampling rate is determined by the available equipment and the temporal effort. A sufficient required time resolution of the laboratory measurements can nevertheless be achieved by interpolation between and/or extrapolating beyond the measuring points.

Figure 40 shows  $q_A(t)$  and  $q_{AR}(t)$  for a single realization of the PBS. As a comparison, the ODEs (136) and (137) are numerically solved as well. It can be observed that the noisy PBS simulation follows the ODE solution.

#### 8.5.10 Parameter Studies

In the following, we examine the performance of the rate estimation approaches introduced in Section 8.5.3 with respect to the parameters. Figure 41 shows the performance of the four different approaches with varying



Figure 41: Numerical results. Normalized mean squared estimation error for the reaction rates with respect to number of binding sites of the cells. The considered parameters are given in Table 7.

number of binding sites N. It should be mentioned that N represents the ratio of binding sites to initial environmental EVs. This can be achieved experimentally by changing the number of binding sites N, for example, by varying the number or type of cells. On the other hand, the initial environmental EV concentration  $Q_0$  can be increased or decreased. From Figure 41, different regions can be identified where each approach is preferable and provides useful rate estimates. Depending on N, all approaches have their justification. For better visualization, we have highlighted three regions in color and labeled them with the approaches that give an NMSEE  $< 10^{\circ}$ at all three rate estimates. The MM approach performs best in the region with low N. In this region, the governing MM kinetics assumption that the substrate concentration is much larger than the enzyme concentration is fulfilled and the quasi-steady-state assumption is satisfied. The maximum of the MM approach observed for  $k_{\rm b}$  and  $N = 10^0$  is due to the upper bound of all rates in the curve fitting algorithm. From  $N = 10^0$  the estimate of  $k_i$ reaches this upper bound, which is compensated by a decreasing NMSEE for  $k_{\rm b}$  as N increases. A low N also means a more dominant nonlinear part in (139). This leads to poor performance of both the iterative approach and the linear approach. Conversely, the influence of the nonlinear part in (139)decreases as N increases. This improves the rate estimation of the iterative approach as well as the rate estimation of the linear approach, which is based only on the linear part in (139). The iterative approach provides the best rate estimates for the parameters under consideration especially in the range  $10^{-1} < N < 10^2$ . Iter. FIT outperforms Iter. CF clearly for  $N < 10^1$ . In this range the spectral signal becomes more noisy. While the closed-form solution depends only on two points in the spectral signal and is therefore sensitive to noise, curve fitting based on numerous points offers more robustness against noise. Furthermore, it can be concluded from Figure 41 that, for the assumed scenario, the estimation of  $k_{\rm b}$  is more accurate than the estimation of  $k_{\rm r}$  and  $k_{i}$ .

In the remaining analysis, we investigate the influence of individual system parameters on the performance of the rate estimation approaches. In doing so, we set N for each individual approach to a value where the respective approach performs well, as listed in Table 7. Figure 42 shows the influence of the initial number of environmental EVs,  $\bar{Q}_0$ , on the NMSEE when estimating  $k_{\rm b}$ ,  $k_{\rm r}$  and  $k_{\rm i}$ . Since N is fixed,  $\bar{N}$  changes accordingly. For all rates, the estimation improves with increasing  $\bar{Q}_0$ . This is to be expected, since the deviation from the expected value decreases with increasing number



Figure 42: Numerical results. Normalized mean squared estimation error for the reaction rates with respect to initial EV quantity in the medium. The considered parameters are given in Table 7. Since N is fixed, the dimensional number of binding sites changes accordingly.

of EVs in PBS. In other words, the PBS result approaches the solution given in (136) and (137) as  $\bar{Q}_0$  increases. For the scenario under investigation, at least  $\bar{Q}_0 = 10^4$  EVs for MM approach, at least  $\bar{Q}_0 = 10^{4.5}$  EVs for Iter. FIT, and at least  $\bar{Q}_0 = 10^5$  EVs for Iter. CF and linear approach should be selected to bring the NMSEE of all rates below  $10^0$ .

Our proposed rate estimation approaches require knowledge of N and  $Q_0$ . While  $\bar{Q}_0$  is comparatively easy to measure directly,  $\bar{N}$  can be determined by additional saturation binding assay experiments and estimated for the final experiment. However, both are experimentally determined values that involve a certain degree of imprecision. Figure 43 illustrates this influence on the rate estimate when  $Q_0$  is overestimated or underestimated indicated by  $\hat{Q}_0$ . Note that  $Q_0$  is dimensionless and N is given relative to it. Thus, an overestimation of  $Q_0$  can also be interpreted as an underestimation of N,



Figure 43: Numerical results. Normalized mean squared estimation error for the overestimation (negative relative estimation error) and underestimation (positive relative estimation error) of the initial EV quantity in the well. The considered parameters are given in Table 7.

and an underestimation of  $Q_0$  as an overestimation of N as well. Figure 43 shows that over- or underestimation mainly affects the NMSEE of  $k_b$ . This observation is consistent with previous analysis [204]. The reason is that the binding process described by  $k_b$  is directly influenced by  $Q_0$ , while  $k_r$  and  $k_i$  depend only on the bound EVs. For the assumed scenario, the NMSEE is below 10<sup>0</sup> for all rate estimates even with an over- or underestimation of  $Q_0$  up to 30%. This result demonstrates that the proposed approaches are robust to an imprecise determination of  $Q_0$  and N.

Besides the determination of  $Q_0$  and N, the experimental measurement of  $q_A(t)$  will also be subject to measurement noise. Reasons for this are, for example, variations of environmental parameters like temperature or imperfection of the used measuring instruments. To investigate the influence of these macroscopic noise sources on the rate estimation, we add a zero-mean



Figure 44: Numerical results. Normalized mean squared estimation error for the reaction rates with respect to additive white Gaussian noise variance. The considered parameters are given in Table 7.

additive white Gaussian noise with a variance of  $\bar{\sigma}^2$  to the PBS samples. Please note that  $\bar{\sigma}^2$  is given in dimensional form. Figure 44 shows the impact of different  $\bar{\sigma}^2$  values on the performance of each approach. As expected, the performance of all rate estimation approaches decreases as the noise power increases. However, noise affects the different estimates differently. The multiplication  $j\omega \tilde{Q}_A(j\omega)$  in the MM and Iter. FIT approach leads to an increased noise power with increasing frequency. Increasing  $\bar{\sigma}^2$  leads to a wider considered frequency range in which 97% of the energy lies. The noise samples falling into the extended frequency range thus degrade the rate estimation using curve fitting. In contrast, Iter. CF and the linear approach are based on closed-form expressions and the relevant frequency range is fixed. The noise here is mainly manifested by a noisy spectral signal, which with increasing  $\bar{\sigma}^2$  affects the amplitude values and the location of the maximum in the imaginary part of  $H(j\omega)$ , which are essential for this estimation ap-



Figure 45: Numerical results. Normalized mean squared estimation error for the reaction rates with respect to reaction rate value. The considered parameters are given in Table 7.

proach. As can be seen from Figure 44, the MM approach shows the highest robustness to the added measurement noise, whereas the Iter. FIT approach is the most vulnerable.

Figure 45 shows the effect on the NMSEE of  $k_{\rm b}$ ,  $k_{\rm r}$ , or  $k_{\rm i}$  when these are varied over a wide range, respectively. For the MM approach, it is noteworthy that the NMSEEs for  $k_{\rm b}$  and  $k_{\rm r}$  have a clear minimum at  $10^{-1}$ . This can be explained by the fact that the initial point of the curve fitting algorithm for these rates is chosen exactly at  $10^{-1}$ . While the MM approach gives poor estimates at low rates for the scenario under investigation, the other three approaches show a more robust behavior, at least for  $k_{\rm b}$  and  $k_{\rm i}$ . Both rates provide an NMSEE of less than  $10^0$  for the considered scenario. Only  $k_{\rm i}$  shows a higher NMSEE for  $k_{\rm i} < 10^{-1}$ . The results demonstrate that the proposed approaches are strongly dependent on the chosen parameters. Especially due to the nonlinearity in the system, certain parameter combinations can lead to



Figure 46: Numerical results. The average number of required iterations of the iterative approach with respect to number of binding sites of the cells. The considered parameters are given in Table 7.

high estimation errors. Likewise, increasing either  $\bar{Q}_0$  (less noise in the PBS),  $N_s$  (wider frequency range) or the total observation time (higher frequency resolution) can lead to an improvement of the rate estimates.

## 8.5.11 Computational Complexity Analysis

In the following, a comparison of the computational complexity of the proposed approaches is given. Among all considered approaches, the curve fitting algorithm is the one with the highest computational complexity, followed by the gradient descent method and the calculation of the closed-form expressions. Consequently, the linear approach has the least computational complexity. If Iter. CF requires multiple iterations, its computational complexity is higher than that of a single curve fitting call in the MM approach. One iteration of the Iter. FIT approach is significantly more complex than an Iter. CF iteration. The number of iterations depends strongly on the chosen error tolerance  $\epsilon$ , as well as on the general scenario. For the scenario under investigation and the parameter study from Figure 41, the average number of required iterations of the iterative approaches are shown in Figure 46. It can be seen that a maximum of 10 and 16 iterations on average are required for the Iter. CF and Iter. FIT, respectively. It can also be observed that the number of required iterations decreases for small and large values of N, and is generally similar for both approaches.

We designed original computational methods for the estimation of chemical reaction rates involved in the EV signaling pathway, particularly associated with the process of EV uptake by recipient cells. They are meant to be combined with a simple experiment, where the recipient cells are cultured in a well and mixed with the known number/concentration of the EVs, from which data are acquired. Our proposed approaches exploit only the monitored number of EVs in the medium where the cells are cultured, without any need to monitor the EVs that either bound to- or internalize into the recipient cells. For the purpose of demonstrating the validity of our approaches, we used particle-based simulations for generating synthetic data that resemble experimental data, thus omitting to conduct actual experiments. The performance of the proposed methods is evaluated and thoroughly discussed using the normalized mean squared estimation error. We managed to obtain the normalized mean squared estimation error for the EV binding rate of less then 10%, even with an overestimation or underestimation of the number of initial EVs up to 30%; this is the highest precision among the estimated rates. Future work will consist in combining our model with real data to identify adequate mathematical models of EV signaling more finely. This will largely help the development of EV-mediated therapy and EV-mediated molecular communication links.

# 9 Part II - Characterization of a cellular communication channel using marked liposomes into a microfluidic systems

In this section we will present an original strategy that uses microfluidic systems to convey valuable information about the variation of colored droplets within an oil matrix. These droplets are analogous to synthetic extracellular vesicles (EVs) or liposomes, sharing the same characteristics highlighted in previous models.

As discussed previously in Section 5.3, the information transmitted is no longer the contents of the EV, but the size of the vesicle itself and its movement. This strategy is based on the idea that knowing how vesicles migrate in our body can help in structuring targeted therapies and/or drugs transported by the liposomes themselves. Furthermore, it would be possible to imagine a microcatheter containing drops in an insoluble and incompressible matrix, isolated from the external environment but sensitive to pressures coming from outside. If implanted in the human body, it could provide real-time and immediate information about blood pressure, heart rate, inflammation, etc. This could be combined with a treatment with liposomes *in loco*, where necessary, reducing possible side effects.

An original methodology will be proposed in Section 9.1, where the existence of low error rate pressure pairs in information transmission is demonstrated, and modeled in a specific context in Section 9.1.5.

# 9.1 Droplet\_speed-shift keying: a modulation scheme for instantaneous microfluidic communications

Molecular Communications promise a plethora of applications in biomedicine [227], [228]. In this context, very interesting opportunities arise, in particular, from the use of microfluidic systems. In fact, microfluidic channels with a diameter in the order of micrometers can be thought as molecular waveguides [229]. An example of such systems is the Lab-on-a-Chip (LoC), which manipulates small volumes of fluids for chemical and biological analysis and synthesis, with application to controlled drug delivery [230] or diagnostic testing [231]. In this perspective, a new line of research has emerged that combines microfluidics with clinical biology, medicine and physics [34].

Moreover, in these range of dimensions, molecular consumption is significantly reduced with respect to other traditional macroscale applications. At the microfluidic sizes, capillary forces allow "free" movement of fluids into the channel, but it is necessary to employ one or more pumps to generate and maintain a specific pressure or to add multiple types of reagents into the system [39], [40].

In this work we propose a solution, called *Droplet\_Speed Shift Keying* (DSSK) which overcomes the limitations caused by propagation delay inside the microfluidic communication medium. More specifically we encode information in the speed at which a train of drops in a continuous phase moves. By exploiting the stable, laminar flow of the drops, we aim at transferring data throughout the channel almost instantaneously.

#### 9.1.1 Droplet speed shift keying (DSSK)

Modulation is a fundamental technique used in telecommunications to transmit information over long distances. It involves changing the characteristics of a signal, such as its frequency, amplitude, or phase, to encode data within it [232]. This technique allows for the efficient use of the limited frequency spectrum available for communication, as multiple signals can be transmitted simultaneously without interfering with each other [233], [234]. Additionally, modulation provides a degree of security, as encoded signals can be difficult to decipher by unauthorized parties. Modulation techniques have continued to evolve over time, allowing for faster data transmission and more efficient use of communication channels.

In this context, modulation refers to the process of changing/tuning the properties of a fluid, such as its flow rate or composition, in order to encode information. The objective of DSSK is to exploit the intrinsic physical properties of the microfluidc systems, i.e., the incompressibility and linearity of the flow in an ideal system [37], [38]), to transmit information almost instantaneously over time in a non ideal channel of non-negligible length. This can be achieved by modifying the pressure or fluid mass rate at the inlet of the microfluidic channel. In fact, we expect that as the inlet pressure of the flows varies, an "instantaneous" variation in the speed of the droplets occurs throughtout the entire length of the channel immediately. Therefore, DSSK encodes information into the pressure or fluid mass rate at the input of the channel.

Since droplets are formed thanks to the interactions between a flow of



Figure 47: Droplet speed shift keying, DSSK, modulator.

the continuous phase and a flow of the dispersed phase, information will be encoded in the variations of the pair of pressures or fluid mass rate of the continuous and dispersed phases.

A scheme of the considered modulation scheme is reported in Figure 47.

In the figure, two immiscible fluids, a continuous phase and a dispersed phase are used to generate droplets in a T-junction. The speed of the droplet is controlled by two pumps that input the corresponding phases with appropriate mass flow rates. We call  $P_D(i)$  and  $P_C(i)$  the mass flow rates of the dispersed and continuous phases, respectively, at the time of transmission of the *i*-th bit,  $b_i$ . More specifically, we assume that  $P_D(i)$  is set equal to  $P_1$ while  $P_C(i)$  is set equal to  $P_2$  if the bit  $b_i$  is 0. Whereas,  $P_D(i)$  and  $P_C(i)$  are set to  $P_3$  and  $P_4$  if the bit  $b_i$  is 1.

The speed of the droplets at the receiver can be detected in several ways. In fact, lasers or cameras can be used. In our experimental setting, as described later in Section 9.1.2, we use a pair of lasers and the corresponding photo-receivers.

In order to select the most convenient pairs  $P_1 - P_2$  and  $P_3 - P_4$ , in the following sections we will execute a set of experiments that allow us to find two pairs of pressures values (or mass fluid rates) that result in droplet speeds such that they are not misinterpreted at the receiver. At the same time we want to identify such pairs in such a way that the values  $P_1 - P_2$  do not differ too much from  $P_3 - P_4$ . In fact, in this way the system will be more stable.

Objective of our study is thus to assess the feasibility of the proposed modulation techniques to identify in the most reliable way the current bit being transmitted (bit 0 or bit 1) based only on the observed instantaneous variation of the flow velocity.

#### 9.1.2 Experimental setup

In this section we describe the experimental setup utilized to assess the feasibility of the proposed approach.

As shown in Figure 48, droplets are generated exploiting a well known microfluidic geometry, i.e., the T-junction. More specifically, the microfluidic chip has been designed using Cinema4D which is a 3D modeling and animation software employed for the creation of complex and detailed shapes. Cinema4D can export the models created in STL format which is compatible with most 3D printing software such as the high resolution Stereolithography (SLA) 3D printing we have then used.

The microfluidic chip has a width of 3 cm, a length of 8 cm, and a height of 0.25 cm. Inside the chip, there are two independent and identical rectangular T-junctions with a diameter of 600  $\mu$ m for the dispersed phase channel and 700  $\mu$ m for the continuous phase flow channel (main channel). The fluids are injected into the microchannels using lateral connectors through flexible plastic tubes. The microfluidic chip is characterized by a T-junction angle of 90°, which enhances the stability of droplet formation. The chip has been designed to reduce the occurrence of blockages in the microchannels, which was a common issue in the microfluidic chip employed in [31].

The fluids are injected into the T-junction by exploiting two NE-1002X pumps. They are syringe pumps that use a stepper motor to drive the plunger of a syringe, allowing for precise and controlled fluid delivery. The NE-1002X has a flow rate ranging between 0.73 µL/min and 10.84 mL/min, with a maximum pressure of 30 psi. One of them pump oil which will act as the continuous phase, whereas the other will pump colored water. In particular, we used sunflower seed oil purified in water vapor current (Organic Oils S.r.l.) (dynamic viscosity  $\mu \approx 0.049$  Pa s and density  $\rho \approx 916$  Kg  $m^{-3}$ ) as continuous phase, hereinafter referred to as  $(Q_o)$ , while water colored blue with food coloring ( $\mu \approx 0.001$  Pa s,  $\rho \approx 1000$  Kg  $m^{-3}$ ) as dispersed phase, hereinafter referred to as  $(Q_c)$ . No surfactants were added to the two phases.

At the receiver side we needed to measure the velocity of the droplet. To this purpose we designed a system consisting of two laser diodes and two photoreceivers as shown in Figure 48. The laser diodes we used are two 635nm red laser diodes (Omicron-Laserage Laserprodukte GmbH, LDM635-5), which have a maximum output power of 5 mW and a beam divergence



Figure 48: Microfluidic components used in our experiments: T-junction.

of 0.6 mrad. The lasers are placed 3.5 cm apart and defocus the beam to enable the detection by the light sensor.

Two plano-convex optical lenses are placed in between the lasers and the microfluidic circuit to further shape and adjust the beam as needed. The light sensor is an AMBI sensor board with a Sharp GA1A1S201WP light sensor, which is used to detect the defocused laser beam as it passes through the microfluidic circuit. These sensors use a phototransistor to detect changes in the amount of light received when the laser is deflected by the droplets passing through the microfluidic circuit.

Lasers and photoreceivers are controlled by a Arduino board employing the ATmega328P microcontroller.

The output of the photoreceivers has been acquired and processed by a software which we implemented for this work exploiting Node-RED. The software allows to achieve relevant information such as droplet size, space between two droplets, and droplet speed. In our work, Node-RED was used to read data from the light sensor through the serial port at 115200 baud. Processed data was saved in CSV format and displayed on a web-based user interface, providing immediate visual feedback.



Figure 49: Numerical results. Droplet speed measured at the receiver vs. the droplet sequence number for two pairs of mass flow rates.

#### 9.1.3 Experimental results

In our experiments we have considered the following mass flow rates pairs: 5-3, 8-6, and  $10-6\mu l/\text{min}$ , where the first value refers to the dispersed phase (colored) and the second value refers to the continuous phase (oil).

The measurements were carried out for 3 minutes and repeated in three independent sets of experiments, in order to have a richer dataset and decrease the value of the standard deviation. The global dataset consists of approximately  $270 \cdot 10^3$  measurements.

In Figures 49 we introduce three plots. In each of them we show the values of speed measured for several droplets by considering two different pairs of mass flow rates. More specifically,

- in the top plot we show in red the speed values obtained when the mass flow rates are 5 and 3  $\mu$ l/min and in blue the speed values obtained when the mass flow rates are 8 and 6  $\mu$ l/min;
- in the central plot we show in red the speed values obtained when the mass flow rates are 5 and 3  $\mu$ l/min and in blue the speed values obtained when the mass flow rates are 10 and 6  $\mu$ l/min;
- in the bottom plot we show in red the speed values obtained when the mass flow rates are 8 and 6  $\mu$ l/min and in blue the speed values obtained when the mass flow rates are 10 and 6  $\mu$ l/min;

In all plots, obviously, on average higher values of speed are measured for pairs with higher mass flow rates (blue markers). We assume that symbol 1 is transmitted by using the pair with higher mass flow rates, whereas the symbol 0 is transmitted by using the pair with the lower mass flow rates.

Therefore, the receiver can distinguish the transmitted symbol by applying a threshold-based demodulation scheme,

- if the droplet speed is higher than the threshold  $\sigma_{\rm Th}$ , then 1 is given as output;
- if the droplet speed is lower than the threshold,  $\sigma_{\rm Th},$  then 0 is given as output.

In order to identify the most convenient value of the threshold  $\sigma_{\rm Th}$ , we assume that the speed measurements are distributed according to Gaussian distributions each characterized by the mean and variance that we measured in the related experiments. In Table 8 we report the average and standard deviation obtained for each considered pair of mass flow rates.

If this is the case, it is well known that the optimal value of the threshold  $\sigma_{\rm Th}$  is the one in which the probability density functions (pdf's) of the speed for the two pairs have the same value.

In Figures 50 we show the resulting Gaussian distribution pdfs. More specifically, in each of the three plots we report the pdfs for two pairs of flow mass rates. In particular,

in the top plot we show in red the pdf when the mass flow rates are 5 and 3 µl/min and in blue the pdf when the mass flow rates are 8 and 6 µl/min. Note that this plot corresponds to the top plot of Figure 49;

Pair	Average	Standard deviation
5-3 $\mu$ l/min	0.115	0.028
8-6 $\mu$ l/min	0.159	0.024
10-6 $\mu$ l/min	0.174	0.018

 Table 8: Statistical characteristics of the droplet speeds for different pairs of mass flow rates.

- in the central plot we show in red the pdf obtained when the mass flow rates are 5 and 3 μl/min and in blue the pdf obtained when the mass flow rates are 10 and 6 μl/min. Note that this plot corresponds to the central plot of Figure 49;
- in the bottom plot we show in red the pdf obtained when the mass flow rates are 8 and 6  $\mu$ l/min and in blue the pdf obtained when the mass flow rates are 10 and 6  $\mu$ l/min. Note that this plot corresponds to the bottom plot of Figure 49.

Note that in Figure 50 the values of the threshold,  $\sigma_{Th}$ , to be considered and the corresponding theoretical error probability  $p_E$  are those reported in the third and fourth column of Table 9. The values of the threshold in the three cases are reported in the corresponding plot of Figure 49. By using such values of the thresholds to discriminate the transmitted symbol with the experimental values we have measured the error probability reported in the fifth column of Table 9

Bit 0 pair	Bit 1 pair	$\sigma_{Th}$	Theor. $p_E$	Exper. $p_E$
5-3 $\mu$ l/min	8-6 $\mu$ l/min	0.136	0.2015	0.235
5-3 $\mu$ l/min	10-6 $\mu$ l/min	0.147	0.0995	0.0990
8-6 $\mu$ l/min	10-6 $\mu$ l/min	0.159	0.351	0.3395

Table 9: Threshold,  $\sigma_{Th}$ , and theoretical and experimental error probabilities,  $p_E$ , for different associations between symbols and mass flow rates pairs.

By comparing the error probabilities obtained theoretically and experimentally, as reported in the fourth and fifth column of Table 9, we observe that the predicted, theoretical values of the error probability are enough accurate as the difference with the experimental results is at most 3.35%. Also, we observe that, as expected the maximum error probability is obtained in the case in which the two pressure pairs are closer together, that is, when



Figure 50: **Probability distributions of droplet stream velocities.** Each distribution is calculated based on the mean and variance of the dataset, on which a normal distribution is derived.

symbol 0 is encoded with the 8-6  $\mu$ l/min pair and symbol 1 is encoded with the 10-6  $\mu$ l/min pair; conversely the minimum error probability is obtained in the case in which the two pressure pairs are further apart, that is, when symbol 0 is encoded with the 5-3  $\mu$ l/min pair and symbol 1 is encoded with the 10-6  $\mu$ l/min pair. Let us observe, however, that a good tradeoff is achieved in the case in which symbol 0 is encoded with the 5-3  $\mu$ l/min pair and symbol 1 is encoded with the 8-6  $\mu$ l/min pair.

### 9.1.4 Limitations and potential improvements

In the proposed microfluidic systems, one of the main challenges is achieving fast response times in order to ensure that the modulation technique is effective. This requires the system to be capable of quickly and accurately changing the fluid properties in response to a signal, as well as detecting these changes with high sensitivity.

One limitation of our proposed system is that the modulation speed is limited by the response time of the pumps used to generate the pressure variations. This means that there is a maximum frequency at which the system can reliably encode information. However, the exact limit will depend on various factors, such as the specific pumps used, the size of the microfluidic channels, and the properties of the fluids being used.

Another factor to consider is the maximum frequency at which the droplets can be generated and detected. This is determined by the physical properties of the fluids and the microfluidic channels, and is related to the maximum velocity at which the droplets can be reliably detected by the lasers.

Despite these limitations, there are many examples of microfluidic systems capable of achieving very high modulation speeds. For example, in the field of automotive fuel injection systems, piezoelectric actuators are used to generate pressure changes with response times on the order of microseconds [235]. Similarly, in the field of inkjet printing, droplets can be generated and manipulated at frequencies of several kHz [236].

Overall, the limitations of our proposed system must be carefully considered when designing and implementing modulation techniques in microfluidic systems. However, with careful optimization of system parameters, it may be possible to achieve very high and reliable modulation rates for a wide range of applications. For this purpose we tried to optimize the system by creating a model that is inspired by an RLC circuit, adding a new pair that lowers the probability of error in confusing bit 0 with bit 1 and vice versa. This new approach will be presented in the next paragraph.

## 9.1.5 Modeling of droplet speed shift keying in microfluidic communications

Droplet microfluidics enables fluid manipulation and facilitates the study of specific reactions within controlled environments. The small dimensions of the chambers and channels in the micrometer range, combined with the physical characteristics resulting from fluid confinement in narrow spaces, allow for various chemical and biological processes to be performed using minimal liquid volumes [35]. Furthermore, at these scales, molecular consumption is significantly reduced compared to traditional macroscale applications. Capillary forces enable fluid movement within the channels, although one or more pumps are required to generate and maintain specific pressures or introduce multiple types of reagents into the system [39], [40].

A major limitation of current microfluidic systems is the slow propagation speed of fluids within the channels. Consequently, the ability to transfer information is constrained by the sluggish motion of droplets, imposing limitations on the amount of information in effective manner. In fact, droplets may need to cover distances of tens of centimeters. This may introduce a delay which may be incompatible with the requirement for prompt responses in controlled environments of certain applications.

To address these challenges, we propose a solution called DSSK, which overcomes the limitations imposed by propagation delays in the communication medium. Specifically, we encode information in the speed at which a series of dye drops move within a continuous oil phase. By leveraging the stable and laminar flow of the drops, our aim is to achieve instantaneous data transfer throughout the channel.

In order to enhance our ability to predict and explain the dynamics of microfluidic channels, we create a model of the response of the microfluidic system to variations in the flow rates. Through this model, we uncover a tradeoff between the probability of errors and the capacity of the channel.

#### 9.1.6 Droplet Speed Shift Keying a new way to send information

In the context of microfluidic communication systems, modulation refers to the process of changing or tuning the properties of a fluid, such as flow rate or composition, to encode information. The objective of DSSK, as discussed in Section 9.1.1, is to leverage the inherent physical properties of microfluidic systems, specifically the ideal incompressibility and linearity of flows [37], [38]), to achieve almost instantaneous information transmission over a nonideal channel with a considerable length.

Since droplets are formed through interactions between a continuous phase and a dispersed phase, information is encoded in variations of the pair of pressures or fluid mass rate of these phases. Figure 51 provides a sketch of the modulation scheme under consideration.



Figure 51: Droplet Speed Shift Keying, DSSK, modulator.

The droplet speed is controlled by two pumps that input the corresponding phases with appropriate mass flow rates. Let  $Q_C(i)$  and  $Q_D(i)$  represent the mass flow rates of the continuous and dispersed phases, respectively, at the time of transmitting the *i*-th bit,  $b_i$ .

Specifically, we assume that if bit  $b_i$  is 0,  $Q_D(i) = Q_1$  and  $Q_C(i) = Q_2$ . Conversely, if bit  $b_i$  is 1,  $Q_D(i) = Q_3$  and  $Q_C(i) = Q_4$ , respectively.

The speed of the droplets at the receiver can be detected using various methods, such as lasers or cameras. In our experimental setup, as described in the following sections, we employ a pair of lasers and corresponding photoreceivers.

To determine the most suitable pairs Q1-Q2 and Q3-Q4, we will conduct a series of experiments in the subsequent sections. These experiments aim to identify pressure values (or similarly mass flow rates) for the two pairs that result in droplet speeds that can be accurately interpreted at the receiver. Simultaneously, we strive to select pairs that minimize the difference between Q1-Q2 and Q3-Q4 to enhance system stability.

The objective of our study, therefore, is to assess the feasibility of DSSK considering a receiver which can only observe the instantaneous variation of flow velocity by measuring the time interval between the arrivals of two consecutive droplets.

#### 9.1.7 Microfluidic Setup

In this section, we will discuss the experimental setup used to test the feasibility of the proposed approach. About microfluidic chip, making, experimental setup and pumps used for this work, are the same reported in Section 9.1.2.

To measure the velocity of the droplet at the receiving side termination,

we developed a system illustrated in Figure 48. This system comprises two laser diodes and two photoreceivers, the same described in Section 9.1.2.

The output from the photoreceivers was captured and processed using custom software developed specifically for this purpose using Node-RED. This software facilitated the acquisition of relevant information such as droplet size, spacing between two droplets, and droplet speed. In our study, Node-RED was employed to read data from the light sensor through the serial port at a baud rate of 115200. The processed data was saved in CSV format.

#### 9.1.8 Experimental results and interpretation

In this section we present some early experimental results that can be used to assess the feasibility of DSSK. To this aim, we begin by considering the possibility to employ different pairs of input flow rates to lead to diverse sets of droplets speed pairs that can be used to encode data. Then, we observe what is the behavior of the microfluidic chip upon releasing dispersed droplets inside the continuous phase. By noting that the release process leads to oscillations, as also observed in [237], we will provide in the following section a model of the release process and a consequent analysis of conditions when a time-varying change in the used flow rates allows to encode dynamically a string of bits.

In our experimental setup, we examined various mass flow rate combinations, specifically 5-3, 8-6, 10-6 and 16-12  $\mu$ l/min. These pairs represented the pressure values for the dispersed phase (colored) and the continuous phase (oil), respectively.

Each measurement was conducted over a duration of 3 minutes and repeated in three separate sets of experiments. In this way we have generated an extensive dataset and we found average values affected by low standard deviation. Overall, the whole dataset consists of approximately 270,000 measurements, which were subsequently divided into three smaller subsets, each corresponding to one of the mass flow rate pairs.

Figures 52 present three plots, each displaying speed measurements for multiple droplets based on two different pairs of mass flow rates. The following details can be observed.

The top plot on the left shows speed values in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 8-6  $\mu$ l/min mass flow rate pair. The bottom plot on the left shows speed values in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 16-12  $\mu$ l/min mass flow rate pair. The



Figure 52: Numercial results. Droplet speed measured at the receiver vs. the droplet sequence number for two pairs of mass flow rates.

top plot on the right shows speed values in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. The bottom plot in the right shows speed values in red for the 8-6  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. Across all plots, it is evident that higher mass flow rates (indicated by blue markers) generally result in higher average speed measurements. We assume that the symbol "1" is transmitted using the pair with the higher mass flow rates, while the symbol "0" is transmitted using the pair with the lower mass flow rates.

To distinguish the transmitted symbol, the receiver applies a thresholdbased demodulation scheme, as follows.

If the droplet speed exceeds the threshold value  $\sigma_{Th}$ , the output is "1." If the droplet speed is below the threshold value  $\sigma_{Th}$ , the output is "0." To determine the optimal threshold value  $\sigma_{Th}$ , we make the assumption that the speed measurements follow Gaussian distributions. In such case, it is well-known that the optimal threshold value  $\sigma_{Th}$  is the one at which the probability density functions (PDFs) of the speed for the two pairs are equal to each other.

Therefore, we estimate the average and standard deviation of the speed for the different pairs, utilize these values to characterize the PDFs, and identify the speed value at which the two PDFs intersect.

Table 10 shows average and standard deviation obtained for each considered pair of mass flow rates.

Pair	Average	Standard deviation
5-3 $\mu$ l/min	0.115	0.028
8-6 $\mu$ l/min	0.159	0.024
$10-6 \ \mu l/min$	0.174	0.018
16-12 $\mu$ l/min	0.295	0.063

Table 10: Statistical characteristics of the droplet speeds for different pairs of mass flow rates.

However, there are instances where the speed obtained from the pairs with lower mass flow rates (red dots) is also high, which can lead to errors.

Figures 53 display the resulting PDFs (Gaussian distributions). Each of the plots represents the PDFs for two pairs of flow mass rates. The details are as follows.

The top plot on the left exhibits the PDF in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 8-6  $\mu$ l/min mass flow rate pair. It corresponds to the top plot of Figure 52. The bottom plot on the left presents the PDF in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 16-12  $\mu$ l/min mass flow rate pair. It corresponds to the central plot of Figure 52. The top plot on the right presents the PDF in red for the 5-3  $\mu$ l/min mass flow rate pair. It corresponds to the central plot of Figure 52. The top plot on the right presents the PDF in red for the 5-3  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. It corresponds to the central plot of Figure 52. The bottom plot on the right illustrates the PDF in red for the 8-6  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. It corresponds to the central plot of Figure 52. The bottom plot on the right illustrates the PDF in red for the 8-6  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. It corresponds to the central plot of Figure 52. The bottom plot on the right illustrates the PDF in red for the 8-6  $\mu$ l/min mass flow rate pair and in blue for the 10-6  $\mu$ l/min mass flow rate pair. It corresponds to the bottom plot of Figure 52.

Note that in Figure 53 the values of the threshold,  $\sigma_{Th}$ , to be considered and the corresponding error probability  $p_E$  are those reported in the third and fourth column of Table 11. The values of the threshold in the different cases are reported in the corresponding plot of Figure 52. By using such values of the thresholds to discriminate the transmitted symbol with the experimental



Figure 53: **Probability distributions of droplet stream velocities.** The PDFs report the number of samples for each flow velocity class. Each distribution is calculated based on the mean and variance of the dataset, on which a normal distribution is derived.

values we obtain the error probability reported in the fifth column of Table 11

Upon comparing the theoretically predicted error probabilities with the experimental results, as presented in the fourth and fifth columns of Table 11, we observe that the theoretical values of the error probability are reasonably accurate, with a maximum difference of 3.35% from the experimental results.

Furthermore, as anticipated, the maximum error probability occurs when symbol 0 is encoded with the 8-6  $\mu$ l/min pair, while symbol 1 is encoded with the 10-6  $\mu$ l/min pair. Conversely, the minimum error probability is observed when symbol 0 is encoded with the 5-3  $\mu$ l/min pair, and symbol 1 is encoded with the 16-12  $\mu$ l/min pair.

Bit 0 pair	Bit 1 pair	$\sigma_{Th}$	Theor. $p_E$	Exper. $p_E$
5-3 $\mu$ l/min	8-6 $\mu$ l/min	0.136	0.2015	0.235
5-3 $\mu$ l/min	$10-6 \ \mu l/min$	0.147	0.0995	0.0990
5-3 $\mu$ l/min	16-12 $\mu$ l/min	0.178	0.04	0.017
8-6 $\mu$ l/min	10-6 $\mu$ l/min	0.159	0.351	0.3395

Table 11: Threshold,  $\sigma_{Th}$ , and theoretical and experimental error probabilities,  $p_E$ , for different associations between symbols and mass flow rates pairs.

#### 9.1.9 DSSK from theory to practice

DSSK requires the system to be capable of quickly and accurately change the fluid properties in response to a signal, as well as detecting these changes with high sensitivity.

When realizing a DSSK based communication system in practice, it is necessary to consider that the description of the operations given Section 9.1.1 is based on the following assumptions:

- the changes in the flow rate are realized by the pumps immediately, that is, in a step-wise fashion. Instead, it is well known that this is not the case when the pumps are controlled by step motors. More specifically, there are studies showing the dynamics of such types of pumps are extremely complex [238]. In general, using the Laplace transform, we can model the response of such pumps, which we call the *pumps subsystem*, with a second order transfer function,  $H_{pumps}(s)$ ;
- the incompressibility of the fluids and the rigidity of the materials utilized to create the microfluidic chips are such that the flow rate changes impressed by the pumps are immediately propagated throughout the entire network of channels and thus to the receiver also. Unfortunately, this is not the case because the syringe is connected to the microfluidic device by means of small flexible tubes that have some elasticity. Furthermore, fluids are incompressible in theory only; in reality their density can change and they exhibit even some elasticity. In general we can model the resulting response of the *microfluidic subsystem* with a second order transfer function,  $H_{\text{micro}}(s)$ .

As a result, the overall system can be modeled as the series of the pump subsystem and microfluidic subsystems. This will be a fourth order system



Figure 54: Numerical results. Exemplary evolution of the droplet speed versus time in the case when the flow rates change from the pair 5-3  $\mu$ l/min to the pair 16-12  $\mu$ l/min.

and the corresponding transfer function,  $H_{\text{tot}}(s)$ , can be calculated as the product of the two, i.e.,  $H_{\text{tot}}(s) = H_{\text{pumps}}(s) \cdot H_{\text{micro}}(s)$ .

In the following of this section we present a procedure that can be used to characterize the transfer function of the whole system  $H_{\text{tot}}$ . This is important because knowledge of the system response is crucial for the implementation of the DSSK.

Then, we will present how to derive the electrical equivalent of the overall system. This is very useful to exploit the analogy between the Navier-Stokes equations regulating fluidodynamics and the equations regulating the flow of current in electrical circuits. By exploiting the electrical equivalent of a microfluidic system it becomes possible to easily predict the behavior of the system, which would be extremely complex otherwise [239].

In order to understand the rationale of the proposed approach, observe Figure 54 where we show the velocity of the droplets measured at the receiver versus time when the flow rates change from the pair 5-3  $\mu$ l/min to the pair 16-12  $\mu$ l/min. More specifically, initially the pumps are off and therefore the flow rates are described by the pair 0-0  $\mu$ l/min. Then the pumps are turned off and after a transient the flow rates become 5-3  $\mu$ l/min. Then the rates are increased to 16-12  $\mu$ l/min and finally, decreased back to the pair 5-3  $\mu$ l/min.

Now let us focus on the time interval when the flow rates change from 5-3  $\mu$ l/min to 16-12  $\mu$ l/min. In Figure 55 we show the system response, normalized in such a way that its initial value is zero and it goes to one at



Figure 55: System response when the flow rates change from 5-3  $\mu$ l/mi to 16-12  $\mu$ l/min and curve obtained by applying the fitting procedure.

the end of the transient. We observe that such response is characterized by two peaks. Therefore, we write  $H_{\text{tot}}(s)$  as the sum:

$$H_{\text{tot}}(s) = \alpha \cdot H_1(s) + (1 - \alpha)H_2(s)$$
 (166)

where  $\alpha$  is a constant in the interval ]0, 1[,

$$H_1(s) = \frac{\omega_1^2}{s^2 + 2\xi_1 \omega_1 s + \omega_1^2} \tag{167}$$

and

$$H_2(s) = \frac{\omega_2^2}{s^2 + 2\xi_2\omega_2 s + \omega_2^2}$$
(168)

The parameters of  $H_1(s)$  are set in such a way that the response fits the first peak, whereas the parameters of  $H_2(s)$  are set in such a way that the corresponding response fits the second peak, i.e.,

$$t_1^{(p)} = \frac{\pi}{\omega_1 \sqrt{1 - \xi_1^2}}$$
 and  $t_2^{(p)} = \frac{\pi}{\omega_2 \sqrt{1 - \xi_2^2}}$  (169)



Figure 56: Exemplary RLC circuit.

In eq. (169), the values  $t_1^{(p)}$  and  $t_2^{(p)}$  are the time instants of the two peaks and can be observed directly from Figure 55. In this way, the fitting procedure only requires to estimate the most appropriate values for three parameters only, i.e.,  $\xi_1$ ,  $\xi_2$ , and  $\alpha$ ; in fact,  $\omega_1$  and  $\omega_2$  can be replaced with

$$\omega_1 = \frac{\pi}{t_1^{(p)}\sqrt{1-\xi_1^2}} \quad \text{and} \quad \omega_2 = \frac{\pi}{t_2^{(p)}\sqrt{1-\xi_2^2}} \tag{170}$$

in eqs. (167) and (168), respectively.

In Figure 55 we show the curve obtained by fitting the response function applying the procedure explained above.

Now we will develop the electrical equivalent models of  $H_1(s)$  and  $H_2(s)$ . To this purpose, consider the circuit in Figure 56. It represents a standard series RLC circuit whose behavior can be described by means of the well knows transfer function given as

$$H(s) = \frac{1/(L_{eq}C_{eq})}{s^2 + sR_{eq}/L_{eq} + 1/(L_{eq}C_{eq})}$$
(171)

where

$$\frac{R_{eq}}{L_{eq}} = 2\chi \cdot w_n \frac{1}{L_{eq}C_{eq}} = w_n^2 \tag{172}$$

and  $\xi$  and w are the parameters evaluated as explained previously.

# 10 Conclusions and Future prospective

This doctoral thesis explores the intricate realm of molecular communication applied to biology, in particular, EV-mediated cellular communication. It tries to shed light on the multiple uptake mechanisms at play within target cells. To address this challenge, our research efforts focus on deciphering the complexity of interaction strategies between EVs and the plasma membrane of the target cell. To explore this little-known territory, we have developed several mathematical models, defined by a system of ordinary differential equations (ODEs). These equations map the temporal evolution of the concentrations of EVs and receptors (resulting from fusion and/or endocytosis) on the target cell membrane during these processes. Furthermore, we estimated reaction rates that align with the various endocytic and fusogenic mechanisms of EVs. The estimated parameters are very promising for channel modeling and receiver engineering in the field of EV-mediated molecular communications, demonstrating robustness in simulations. However, there is room for improvement, particularly in the context of receptor saturation and the range of changes in the rate of the chemical reaction. This research paves the way for practical applications with the aim of simplifying experimental setups and improving future efficiency of data analysis.

The paucity of literature on relevant parameters requires an innovative approach. Due to this, it is not possible to create a single universal model that can describe the uptake of vesicles by target cells, but it is necessary to create different ones for each specific key study and only then test their effectiveness in other contexts. Furthermore, the detection methods of EVs in the extracellular environment and during cellular treatments are multiple (NTA, plate reader, CytoFlow, etc.), significantly influencing the EVs count and the estimation of the parameters themselves due to the intrinsic limitations of such technologies. We propose to fill the gap by comparing the analytical solutions of our model with upcoming biological experimental results. Although this method presents challenges, our work provides a mathematical framework that can facilitate a more comprehensive exploration of EV communication mechanisms, improve the efficiency of experimental design, and contribute to the elucidation of crucial biological parameters. The parameters reported in all the tables were obtained from the literature, they were not reported with error bands because they were not present in the articles and for this reason they are reported without approximation. The study also focuses on the critical role of diffusion and recycling coefficients which play
a minor role. The information obtained from the model is valuable in the context of drug delivery, where engineered EVs carrying anti-tumor proteins can activate apoptosis pathways in tumor cells and influence EV signaling within the tumor microenvironment. Future research will focus on developing a drug response function. However, it must be underlined that all these models present important limitations due to the lack of knowledge and technological limits of the instruments, as already underlined, but useful for planning experiments and reducing the materials used.

Next, we focused on the possibility of using the lipid envelope of a liposome as a source of information, instead of its contents. This is because mapping EV movements in blood can implement knowledge about their internalization and local effects. This idea inspired a possible application in the field of diagnosis and prevention, exploiting the transmission of information due to the change in speed of colored droplets, which emulate liposomes, within a microfluidic system. Our research is based on using colored droplets in an oil matrix to transmit information via Droplet Speed Shift Keying (DSSK), a modulation scheme applicable in droplet microfluidic communications that makes the delay independent of channel length. In this work, we motivated the need for such an approach, clarified the main characteristics of DSSK, and presented some preliminary results obtained through a physical testbed that we built to validate the feasibility of DSSK. Indeed, the experimental results are encouraging, demonstrating the possibility of transmitting information through changes in droplet velocity induced by pressure alterations.

## 11 Acknowledgments

I would like to express sincere thanks to my tutor, Prof. Alfio Lombardo, and to my co-tutors, Prof. Giacomo Morabito, Prof. Nunzio Iraci, and Doc. Loredana Leggio. Without their motivation and continuous encouragement, this research would not have been successful. I also extend my sincere thanks to Prof. Laura Galluccio, Prof. Ilangko Balasingham, to my colleagues Salvo Quattropani, Carmelo Ricci, Roberta Avanzato, Christian Grasso, Andrea Panebianco, Martin Damrath, Mohammad Zoofaghari, Hamid Khoshfekr Rudsari, Naimahmed Nesaragi and Carla Panarello for the support and help in writing this thesis. I would also like to extend my thanks to my parents, friends, and last but not least to Elizabeth, who has been my "hook in the middle of the sky". They all played an important part during my research journey and provided me with moral support and encouragement when necessary.

## References

- Fei Teng and Martin Fussenegger. Shedding light on extracellular vesicle biogenesis and bioengineering. Advanced Science, 8(1):2003505, 2021.
- [2] Dilip Kumar Sharma, Gopala Krishna Rapaka, Ajay Prakash Pasupulla, Sushma Jaiswal, Kishori Abadar, and Harpreet Kaur. A review on smart grid telecommunication system. *Materials Today: Proceed*ings, 51:470–474, 2022.
- [3] Nariman Farsad, Na-Rae Kim, Andrew W Eckford, and Chan-Byoung Chae. Channel and noise models for nonlinear molecular communication systems. *IEEE Journal on Selected Areas in Communications*, 32(12):2392–2401, 2014.
- [4] Nariman Farsad, H Birkan Yilmaz, Andrew Eckford, Chan-Byoung Chae, and Weisi Guo. A comprehensive survey of recent advancements in molecular communication. *IEEE Communications Surveys & Tutorials*, 18(3):1887–1919, 2016.
- [5] Isabella A Guedes, Camila S de Magalhães, and Laurent E Dardenne. Receptor-ligand molecular docking. *Biophysical reviews*, 6:75–87, 2014.
- [6] Jean-Marc Zingg. Vitamin e: regulatory role on signal transduction. *IUBMB life*, 71(4):456–478, 2019.
- [7] Inge Katrin Herrmann, Matthew John Andrew Wood, and Gregor Fuhrmann. Extracellular vesicles as a next-generation drug delivery platform. *Nature nanotechnology*, 16(7):748–759, 2021.
- [8] Raymond Cheong, Alex Rhee, Chiaochun Joanne Wang, Ilya Nemenman, and Andre Levchenko. Information transduction capacity of noisy biochemical signaling networks. *science*, 334(6054):354–358, 2011.
- [9] Andre Levchenko and Ilya Nemenman. Cellular noise and information transmission. *Current opinion in biotechnology*, 28:156–164, 2014.
- [10] Yuki Moritani, Satoshi Hiyama, and Tatsuya Suda. A molecular communication system. In Natural Computing: 4th International Workshop on Natural Computing Himeji, Japan, September 2009 Proceedings, pages 82–89. Springer, 2010.

- [11] Nariman Farsad, Weisi Guo, and Andrew W Eckford. Tabletop molecular communication: Text messages through chemical signals. *PloS* one, 8(12):e82935, 2013.
- [12] Mladen Veletić, Michael Taynnan Barros, Ilangko Balasingham, and Sasitharan Balasubramaniam. A molecular communication model of exosome-mediated brain drug delivery. In Proceedings of the Sixth Annual ACM International Conference on Nanoscale Computing and Communication, pages 1–7, 2019.
- [13] Federico Calì, Giovanni Li-Destri, and Nunzio Tuccitto. Interfacial shift keying allows a high information rate in molecular communication: Methods and data. *IEEE Transactions on Molecular, Biological and Multi-Scale Communications*, 2023.
- [14] Giordano Rampioni, Francesca D'Angelo, Livia Leoni, and Pasquale Stano. Gene-expressing liposomes as synthetic cells for molecular communication studies. *Frontiers in bioengineering and biotechnology*, 7:1, 2019.
- [15] G Wayne Brodland. How computational models can help unlock biological systems. In Seminars in cell & developmental biology, volume 47, pages 62–73. Elsevier, 2015.
- [16] John A Sokolowski and Catherine M Banks. *Principles of modeling and simulation: a multidisciplinary approach.* John Wiley & Sons, 2011.
- [17] Mario Vailati-Riboni, Valentino Palombo, and Juan J Loor. What are omics sciences? *Periparturient diseases of dairy cows: a systems biology approach*, pages 1–7, 2017.
- [18] Vasily Osipov, Mikhail Lushnov, Elena Stankova, Alexander Vodyaho, and Nataly Zukova. Inductive synthesis of the models of biological systems according to clinical trials. In Computational Science and Its Applications-ICCSA 2017: 17th International Conference, Trieste, Italy, July 3-6, 2017, Proceedings, Part I 17, pages 103–115. Springer, 2017.
- [19] Ryszard Tadeusiewicz. Neural networks as a tool for modeling of biological systems. Bio-Algorithms and Med-Systems, 11(3):135–144, 2015.

- [20] Yanyan Liu, Lianhai Wu, John A Baddeley, and Christine A Watson. Models of biological nitrogen fixation of legumes. *Sustainable Agriculture Volume 2*, pages 883–905, 2011.
- [21] Andreas Hilfinger and Johan Paulsson. Separating intrinsic from extrinsic fluctuations in dynamic biological systems. *Proceedings of the National Academy of Sciences*, 108(29):12167–12172, 2011.
- [22] Albert-Laszlo Barabasi and Zoltan N Oltvai. Network biology: understanding the cell's functional organization. *Nature reviews genetics*, 5(2):101–113, 2004.
- [23] Tadashi Nakano, Michael J Moore, Fang Wei, Athanasios V Vasilakos, and Jianwei Shuai. Molecular communication and networking: Opportunities and challenges. *IEEE transactions on nanobioscience*, 11(2):135–148, 2012.
- [24] Frank J Bruggeman and Hans V Westerhoff. The nature of systems biology. TRENDS in Microbiology, 15(1):45–50, 2007.
- [25] Wassim M Haddad, V Chellaboina, and Elias August. Stability and dissipativity theory for nonnegative dynamical systems: A thermodynamic framework for biological and physiological systems. In Proceedings of the 40th IEEE Conference on Decision and Control (Cat. No. 01CH37228), volume 1, pages 442–458. IEEE, 2001.
- [26] SERGIO Cerutti, G Carrault, PJM Cluitmans, A Kinie, T Lipping, N Nikolaidis, I Pitas, and MARIA GABRIELLA Signorini. Non-linear algorithms for processing biological signals. *Computer methods and programs in biomedicine*, 51(1-2):51–73, 1996.
- [27] Michael Schaub and Fitsum Abadi. Integrated population models: a novel analysis framework for deeper insights into population dynamics. *Journal of Ornithology*, 152:227–237, 2011.
- [28] Hamid Khoshfekr Rudsari, Mladen Veletić, Jacob Bergsland, and Ilangko Balasingham. Cardiac bio-nanonetwork: Extracellular vesicles release modeling for engineered stem cell-derived cardiomyocyte. In Proceedings of the Eight Annual ACM International Conference on Nanoscale Computing and Communication, pages 1–7, 2021.

- [29] Mohammad Zoofaghari, Fabrizio Pappalardo, Martin Damrath, and Balasingham Ilangko. Modeling extracellular vesicles-mediated interactions of cells in the tumor microenvironment. 2023.
- [30] Ting Lu, Michael Ferry, Ron Weiss, and Jeff Hasty. A molecular noise generator. *Physical biology*, 5(3):036006, 2008.
- [31] Lidia Donvito, Laura Galluccio, Alfio Lombardo, Giacomo Morabito, Alfio Nicolosi, and Marco Reno. Experimental validation of a simple, low-cost, t-junction droplet generator fabricated through 3d printing. Journal of Micromechanics and Microengineering, 25(3):035013, 2015.
- [32] David N Breslauer, Philip J Lee, and Luke P Lee. Microfluidics-based systems biology. *Molecular Biosystems*, 2(2):97–112, 2006.
- [33] Laura Galluccio, Alfio Lombardo, Giacomo Morabito, Fabrizio Pappalardo, and Salvatore Quattropani. Droplet speed-shift keying: a modulation scheme for instantaneous microfluidic communications. In Proceedings of the 10th ACM International Conference on Nanoscale Computing and Communication, pages 110–115, 2023.
- [34] Leslie Y Yeo, Hsueh-Chia Chang, Peggy PY Chan, and James R Friend. Microfluidic devices for bioapplications. *small*, 7(1):12–48, 2011.
- [35] Nam-Trung Nguyen, Steven T Wereley, and SAM Shaegh. Fundamentals and applications of microfluidics, artech house. *Inc.: Boston, MA*, *USA*, 2006.
- [36] Osborne Reynolds. Xxix. an experimental investigation of the circumstances which determine whether the motion of water shall be direct or sinuous, and of the law of resistance in parallel channels. *Philosophical Transactions of the Royal society of London*, (174):935–982, 1883.
- [37] Yingying Dou, Bingsheng Wang, Mingliang Jin, Ying Yu, Guofu Zhou, and Lingling Shui. A review on self-assembly in microfluidic devices. *Journal of Micromechanics and Microengineering*, 27(11):113002, 2017.
- [38] Todd M Squires and Stephen R Quake. Microfluidics: Fluid physics at the nanoliter scale. *Reviews of modern physics*, 77(3):977, 2005.

- [39] Weian Zhao and Albert van den Berg. Lab on paper. Lab on a Chip, 8(12):1988–1991, 2008.
- [40] Claudio Parolo and Arben Merkoçi. based nanobiosensors for diagnostics. Chemical Society Reviews, 42(2):450–457, 2013.
- [41] Alfred H Free, Ernest C Adams, Mary Lou Kercher, Helen M Free, and Marion H Cook. Simple specific test for urine glucose. *Clinical chemistry*, 3(3):163–168, 1957.
- [42] Chao-Min Cheng, Andres W Martinez, Jinlong Gong, Charles R Mace, Scott T Phillips, Emanuel Carrilho, Katherine A Mirica, and George M Whitesides. Paper-based elisa. Angewandte Chemie, 122(28):4881– 4884, 2010.
- [43] Shahin Aghamiri, Navid Rabiee, Sepideh Ahmadi, Mohammad Rabiee, Mojtaba Bagherzadeh, and Mahdi Karimi. Microfluidic devices: Synthetic approaches. In *Biomedical Applications of Microfluidic Devices*, pages 23–36. Elsevier, 2021.
- [44] Hadi Rouhani, Fatola Farhadi, Mahsa Akbari Kenari, Effat Eskandari, and Seeram Ramakrishna. Selection of suitable bentonite and the influence of various acids on the preparation of a special clay for the removal of trace olefins from aromatics. *Clay Minerals*, 56(3):185–196, 2021.
- [45] Enoch Kim, Younan Xia, and George M Whitesides. Polymer microstructures formed by moulding in capillaries. Nature, 376(6541):581–584, 1995.
- [46] J Cooper McDonald and George M Whitesides. Poly (dimethylsiloxane) as a material for fabricating microfluidic devices. Accounts of chemical research, 35(7):491–499, 2002.
- [47] Arturo Urrios, Cesar Parra-Cabrera, Nirveek Bhattacharjee, Alan M Gonzalez-Suarez, Luis G Rigat-Brugarolas, Umashree Nallapatti, Josep Samitier, Cole A DeForest, Francesc Posas, José L Garcia-Cordero, et al. 3d-printing of transparent bio-microfluidic devices in peg-da. *Lab on a Chip*, 16(12):2287–2294, 2016.

- [48] Andres W Martinez, Scott T Phillips, Manish J Butte, and George M Whitesides. Patterned paper as a platform for inexpensive, low-volume, portable bioassays. Angewandte Chemie, 119(8):1340–1342, 2007.
- [49] Yuxiao Liu, Yi Cheng, Cheng Zhao, Huan Wang, and Yuanjin Zhao. Nanomotor-derived porous biomedical particles from droplet microfluidics. Advanced Science, 9(4):2104272, 2022.
- [50] R Hague, G D'costa, and PM Dickens. Structural design and resin drainage characteristics of quickcast 2.0. *Rapid Prototyping Journal*, 7(2):66–73, 2001.
- [51] Anthony K Au, Wonjae Lee, and Albert Folch. Mail-order microfluidics: evaluation of stereolithography for the production of microfluidic devices. *Lab on a Chip*, 14(7):1294–1301, 2014.
- [52] J Brian Hutchison, K Tommy Haraldsson, Brian T Good, Robert P Sebra, Ning Luo, Kristi S Anseth, and Christopher N Bowman. Robust polymer microfluidic device fabrication via contact liquid photolithographic polymerization (clipp). Lab on a Chip, 4(6):658–662, 2004.
- [53] Roozbeh Safavieh, Gina Z Zhou, and David Juncker. Microfluidics made of yarns and knots: from fundamental properties to simple networks and operations. *Lab on a Chip*, 11(15):2618–2624, 2011.
- [54] Loredana Leggio, Francesca L'Episcopo, Andrea Magrì, María José Ulloa-Navas, Greta Paternò, Silvia Vivarelli, Carlos AP Bastos, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, et al. Small extracellular vesicles secreted by nigrostriatal astrocytes rescue cell death and preserve mitochondrial function in parkinson's disease. Advanced healthcare materials, 11(20):2201203, 2022.
- [55] Nunzio Iraci, Loredana Leggio, Alfio Lombardo, Carla Panarello, Fabrizio Pappalardo, and Greta Paternó. An analytical model for the inference of the ev reception process parameters in cell-to-cell communication. In Proceedings of the 10th ACM International Conference on Nanoscale Computing and Communication, pages 136–141, 2023.
- [56] Dorothee Murat, Meghan Byrne, and Arash Komeili. Cell biology of prokaryotic organelles. *Cold Spring Harbor perspectives in biology*, 2(10):a000422, 2010.

- [57] Michael P Rout and Mark C Field. The evolution of organellar coat complexes and organization of the eukaryotic cell. Annual review of biochemistry, 86:637–657, 2017.
- [58] Graça Raposo and Philip D Stahl. Extracellular vesicles: a new communication paradigm? *Nature Reviews Molecular Cell Biology*, 20(9):509– 510, 2019.
- [59] Emanuele Cocucci and Jacopo Meldolesi. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends in cell biology*, 25(6):364–372, 2015.
- [60] Rose M Johnstone, Mohammed Adam, JR Hammond, L Orr, and Claire Turbide. Vesicle formation during reticulocyte maturation. association of plasma membrane activities with released vesicles (exosomes). Journal of Biological Chemistry, 262(19):9412–9420, 1987.
- [61] C Thery. Boussac m, veron p, ricciardi-castagnoli p, raposo g, garin j, amigorena s. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol, 166:7309–7318, 2001.
- [62] Loredana Leggio, Francesca L'Episcopo, Andrea Magrì, María José Ulloa-Navas, Greta Paternò, Silvia Vivarelli, Carlos AP Bastos, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, et al. Small extracellular vesicles secreted by region-specific astrocytes ameliorate the mitochondrial function in a cellular model of parkinson's disease. *bioRxiv*, 2021.
- [63] Elena Taverna, Magdalena Götz, and Wieland B Huttner. The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. Annual review of cell and developmental biology, 30:465–502, 2014.
- [64] Giuseppina Bozzuto and Agnese Molinari. Liposomes as nanomedical devices. International journal of nanomedicine, 10:975, 2015.
- [65] Temidayo OB Olusanya, Rita Rushdi Haj Ahmad, Daniel M Ibegbu, James R Smith, and Amal Ali Elkordy. Liposomal drug delivery systems and anticancer drugs. *Molecules*, 23(4):907, 2018.

- [66] Melody D Fulton and Wided Najahi-Missaoui. Liposomes in cancer therapy: How did we start and where are we now. *International Journal* of Molecular Sciences, 24(7):6615, 2023.
- [67] Guillaume Van Niel, Gisela d'Angelo, and Graça Raposo. Shedding light on the cell biology of extracellular vesicles. *Nature reviews Molecular cell biology*, 19(4):213–228, 2018.
- [68] Thomas Juan and Maximilian Fürthauer. Biogenesis and function of escrt-dependent extracellular vesicles. In Seminars in cell & developmental biology, volume 74, pages 66–77. Elsevier, 2018.
- [69] Marina Colombo, Graça Raposo, and Clotilde Théry. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual review of cell and developmental biology, 30:255–289, 2014.
- [70] Hina Kalra, Gregor PC Drummen, and Suresh Mathivanan. Focus on extracellular vesicles: introducing the next small big thing. *International journal of molecular sciences*, 17(2):170, 2016.
- [71] Edouard M Bevers and Patrick L Williamson. Getting to the outer leaflet: physiology of phosphatidylserine exposure at the plasma membrane. *Physiological reviews*, 96(2):605–645, 2016.
- [72] John F Oram and Ashley M Vaughan. Abca1-mediated transport of cellular cholesterol and phospholipids to hdl apolipoproteins. *Current* opinion in lipidology, 11(3):253–260, 2000.
- [73] Vandhana Muralidharan-Chari, James Clancy, Carolyn Plou, Maryse Romao, Philippe Chavrier, Graca Raposo, and Crislyn D'Souza-Schorey. Arf6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Current Biology*, 19(22):1875–1885, 2009.
- [74] Joseph F Nabhan, Ruoxi Hu, Raymond S Oh, Stanley N Cohen, and Quan Lu. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (armms) at plasma membrane by recruitment of tsg101 protein. *Proceedings of the National Academy of Sciences*, 109(11):4146–4151, 2012.

- [75] Susanne Rauch and Juan Martin-Serrano. Multiple interactions between the escrt machinery and arrestin-related proteins: implications for ppxy-dependent budding. *Journal of virology*, 85(7):3546–3556, 2011.
- [76] Kelly J McKelvey, Katie L Powell, Anthony W Ashton, Jonathan M Morris, and Sharon A McCracken. Exosomes: mechanisms of uptake. *Journal of circulating biomarkers*, 4:7, 2015.
- [77] Ilaria Prada and Jacopo Meldolesi. Binding and fusion of extracellular vesicles to the plasma membrane of their cell targets. *International journal of molecular sciences*, 17(8):1296, 2016.
- [78] Valentina R Minciacchi, Michael R Freeman, and Dolores Di Vizio. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. In *Seminars in cell & developmental biology*, volume 40, pages 41–51. Elsevier, 2015.
- [79] Ashley E Russell, Alexandra Sneider, Kenneth W Witwer, Paolo Bergese, Suvendra N Bhattacharyya, Alexander Cocks, Emanuele Cocucci, Uta Erdbrügger, Juan M Falcon-Perez, David W Freeman, et al. Biological membranes in ev biogenesis, stability, uptake, and cargo transfer: an isev position paper arising from the isev membranes and evs workshop. Journal of Extracellular Vesicles, 8(1):1684862, 2019.
- [80] Benjamin Podbilewicz. Virus and cell fusion mechanisms. Annual review of cell and developmental biology, 30:111–139, 2014.
- [81] AJG Pötgens, S Drewlo, M Kokozidou, and P Kaufmann. Syncytin: the major regulator of trophoblast fusion? recent developments and hypotheses on its action. *Human reproduction update*, 10(6):487–496, 2004.
- [82] JM Tolosa, JE Schjenken, VL Clifton, A Vargas, B Barbeau, P Lowry, K Maiti, and R Smith. The endogenous retroviral envelope protein syncytin-1 inhibits lps/pha-stimulated cytokine responses in human blood and is sorted into placental exosomes. *Placenta*, 33(11):933–941, 2012.

- [83] B Bjerregaard, JG Lemmen, MR Petersen, E Østrup, LH Iversen, K Almstrup, L-I Larsson, and S Ziebe. Syncytin-1 and its receptor is present in human gametes. *Journal of assisted reproduction and genetics*, 31(5):533-539, 2014.
- [84] Rolf Buslei, Pamela L Strissel, Christine Henke, Regina Schey, Nadine Lang, Matthias Ruebner, Claus C Stolt, Ben Fabry, Michael Buchfelder, and Reiner Strick. Activation and regulation of endogenous retroviral genes in the human pituitary gland and related endocrine tumours. Neuropathology and Applied Neurobiology, 41(2):180–200, 2015.
- [85] Pilvi Maliniemi, Michelle Vincendeau, Jens Mayer, Oliver Frank, Sonja Hahtola, Leena Karenko, Emilia Carlsson, Francois Mallet, Wolfgang Seifarth, Christine Leib-Mösch, et al. Expression of human endogenous retrovirus-w including syncytin-1 in cutaneous t-cell lymphoma. *PloS* one, 8(10):e76281, 2013.
- [86] Gilles RX Hickson, Luke H Chamberlain, Valerie H Maier, and Gwyn W Gould. Quantification of snare protein levels in 3t3-l1 adipocytes: implications for insulin-stimulated glucose transport. *Biochemical and biophysical research communications*, 270(3):841–845, 2000.
- [87] Reinhard Jahn and Richard H Scheller. Snares—engines for membrane fusion. Nature reviews Molecular cell biology, 7(9):631–643, 2006.
- [88] Tyler DR Vance and Jeffrey E Lee. Virus and eukaryote fusogen superfamilies. *Current Biology*, 30(13):R750–R754, 2020.
- [89] Laura Ann Mulcahy, Ryan Charles Pink, and David Raul Francisco Carter. Routes and mechanisms of extracellular vesicle uptake. *Journal* of extracellular vesicles, 3(1):24641, 2014.
- [90] Angela Montecalvo, Adriana T Larregina, William J Shufesky, Donna Beer Stolz, Mara LG Sullivan, Jenny M Karlsson, Catherine J Baty, Gregory A Gibson, Geza Erdos, Zhiliang Wang, et al. Mechanism of transfer of functional micrornas between mouse dendritic cells via exosomes. *Blood, The Journal of the American Society of Hematology*, 119(3):756–766, 2012.

- [91] Cristina Escrevente, Sascha Keller, Peter Altevogt, and Júlia Costa. Interaction and uptake of exosomes by ovarian cancer cells. *BMC cancer*, 11:1–10, 2011.
- [92] Du Feng, Wen-Long Zhao, Yun-Ying Ye, Xiao-Chen Bai, Rui-Qin Liu, Lei-Fu Chang, Qiang Zhou, and Sen-Fang Sui. Cellular internalization of exosomes occurs through phagocytosis. *Traffic*, 11(5):675–687, 2010.
- [93] Mattia I Morandi, Petro Busko, Efrat Ozer-Partuk, Suman Khan, Giulia Zarfati, Yael Elbaz-Alon, Paula Abou Karam, Tina Napso Shogan, Lana Ginini, Ziv Gil, et al. Extracellular vesicle fusion visualized by cryo-em. *BioRxiv*, pages 2022–03, 2022.
- [94] Saray Tabak, Sofia Schreiber-Avissar, and Elie Beit-Yannai. Influence of anti-glaucoma drugs on uptake of extracellular vesicles by trabecular meshwork cells. *International Journal of Nanomedicine*, 16:1067, 2021.
- [95] Hong-rong Xie, Lin-sen Hu, and Guo-yi Li. Sh-sy5y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in parkinson's disease. *Chinese medical journal*, 123(08):1086–1092, 2010.
- [96] Annett Blochberger. clinical features and diagnosis.
- [97] Dennis W Dickson. Parkinson's disease and parkinsonism: neuropathology. Cold Spring Harbor perspectives in medicine, 2(8), 2012.
- [98] Francesca L'episcopo, Cataldo Tirolo, Luca Peruzzotti-Jametti, Maria F Serapide, Nunzio Testa, Salvatore Caniglia, Beatrice Balzarotti, Stefano Pluchino, and Bianca Marchetti. Neural stem cell grafts promote astroglia-driven neurorestoration in the aged parkinsonian brain via wnt/β-catenin signaling. Stem Cells, 36(8):1179–1197, 2018.
- [99] Kaizhe Wang, Ling Ye, Hongfang Lu, Huili Chen, Yanyan Zhang, Yunlong Huang, and Jialin C Zheng. Tnf-α promotes extracellular vesicle release in mouse astrocytes through glutaminase. *Journal of neuroinflammation*, 14(1):1–10, 2017.
- [100] Chiara Cossetti, Nunzio Iraci, Tim R Mercer, Tommaso Leonardi, Emanuele Alpi, Denise Drago, Clara Alfaro-Cervello, Harpreet K Saini, Matthew P Davis, Julia Schaeffer, et al. Extracellular vesicles from

neural stem cells transfer ifn- $\gamma$  via ifngr1 to activate stat1 signaling in target cells. *Molecular cell*, 56(2):193–204, 2014.

- [101] Nunzio Iraci, Edoardo Gaude, Tommaso Leonardi, Ana SH Costa, Chiara Cossetti, Luca Peruzzotti-Jametti, Joshua D Bernstock, Harpreet K Saini, Maurizio Gelati, Angelo Luigi Vescovi, et al. Extracellular vesicles are independent metabolic units with asparaginase activity. *Nature chemical biology*, 13(9):951–955, 2017.
- [102] Pierpaolo Risiglione, Loredana Leggio, Salvatore AM Cubisino, Simona Reina, Greta Paternò, Bianca Marchetti, Andrea Magrì, Nunzio Iraci, and Angela Messina. High-resolution respirometry reveals mpp+ mitochondrial toxicity mechanism in a cellular model of parkinson's disease. International journal of molecular sciences, 21(21):7809, 2020.
- [103] Ali Ertürk, Yuanyuan Wang, and Morgan Sheng. Local pruning of dendrites and spines by caspase-3-dependent and proteasome-limited mechanisms. *Journal of Neuroscience*, 34(5):1672–1688, 2014.
- [104] Dominik Pesta and Erich Gnaiger. High-resolution respirometry: Oxphos protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Mitochondrial bioenergetics: methods and* protocols, pages 25–58, 2012.
- [105] Loredana Leggio, Greta Paternò, Silvia Vivarelli, Francesca L'episcopo, Cataldo Tirolo, Gabriele Raciti, Fabrizio Pappalardo, Carmela Giachino, Salvatore Caniglia, Maria Francesca Serapide, et al. Extracellular vesicles as nanotherapeutics for parkinson's disease. *Biomolecules*, 10(9):1327, 2020.
- [106] Diana Guimarães, Artur Cavaco-Paulo, and Eugénia Nogueira. Design of liposomes as drug delivery system for therapeutic applications. *International journal of pharmaceutics*, 601:120571, 2021.
- [107] Xiaowei Dong. Current strategies for brain drug delivery. Theranostics, 8(6):1481, 2018.
- [108] Maitrayee Sardar Sinha, Anna Ansell-Schultz, Livia Civitelli, Camilla Hildesjö, Max Larsson, Lars Lannfelt, Martin Ingelsson, and Martin

Hallbeck. Alzheimer's disease pathology propagation by exosomes containing toxic amyloid-beta oligomers. *Acta neuropathologica*, 136:41– 56, 2018.

- [109] Judith M Silverman, Sarah M Fernando, Leslie I Grad, Andrew F Hill, Bradley J Turner, Justin J Yerbury, and Neil R Cashman. Disease mechanisms in als: misfolded sod1 transferred through exosomedependent and exosome-independent pathways. *Cellular and molecular neurobiology*, 36:377–381, 2016.
- [110] Andrew D Vogel, Raghavendra Upadhya, and Ashok K Shetty. Neural stem cell derived extracellular vesicles: attributes and prospects for treating neurodegenerative disorders. *EBioMedicine*, 38:273–282, 2018.
- [111] Bianca Marchetti, Cataldo Tirolo, Francesca L'Episcopo, Salvatore Caniglia, Nunzio Testa, Jayden A Smith, Stefano Pluchino, and Maria F Serapide. Parkinson's disease, aging and adult neurogenesis: Wnt/β-catenin signalling as the key to unlock the mystery of endogenous brain repair. Aging cell, 19(3):e13101, 2020.
- [112] Francesca L'Episcopo, Cataldo Tirolo, Maria F Serapide, Salvatore Caniglia, Nunzio Testa, Loredana Leggio, Silvia Vivarelli, Nunzio Iraci, Stefano Pluchino, and Bianca Marchetti. Microglia polarization, geneenvironment interactions and wnt/β-catenin signaling: emerging roles of glia-neuron and glia-stem/neuroprogenitor crosstalk for dopaminergic neurorestoration in aged parkinsonian brain. Frontiers in aging neuroscience, 10:12, 2018.
- [113] Pei-Chun Chen, Marcelo R Vargas, Amar K Pani, Richard J Smeyne, Delinda A Johnson, Yuet Wai Kan, and Jeffrey A Johnson. Nrf2mediated neuroprotection in the mptp mouse model of parkinson's disease: Critical role for the astrocyte. *Proceedings of the National Academy of Sciences*, 106(8):2933–2938, 2009.
- [114] Etienne C Hirsch, Peter Jenner, and Serge Przedborski. Pathogenesis of parkinson's disease. *Movement Disorders*, 28(1):24–30, 2013.
- [115] Oleh Hornykiewicz. Biochemical aspects of parkinson's disease. Neurology, 51(2 Suppl 2):S2–S9, 1998.

- [116] Jacob T Bendor, Todd P Logan, and Robert H Edwards. The function of  $\alpha$ -synuclein. Neuron, 79(6):1044–1066, 2013.
- [117] Victor S Van Laar, Jianming Chen, Alevtina D Zharikov, Qing Bai, Roberto Di Maio, April A Dukes, Teresa G Hastings, Simon C Watkins, J Timothy Greenamyre, Claudette M St Croix, et al. α-synuclein amplifies cytoplasmic peroxide flux and oxidative stress provoked by mitochondrial inhibitors in cns dopaminergic neurons in vivo. *Redox Biology*, 37:101695, 2020.
- [118] Bianca Marchetti and Maria P Abbracchio. To be or not to be (inflamed)-is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? Trends in pharmacological sciences, 26(10):517-525, 2005.
- [119] Jason R Cannon and J Timothy Greenamyre. Gene-environment interactions in parkinson's disease: Specific evidence in humans and mammalian models. *Neurobiology of disease*, 57:38–46, 2013.
- [120] Etienne C Hirsch and David G Standaert. Ten unsolved questions about neuroinflammation in parkinson's disease. Movement Disorders, 36(1):16-24, 2021.
- [121] Patrick P Michel, Etienne C Hirsch, and Stéphane Hunot. Understanding dopaminergic cell death pathways in parkinson disease. *Neuron*, 90(4):675–691, 2016.
- [122] Michael V Sofroniew. Astrocyte reactivity: subtypes, states, and functions in cns innate immunity. Trends in Immunology, 41(9):758–770, 2020.
- [123] Benjamin E Clarke, Doaa M Taha, Giulia E Tyzack, and Rickie Patani. Regionally encoded functional heterogeneity of astrocytes in health and disease: A perspective. *Glia*, 69(1):20–27, 2021.
- [124] Maria Francesca Serapide, Francesca L'Episcopo, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, Carmela Giachino, and Bianca Marchetti. Boosting antioxidant self-defenses by grafting astrocytes rejuvenates the aged microenvironment and mitigates nigrostriatal toxicity in parkinsonian brain via an nrf2-driven wnt/β-catenin prosurvival axis. Frontiers in Aging Neuroscience, 12:24, 2020.

- [125] Ikuko Miyazaki and Masato Asanuma. Neuron-astrocyte interactions in parkinson's disease. *Cells*, 9(12):2623, 2020.
- [126] Clotilde Lauro, Myriam Catalano, Flavia Trettel, and Cristina Limatola. Fractalkine in the nervous system: neuroprotective or neurotoxic molecule? Annals of the New York academy of sciences, 1351(1):141– 148, 2015.
- [127] Flavia Trettel, Maria Amalia Di Castro, and Cristina Limatola. Chemokines: key molecules that orchestrate communication among neurons, microglia and astrocytes to preserve brain function. Neuroscience, 439:230–240, 2020.
- [128] F L'Episcopo, C Tirolo, N Testa, S Caniglia, MC Morale, C Cossetti, P D'Adamo, E Zardini, L Andreoni, AEC Ihekwaba, et al. Corrigendum to "reactive astrocytes and wnt/β-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine model of parkinson's disease" [neurobiol. dis. 41/2 (2011) 508–527]. Neurobiology of Disease, 3(42):539, 2011.
- [129] Francesca L'Episcopo, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, Maria C Morale, Michela Deleidi, Maria F Serapide, Stefano Pluchino, and Bianca Marchetti. Plasticity of subventricular zone neuroprogenitors in mptp (1-methyl-4-phenyl-1, 2, 3, 6tetrahydropyridine) mouse model of parkinson's disease involves cross talk between inflammatory and wnt/β-catenin signaling pathways: functional consequences for neuroprotection and repair. Journal of Neuroscience, 32(6):2062–2085, 2012.
- [130] Francesca L'Episcopo, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, Maria Concetta Morale, Maria Francesca Serapide, Stefano Pluchino, and Bianca Marchetti. Wnt/β-catenin signaling is required to rescue midbrain dopaminergic progenitors and promote neurorepair in ageing mouse model of parkinson's disease. Stem Cells, 32(8):2147– 2163, 2014.
- [131] Luca FR Gebert and Ian J MacRae. Regulation of microrna function in animals. Nature reviews Molecular cell biology, 20(1):21–37, 2019.

- [132] JAD Wattis, B O'Malley, H Blackburn, L Pickersgill, J Panovska, HM Byrne, and KG Jackson. Mathematical model for low density lipoprotein (ldl) endocytosis by hepatocytes. *Bulletin of mathematical biology*, 70(8):2303–2333, 2008.
- [133] Jean-Marc Galan, Andreas Wiederkehr, Jae Hong Seol, Rosine Haguenauer-Tsapis, Raymond J Deshaies, Howard Riezman, and Matthias Peter. Skp1p and the f-box protein rcy1p form a non-scf complex involved in recycling of the snare snc1p in yeast. *Molecular* and cellular biology, 21(9):3105–3117, 2001.
- [134] Sandro Vivona, Daniel J Cipriano, Seán O'Leary, Ye Henry Li, Timothy D Fenn, and Axel T Brunger. Disassembly of all snare complexes by n-ethylmaleimide-sensitive factor (nsf) is initiated by a conserved 1: 1 interaction between α-soluble nsf attachment protein (snap) and snare complex. Journal of Biological Chemistry, 288(34):24984–24991, 2013.
- [135] Dimitrios E Panayotounakos. Exact analytic solutions of unsolvable classes of first and second order nonlinear odes (part i: Abel's equations). Applied mathematics letters, 18(2):155–162, 2005.
- [136] DE Panayotounakos, Th I Zarmpoutis, and P Sotiropoulos. The general solutions of the normal abel's type nonlinear ode of the second kind. *IAENG International Journal of Applied Mathematics*, 43(3):94– 98, 2013.
- [137] Valentin F Zaitsev and Andrei D Polyanin. Handbook of exact solutions for ordinary differential equations. CRC press, 2002.
- [138] Alfio Lombardo, Giacomo Morabito, Carla Panarello, and Fabrizio Pappalardo. Modeling biological receivers: The case of extracellular vesicle fusion to the plasma membrane of the target cell. In Proceedings of the 9th ACM International Conference on Nanoscale Computing and Communication, NANOCOM '22, New York, NY, USA, 2022. Association for Computing Machinery.
- [139] Brian J Jurgielewicz, Yao Yao, and Steven L Stice. Kinetics and specificity of hek293t extracellular vesicle uptake using imaging flow cytometry. *Nanoscale research letters*, 15:1–11, 2020.

- [140] Loredana Leggio, Francesca L'Episcopo, Andrea Magrì, María José Ulloa-Navas, Greta Paternò, Silvia Vivarelli, Carlos AP Bastos, Cataldo Tirolo, Nunzio Testa, Salvatore Caniglia, et al. Small extracellular vesicles secreted by nigrostriatal astrocytes rescue cell death and preserve mitochondrial function in parkinson's disease. Advanced Healthcare Materials, page 2201203.
- [141] Pbs dulbecco's. *PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling.*
- [142] Luminex. Ideas® image data exploration and analysis software user's manual.
- [143] Amnis merck kgaa, darmstadt. FlowSight User's Manual.
- [144] Scientific thermofisher. Varioskan LUX multimode microplate reader.
- [145] Hamidreza Arjmandi, Mohammad Zoofaghari, and Adam Noel. Diffusive molecular communication in a biological spherical environment with partially absorbing boundary. *IEEE Transactions on Communications*, 67(10):6858–6867, 2019.
- [146] Mohammad Zoofaghari, Ali Etemadi, Hamidreza Arjmandi, and Ilangko Balasingham. Modeling molecular communication channel in the biological sphere with arbitrary homogeneous boundary conditions. *IEEE Wireless Communications Letters*, 10(12):2786–2790, 2021.
- [147] María Sancho-Albero, Nuria Navascués, Gracia Mendoza, Víctor Sebastián, Manuel Arruebo, Pilar Martín-Duque, and Jesús Santamaría. Exosome origin determines cell targeting and the transfer of therapeutic nanoparticles towards target cells. Journal of nanobiotechnology, 17(1):1–13, 2019.
- [148] Inbal Hazan-Halevy, Daniel Rosenblum, Shiri Weinstein, Osnat Bairey, Pia Raanani, and Dan Peer. Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant blymphocytes. *Cancer letters*, 364(1):59–69, 2015.
- [149] Karin L Nicholson, Mary Munson, Rebecca B Miller, Thomas J Filip, Robert Fairman, and Frederick M Hughson. Regulation of snare complex assembly by an n-terminal domain of the t-snare sso1p. *Nature* structural biology, 5(9):793–802, 1998.

- [150] John G Walker. Improved nano-particle tracking analysis. Measurement Science and Technology, 23(6):065605, 2012.
- [151] Adaobi Nwaneshiudu, Christiane Kuschal, Fernanda H Sakamoto, R Rox Anderson, Kathryn Schwarzenberger, and Roger C Young. Introduction to confocal microscopy. *Journal of Investigative Dermatol*ogy, 132(12):1–5, 2012.
- [152] Peter J Goodhew, John Humphreys, and Richard Beanland. Electron microscopy and analysis. CRC press, 2000.
- [153] Katrien De Cock, Xueying Zhang, Mónica F. Bugallo, and Petar M. Djurić. Stochastic simulation and parameter estimation of first order chemical reactions. In 2004 12th European Signal Processing Conference, pages 1111–1114, 2004.
- [154] Adel Mhamdi and Wolfgang Marquardt. An inversion approach to the estimation of reaction rates in chemical reactors. In 1999 European Control Conference (ECC), pages 3041–3046. IEEE, 1999.
- [155] Andrea Picco, Markus Mund, Jonas Ries, François Nédélec, and Marko Kaksonen. Visualizing the functional architecture of the endocytic machinery. *Elife*, 4:e04535, 2015.
- [156] Daisy Duan, Meretta Hanson, David O Holland, and Margaret E Johnson. Integrating protein copy numbers with interaction networks to quantify stoichiometry in clathrin-mediated endocytosis. *Scientific reports*, 12(1):1–21, 2022.
- [157] Soheil Aghamohammadzadeh and Kathryn R Ayscough. Differential requirements for actin during yeast and mammalian endocytosis. Nature cell biology, 11(8):1039–1042, 2009.
- [158] B áL Coomber and PA Stewart. Morphometric analysis of cns microvascular endothelium. *Microvascular research*, 30(1):99–115, 1985.
- [159] Thomas F. Coleman and Yuying Li. An interior trust region approach for nonlinear minimization subject to bounds. SIAM Journal on Optimization, 6(2):418–445, 1996.

- [160] Bongseop Kwak, Altug Ozcelikkale, Crystal S Shin, Kinam Park, and Bumsoo Han. Simulation of complex transport of nanoparticles around a tumor using tumor-microenvironment-on-chip. *Journal of Controlled Release*, 194:157–167, 2014.
- [161] Uma Thanigai Arasu, Kai Härkönen, Arto Koistinen, and Kirsi Rilla. Correlative light and electron microscopy is a powerful tool to study interactions of extracellular vesicles with recipient cells. *Experimental Cell Research*, 376(2):149–158, 2019.
- [162] Chonlada Charoenviriyakul, Yuki Takahashi, Masaki Morishita, Akihiro Matsumoto, Makiya Nishikawa, and Yoshinobu Takakura. Cell type-specific and common characteristics of exosomes derived from mouse cell lines: Yield, physicochemical properties, and pharmacokinetics. European Journal of Pharmaceutical Sciences, 96:316–322, 2017.
- [163] B. H. Sung, T. Ketova, D. Hoshino, A. Zijlstra, and A. M. Weaver. Directional cell movement through tissues is controlled by exosome secretion. *Nature Communications*, 6(1), 2015.
- [164] A. Birbrair. *Tumor Microenvironment*. Springer Nature, 2021.
- [165] R. Jaiswal and L. M. Sedger. Intercellular vesicular transfer by exosomes, microparticles and oncosomes - implications for cancer biology and treatments. *Frontiers in Oncology*, 9, 2019.
- [166] et al. Bebelman, Maarten P. Biogenesis and function of extracellular vesicles in cancer. *Pharmacology & therapeutics*, 2018.
- [167] L. Wu. Exosomes derived from gastric cancer cells activate nf-κb pathway in macrophages to promote cancer progression. *Tumor Biology*, 37(9):12169–12180, 2016.
- [168] Feiya Ma, Jensen Vayalil, Grace Lee, Yuqi Wang, and Guangyong Peng. Emerging role of tumor-derived extracellular vesicles in t cell suppression and dysfunction in the tumor microenvironment. *Journal* for immunotherapy of cancer, 9(10), 2021.
- [169] C. Marar B. Starich and D. Wirtz. Extracellular vesicles in immunomodulation and tumor progression. *Nature Immunology*, 22(5):560–570, 2021.

- [170] N. Abu and N. A. A. Rus Bakarurraini. The interweaving relationship between extracellular vesicles and t cells in cancer. *Cancer Letters*, 530:1–7, 2022.
- [171] D. T. Smallwood. Extracellular vesicles released by cd40/il-4-stimulated cll cells confer altered functional properties to cd4+ t cells. *Blood*, 128(4):542–552, 2016.
- [172] Frederick R. Maxfield and Timothy E. McGraw. Endocytic recycling. Nature Reviews Molecular Cell Biology, 2004.
- [173] L. Czernek and M. Düchler. Functions of cancer-derived extracellular vesicles in immunosuppression. Archivum Immunologiae et Therapiae Experimentalis, 65(4):311–323, 2017.
- [174] J. Meldolesi. Extracellular vesicles, news about their role in immune cells: physiology, pathology and diseases. *Clinical and Experimental Immunology*, 196(3):318–327, 2019.
- [175] Y. Zhang J. Tan Y. Miao and Q. Zhang. The effect of extracellular vesicles on the regulation of mitochondria under hypoxia. *Cell Death* & Disease, 12(4), 2021.
- [176] M. Alavi, N. Karimi, and M. Safaei. Application of various types of liposomes in drug delivery systems. Advanced Pharmaceutical Bulletin, 7(1):3–9, 2017.
- [177] I. Herrmann, M. Wood, and G. Fuhrmann. Extracellular vesicles as a next-generation drug delivery platform. *Nature Nanotechnology*, 16(7):748–759, 2021.
- [178] G. Nasiri, N. Azarpira, A. Alizadeh, S. Goshtasbi, and L. Tayebi. Shedding light on the role of keratinocyte-derived extracellular vesicles on skin-homing cells. *Stem Cell Research & Therapy*, 11(1), 2020.
- [179] Miriam Aarsund Larsen. Natural killer cell-derived extracellular vesicles and their anti-tumor capacities. 2023.
- [180] Lingyun Wang, Zhichao Sun, and Hongmei Wang. Extracellular vesicles and the regulation of tumor immunity: Current progress and future directions. Journal of Cellular Biochemistry, 122(7):760–769, 2021.

- [181] W. P. Daley and K. M. Yamada. Ecm-modulated cellular dynamics as a driving force for tissue morphogenesis. *Current Opinion in Genetics* & Development, 23(4):408–414, 2013.
- [182] V. Jamali, A. Ahmadzadeh, W. Wicke, A. Noel, and R. Schober. Channel modeling for diffusive molecular communication—a tutorial review. *Proceedings of the IEEE*, 107(7):1256–1301, 2019.
- [183] A. Noel, Y. Deng, D. Makrakis, and A. Hafid. Active versus passive: Receiver model transforms for diffusive molecular communication. 2016.
- [184] et al. Deng, Yansha. Modeling and simulation of molecular communication systems with a reversible adsorption receiver. *IEEE Transactions* on Molecular, Biological and Multi-Scale Communications, 1(4), 2015.
- [185] A. Ahmadzadeh, H. Arjmandi, A. Burkovski, and R. Schober. Comprehensive reactive receiver modeling for diffusive molecular communication systems: Reversible binding, molecule degradation, and finite number of receptors. *IEEE Transactions on NanoBioscience*, 15(7):713–727, 2016.
- [186] M. Zoofaghari, A. Etemadi, H. Arjmandi, and I. Balasingham. Modeling molecular communication channel in the biological sphere with arbitrary homogeneous boundary conditions. *IEEE Wireless Communications Letters*, 10(12):2786–2790, 2021.
- [187] C. Brandenberger. Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles. *Small*, 6(15):1669–1678, 2010.
- [188] U. T. Arasu, K. Härkönen, A. Koistinen, and K. Rilla. Correlative light and electron microscopy is a powerful tool to study interactions of extracellular vesicles with recipient cells. *Experimental Cell Research*, 376(2):149–158, 2019.
- [189] K. L. Nicholson, M. Munson, R. B. Miller, T. J. Filip, R. Fairman, and F. M. Hughson. Regulation of snare complex assembly by an n-terminal domain of the t-snare sso1p. *Nature Structural Biology*, 5(9):793–802, 1998.

- [190] S. Casado, M. del V. T. Lobo, and C. L. Paíno. Dynamics of plasma membrane surface related to the release of extracellular vesicles by mesenchymal stem cells in culture. *Scientific Reports*, 7(1), 2017.
- [191] Y. Xie. Tumor apoptotic bodies inhibit ctl responses and antitumor immunity via membrane-bound transforming growth factor-β1 inducing cd8+ t-cell anergy and cd4+ tr1 cell responses. Cancer Research, 69(19):7756–7766, 2009.
- [192] E. Syková. Glia and extracellular space diffusion parameters in the injured and aging brain. pages 77–098, 2008.
- [193] S.-J. Hao, Y. Wan, Y.-Q. Xia, X. Zou, and S.-Y. Zheng. Size-based separation methods of circulating tumor cells. Advanced Drug Delivery Reviews, 125:3–20, 2018.
- [194] B. Kwak, A. Ozcelikkale, C. S. Shin, K. Park, and B. Han. Simulation of complex transport of nanoparticles around a tumor using tumormicroenvironment-on-chip. *Journal of Controlled Release*, 194:157–167, 2014.
- [195] S.-W. Wu, L. Li, Y. Wang, and Z. Xiao. Ctl-derived exosomes enhance the activation of ctls stimulated by low-affinity peptides. *Frontiers in Immunology*, 10, 2019.
- [196] D. H. Kim. Exosomal pd-11 promotes tumor growth through immune escape in non-small cell lung cancer. Experimental & Molecular Medicine, 51(8):1–13, 2019.
- [197] H. Tadokoro. Adenosine leakage from perforin-burst extracellular vesicles inhibits perforin secretion by cytotoxic t-lymphocytes. *PLOS ONE*, 15(4), 2020.
- [198] N. Seo. Activated cd8+ t cell extracellular vesicles prevent tumour progression by targeting of lesional mesenchymal cells. *Nature Communications*, 9(1), 2018.
- [199] C. Ciardiello, R. Migliorino, A. Leone, and A. Budillon. Large extracellular vesicles: Size matters in tumor progression. *Cytokine & Growth Factor Reviews*, 51:69–74, 2020.

- [200] et al. Van Niel, Guillaume. Challenges and directions in studying cell-cell communication by extracellular vesicles. *Nature Reviews Molecular Cell Biology*, 23(5):369–382, 2022.
- [201] et al. Mathieu, Mathilde. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nature cell biology*, 21(1):9–17, 2019.
- [202] Scott Ferguson and Ralph Weissleder. Modeling EV kinetics for use in early cancer detection. *Advanced biosystems*, 4(12):1900305, 2020.
- [203] Emeline Bonsergent, Eleonora Grisard, Julian Buchrieser, Olivier Schwartz, Clotilde Théry, and Grégory Lavieu. Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells. *Nature communications*, 12(1):1–11, 2021.
- [204] Mohammad Zoofaghari, Martin Damrath, Hamid Khoshfekr Rudsari, Fabrizio Pappalardo, Mladen Veletić, and Ilangko Balasingham. Reaction rates estimation for the endocytic reception in extracellular vesicles-mediated communications. In *Proceedings of the 9th ACM International Conference on Nanoscale Computing and Communication*, NANOCOM '22, page 1–6, New York, NY, USA, 2022. Association for Computing Machinery.
- [205] Ling-Gang Wu. Kinetic regulation of vesicle endocytosis at synapses. Trends in neurosciences, 27(9):548–554, 2004.
- [206] Yanan Gao, You Qin, Chao Wan, Yajie Sun, Jingshu Meng, Jing Huang, Yan Hu, Honglin Jin, and Kunyu Yang. Small extracellular vesicles: a novel avenue for cancer management. *Frontiers in Oncol*ogy, 11:638357, 2021.
- [207] Mladen Veletić, Michael Taynnan Barros, Ilangko Balasingham, and Sasitharan Balasubramaniam. A molecular communication model of exosome-mediated brain drug delivery. In *Proceedings of the Sixth Annual ACM International Conference on Nanoscale Computing and Communication*, NANOCOM '19, page 1–7, New York, NY, USA, 2019. Association for Computing Machinery.

- [208] Hamid Khoshfekr Rudsari, Mohammad Zoofaghari, Mladen Veletić, Jacob Bergsland, and Ilangko Balasingham. The end-to-end molecular communication model of extracellular vesicle-based drug delivery. *IEEE Transactions on NanoBioscience*, 2022.
- [209] Yan Guan, Xiaonan Shan, Fenni Zhang, Shaopeng Wang, Hong-Yuan Chen, and Nongjian Tao. Kinetics of small molecule interactions with membrane proteins in single cells measured with mechanical amplification. *Science Advances*, 1(9):e1500633, 2015.
- [210] Marta K Domanska, Volker Kiessling, Alexander Stein, Dirk Fasshauer, and Lukas K Tamm. Single vesicle millisecond fusion kinetics reveals number of snare complexes optimal for fast snare-mediated membrane fusion. The Journal of Biological Chemistry, 285(15):11753, 2010.
- [211] Craig M McKittrick, Sean McKee, Simon Kennedy, Keith Oldroyd, Marcus Wheel, Giuseppe Pontrelli, Simon Dixon, Sean McGinty, and Christopher McCormick. Combining mathematical modelling with in vitro experiments to predict in vivo drug-eluting stent performance. Journal of Controlled Release, 303:151–161, 2019.
- [212] Steffi Hansen, Andreas Henning, Arne Naegel, Michael Heisig, Gabriel Wittum, Dirk Neumann, Karl-Heinz Kostka, Jarmila Zbytovska, Claus-Michael Lehr, and Ulrich F Schaefer. In-silico model of skin penetration based on experimentally determined input parameters. part i: Experimental determination of partition and diffusion coefficients. *European Journal of Pharmaceutics and Biopharmaceutics*, 68(2):352– 367, 2008.
- [213] Ning Wu, Li-Shan Wang, Darren Cherng-Wen Tan, Shabbir M Moochhala, and Yi-Yan Yang. Mathematical modeling and in vitro study of controlled drug release via a highly swellable and dissoluble polymer matrix: polyethylene oxide with high molecular weights. *Jour*nal of controlled release, 102(3):569–581, 2005.
- [214] Manar A Alqudah. Cancer treatment by stem cells and chemotherapy as a mathematical model with numerical simulations. *Alexandria Engineering Journal*, 59(4):1953–1957, 2020.

- [215] Berta Mendoza-Juez, Alicia Martínez-González, Gabriel F Calvo, and Víctor M Pérez-García. A mathematical model for the glucose-lactate metabolism of in vitro cancer cells. *Bulletin of mathematical biology*, 74(5):1125–1142, 2012.
- [216] Emmanuel Luján, Liliana N Guerra, Alejandro Soba, Nicolás Visacovsky, Daniel Gandía, Juan C Calvo, and Cecilia Suárez. Mathematical modelling of microtumour infiltration based on in vitro experiments. *Integrative Biology*, 8(8):879–885, 2016.
- [217] Andrew M Stein, Tim Demuth, David Mobley, Michael Berens, and Leonard M Sander. A mathematical model of glioblastoma tumor spheroid invasion in a three-dimensional in vitro experiment. *Biophysical journal*, 92(1):356–365, 2007.
- [218] Pavel Loskot, Komlan Atitey, and Lyudmila Mihaylova. Comprehensive review of models and methods for inferences in bio-chemical reaction networks. *Frontiers in genetics*, page 549, 2019.
- [219] Arne Naegel, Steffi Hansen, Dirk Neumann, Claus-Michael Lehr, Ulrich F Schaefer, Gabriel Wittum, and Michael Heisig. In-silico model of skin penetration based on experimentally determined input parameters. part ii: Mathematical modelling of in-vitro diffusion experiments. identification of critical input parameters. *European journal of pharmaceutics and biopharmaceutics*, 68(2):368–379, 2008.
- [220] Awatif Jahman Alqarni, Azmin Sham Rambely, and Ishak Hashim. Dynamic modelling of interactions between microglia and endogenous neural stem cells in the brain during a stroke. *Mathematics*, 8(1):132, 2020.
- [221] Sana Abdulkream Alharbi and Azmin Sham Rambely. A new odebased model for tumor cells and immune system competition. *Mathematics*, 8(8):1285, 2020.
- [222] Carrie A Franzen, Patricia E Simms, Adam F Van Huis, Kimberly E Foreman, Paul C Kuo, and Gopal N Gupta. Characterization of uptake and internalization of exosomes by bladder cancer cells. *BioMed research international*, 2014, 2014.

- [223] Jakub M. Tomczak and Ewelina Weglarz-Tomczak. Estimating kinetic constants in the Michaelis-Menten model from one enzymatic assay using approximate Bayesian computation. *FEBS letters*, 593:2742– 2750, Oct 2019.
- [224] Steven S Andrews and Dennis Bray. Stochastic simulation of chemical reactions with spatial resolution and single molecule detail. *Physical biology*, 1(3):137, 2004.
- [225] J A D Wattis, B O'Malley, H Blackburn, L Pickersgill, J Panovska, H M Byrne, and K G Jackson. Mathematical model for low density lipoprotein (LDL) endocytosis by hepatocytes. *Bulletin of Mathematical Biology*, 70(8):2303–2333, Aug. 2008.
- [226] H. James Harwood and Lorraine D. Pellarin. Kinetics of low-density lipoprotein receptor activity in Hep-G2 cells: derivation and validation of a Briggs-Haldane-based kinetic model for evaluating receptormediated endocytotic processes in which receptors recycle. *Biochem* J., 323:649–659, May 1997.
- [227] Ian F Akyildiz, Fernando Brunetti, and Cristina Blázquez. Nanonetworks: A new communication paradigm. Computer Networks, 52(12):2260–2279, 2008.
- [228] Weisi Guo, Mahmoud Abbaszadeh, Lin Lin, Jerome Charmet, Peter Thomas, Zhuangkun Wei, Bin Li, and Chenglin Zhao. Molecular physical layer for 6g in wave-denied environments. *IEEE Communications Magazine*, 59(5):33–39, 2021.
- [229] George M Whitesides. The origins and the future of microfluidics. nature, 442(7101):368–373, 2006.
- [230] Richard B Fair, Andrey Khlystov, Tina D Tailor, Vladislav Ivanov, Randall D Evans, Vijay Srinivasan, Vamsee K Pamula, Michael G Pollack, Peter B Griffin, and Jack Zhou. Chemical and biological applications of digital-microfluidic devices. *IEEE Design & Test of Computers*, 24(1):10–24, 2007.
- [231] Dominic Eicher and Christoph A Merten. Microfluidic devices for diagnostic applications. Expert review of molecular diagnostics, 11(5):505– 519, 2011.

- [232] Jitendra Singh and Naresh Kumar. Performance analysis of different modulation format on free space optical communication system. Optik, 124(20):4651–4654, 2013.
- [233] GL Woods, P Papaparaskeva, M Shtaif, I Brener, and DA Pitt. Reduction of cross-phase modulation-induced impairments in long-haul wdm telecommunication systems via spectral inversion. *IEEE Photonics Technology Letters*, 16(2):677–679, 2004.
- [234] M Lucki, R Agalliu, and R Zeleny. Limits of advanced modulation formats for transition in fiber optic telecommunication systems to increase speeds from 10, 40, 100 gbps to higher bit rates. In *Optical Modelling* and Design III, volume 9131, pages 591–599. SPIE, 2014.
- [235] R. S. Wijetunge, C. J. Brace, J. G. Hawley, N. D. Vaughan, R. W. Horrocks, and G. L. Bird. Dynamic behaviour of a high speed direct injection diesel engine. *SAE Transactions*, 108:1120–1129, 1999.
- [236] Patrick Cooley, David Wallace, and Bogdan Antohe. Applicatons of ink-jet printing technology to biomems and microfluidic systems. JALA: Journal of the Association for Laboratory Automation, 7(5):33– 39, 2002.
- [237] C. Yao, Y. Liu, C. Xu, Zhao, S., and G. Chen. Formation of liquid–liquid slug flow in a microfluidic t-junction: Effects of fluid properties and leakage flow. *AIChE Journal*, 64, 2018.
- [238] Wen Zeng, Songjing Li, and Zuwen Wang. Characterization of syringepump-driven versus pressure-driven microfluidic flows. In 2015 International Conference on Fluid Power and Mechatronics (FPM), pages 711–715. IEEE, 2015.
- [239] Fatih Dinc, Bayram Cevdet Akdeniz, Ali Emre Pusane, and Tuna Tugcu. A general analytical approximation to impulse response of 3-d microfluidic channels in molecular communication. *IEEE transactions* on nanobioscience, 18(3):396–403, 2019.