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A novel cationic β-cyclodextrin decorated with a choline-like pendant exhibits Iodophor, Mucoadhesive and bactericidal properties

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ABSTRACT

Iodine is a vital microelement and a powerful antiseptic with a rapid and broad spectrum of action. The development of iodophor compounds to improve the solubility and stability of iodine is still challenging. Here, we report the synthesis of a novel cationic β-cyclodextrin bearing a choline-like pendant (β-CD-Chol) designed to complex and deliver iodine to bacterial cells. The characterization of β-CD-Chol and the investigation of the inclusion complex with iodine were performed by NMR spectroscopy, mass spectrometry, UV–vis spectrophotometry, isothermal titration calorimetry, and dynamic light scattering. The functionalization with the positively charged unit conferred improved water-solubility, mucoadhesivity, and iodine complexation efficiency to the β-CD scaffold. The water-soluble β-CD-Chol/iodine complex efficiently formed both in solution and by solid-vapor reaction. The solid complex exhibited a significant stability for months. Iodine release from the inclusion complex was satisfactory and the bactericidal activity was proved against a *Staphylococcus epidermidis* strain. The absence of cytotoxicity tested on human keratinocytes and the improved mucoadhesivity make β-CD-Chol a promising drug delivery system and an appealing iodophor candidate for iodine-based antisepsis including mucosa disinfection.

1. Introduction

The attack of pathogen microorganisms is a serious threat to all living beings and developing effective bactericidal systems is an urgent need. Iodine is a well-known, inexpensive, easily available, and effective antiseptic with a broad spectrum of action (anti-virus, -bacteria, -fungi, and -protozoa). Iodine destroys microbial biomolecules, irreversibly damages cellular membranes and alters the metabolic functions of pathogens (Lawrence, 1998; Selvaggi et al., 2003). However, iodine is toxic, poorly soluble in water and easily sublimates. To overcome these weaknesses, iodophors capable of complexing and controlling iodine release have been developed (Capriotti & Capriotti, 2012; Makhayeva et al., 2020). Nevertheless, the use of iodine as an antiseptic is still challenging. Currently, polyvinylpyrrolidone-iodine (PVP-I) is the most used iodophor in pharmaceuticals (Luo et al., 2021). However, PVP is only poorly biodegradable (Wang et al., 2006) and the antimicrobial effect of PVP-I strongly depends on the release of iodine from the complex. Furthermore, due to the lack of mucoadhesive properties of PVP, iodine remains on the mucosal membranes for a short time, limiting its antimicrobial efficacy (Au-Duong & Lee, 2017).

Cyclodextrins (CDs) and their derivatives have been proposed as biodegradable and mucoadhesive iodophors exhibiting antiseptic properties (Asim et al., 2019; Kali et al., 2023). CDs are cyclic oligosaccharides characterized by inexpensive price, high accessibility, and the presence of a hydrophobic cavity of well-defined dimensions depending on the number of glucopyranose units forming the cycle (6, 7, and 8 for α-, β- and γ-CDs, respectively) (Periasamy, 2020). Nowadays, CDs are used as excipients in numerous pharmaceutical formulations (Aiassa et al., 2021) since the host properties of their cavities can improve the water solubility and stability of a variety of hydrophobic

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drugs by forming inclusion complexes. The reversibility of the inclusion phenomena allows the release of the drug and regeneration of the CD molecular container (Morin-Crini et al., 2021).

CDs as starch-derived compounds form very stable inclusion complexes with iodine and triiodide ions and this property has been known since the late 1940s. Iodine molecule with a diameter of 4.94 Å can fit in the internal cavity of a β-CD having a size of 6.0–6.5 Å (Mura, 2014). Stable β-CD/iodine complexes with pharmacological activities have been reported (Betzel et al., 1983; Noltemeyer & Saenger, 1976; Wang et al., 2011). A β-CD/iodine complex was also patented to be used as preservative in fish paste (Hirose & Miwa, 1976) and frozen marine products (Hirose et al., 1976), as well as bactericide in aerosols and human body deodorants (Yoshitomi et al., 1977).

Among CD derivatives, cationic CDs represent a remarkable family of drug delivery systems with great potential for pharmaceutical applications (Ahmadi et al., 2020; Zhang et al., 2022). Positively charged CDs displayed favorable mucoadhesive (Feng et al., 2020; Kali et al., 2023), cell penetration, drug (Feng et al., 2020; Gil et al., 2009) and gene delivery (Yannakopoulou, 2012) properties.

Choline is an appropriate ligand to target cells expressing choline transporters, such as tumor cells (Li et al., 2013), unicellular pathogens (Biagini et al., 2004) and bacteria (Malek et al., 2011; Neves et al., 2022).

In the search of more effective iodine-based antiseptic agents, with the further aim of improving the iodophor properties of suitable carriers such as β-CD macrocycles, here we propose the design and synthesis of a novel cationic β-CD derivative bearing a choline ligand unit (β-CD-Chol). We hypothesized that the linkage of a positively charged choline-like group to the β-CD backbone (i) dramatically enhances the β-CD water-solubility by disruption of the hydrogen-bond network typical of the native β-CD; (ii) increases the mucoadhesivity and favors the interaction with the negatively charged bacterial membrane; (iii) improves the affinity for iodine thus providing a water soluble iodine inclusion complex with antibacterial activity; (iv) can offer a novel antiseptic agent as a stable solid-state β-CD/iodine complex.

The β-CD-Chol receptor we designed and synthesized to prove the abovementioned premises. It was characterized by NMR spectroscopy, mass spectrometry and dynamic light scattering analysis. The β-CD-Chol/iodine complex was prepared both in water (phosphate/citrate buffer solution, pH 6) and at the solid state by solid-vapor reaction. The inclusion complex was characterized by NMR spectroscopy, UV–vis spectrophotometry, and isothermal titration calorimetry. The effect of β-CD-Chol on the solubility, stability, loading and release of iodine was investigated and critically compared to the native β-CD or PVP-I, selected as a model of commercial iodophor. Parameters such as stoichiometry and binding affinity of the β-CD-Chol/iodine complex were determined. The cytotoxicity and mucoadhesivity of the cationic β-CD-Chol were tested on human keratinocyte cells and mouse intestinal mucosa, respectively. The bactericidal activity of the β-CD-Chol/iodine complex was demonstrated by time killing assay against a *Staphylococcus epidermidis* strain. Overall, the converging evidences yield by the complementary use of different techniques and investigation tools led us to ascertain the appealing properties of our devised iodine receptor that may be a promising candidate as an effective antibacterial agent.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma Aldrich and used without further purification. High purity water (Millipore, Milli-Q Element A 10 ultrapure water) and A grade glassware were employed throughout for ITC measurements. *Staphylococcus epidermidis* (ATCC® 12228, Origin Strain No. CECT® 231) and related reagents were obtained from Merck KGaA (Darmstadt, Germany). Bacteria were cultured (37 °C) in 15 % agar plates supplemented with nutrient broth (3 % beef extract, 5 % peptone). HaCaT human keratinocytes were obtained from the IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia dell'Emilia Romagna, Brescia, Italy) and maintained at 37 °C (5 % CO_2) in DMEM (with 10 % FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin). All media and tissue-culture reagents were from Euroclone S.p.A. (Pero, Milan, Italy).

2.2. Instrumentation

UV–vis spectra were recorded on an Agilent 8453 (Agilent Technologies, USA) or Jasco V770 (Jasco Europe, Italy) spectrophotometer. Freeze-drying was performed using a LyoQuest-85 (Telstar, Italy) freeze dryer. NMR and HRMS spectra were recorded on a Bruker 400™ spectrometer (Bruker, Germany) and a LTQ Orbitrap XL (Thermo Scientific, USA) instrument with an ESI ionization, respectively. DLS measurements were performed on a ZetaSizer NanoZS90 (Malvern Instrument, Malvern, UK) equipped with a 633 nm laser, at the scattering angle of 90° and 25 °C temperature. ITC experiments were run at 25 °C using a nano-isothermal titration calorimeter (Nano-ITC, TA Instruments, New Castle, DE, USA) equipped with an active cell of 0.988 mL and a 250 μL injection syringe.

2.3. Methods

2.3.1. Synthesis of β-CD-Chol

Dry monotosylate β-CD (Tosyl-β-CD) (100 mg, 89 μmol), prepared as reported in literature (Tripodo et al., 2013), was dissolved in neat *N,N*-dimethylethanolamine (1.8 mL, 22 μmol) and stirred under argon atmosphere. After complete dissolution, the reaction mixture was heated to 80 °C and stirred overnight. The solvent was removed in vacuo, the solid residue was purified on a C18 reversed-phase silica gel (LiChroprep RP18) column with water as eluent. The fractions containing β-CD-Chol were collected, and the solvent was removed in vacuo. The obtained product was subjected to ion–exchange chromatography (Dowex 50, H^+ form) eluting with 3 wt% aqueous NH_4HCO_3 (450 mL). The eluate was dried in vacuo. The solid residue was dissolved in a minimal amount of H_2O and precipitated by addition of acetone (60 mL). Finally, β-CD-Chol with bicarbonate as the counterion was freeze-dried to give a white solid in 40 % yield (45 mg, 35 μmol); Rf: 0.27 (Propanol/H_2O/NH_4OH/AcOEt, 5:3:2:2, v/v/v/v); $[\alpha]_D^{25} = +116$ (H_2O).

2.3.2. Chemical characterization of β-CD-Chol

NMR assignment. ^{1}H NMR (400.13 MHz, D_2O, 297 K): $\delta = 4.98–4.93$ (m, 7H, 7 × H-1), 4.42 (t, $J = 8.9$ *Hz*, 1H, H-5′), 3.84 (bt, $J = 9.6$ *Hz*, 1H, H-3′), 3.95–3.39 (m, 43H, 7 × H-2, 6 × H-3, 6 × H-4, 6 × H-5, 14 × H-6, 4 × Chol CH_2), 3.51 (m, $J = 9.4$ *Hz*, 1H, H-4′), 3.11, 3.12 (s, 6H, 2 × Chol N-CH_3) ppm. ^{13}C NMR (100.62 MHz, D_2O, 297 K): δ = 102.2, 102.1, 101.8, 101.6, 100.3 (C-1), 83.0, 81.6, 81.2, 81.1, 80.9, 80.8 (C-4), 73.5–71.3 (C-2, C-3, C-5), 67.1 (C-5′), 66.8 (C-6′), 65.9 (Chol CH_2O), 61.2–59.9 (C-6), 55.3 (Chol CH_2N), 52.8, 52.3 (Chol CH_3N). *HR-MS spectrum*: Calculated for $C_{46}H_{80}O_{35}$ *m/z* 1206.451, found 1206.456 $[M]^+$

2.3.3. DLS measurements

Aqueous solutions (4 mM) of β-CD and β-CD-Chol were prepared and passed through a 0.2 μm filter before DLS measurements.

2.3.4. Preparation of β-CD-Chol/iodine complex in solution

To a water solution of β-CD-Chol (2 mg/mL, 1.57 mM) an excess of solid iodine (1.59 mg/mL, 6.3 mM) was added. The mixture was stirred at 25 °C for 3 days, then it was placed in a refrigerator for 12 h. Then, the sample was centrifuged at 10.000 rpm for 15 min to remove undissolved iodine, and the yellow supernatant was recovered.

2.3.5. Preparation of β-CD-Chol/iodine complex by iodine vapors

Lyophilized β-CD-Chol (5 mg, 3.95 μmol) in an opened vial was placed in a closed glass vessel containing solid iodine. The β-CD-Chol

powder was magnetically stirred at 50 °C for 5 h. In the presence of iodine vapors, the color of the powder changed from white to brown. The vial was removed from the glass vessel and the powder was kept in the opened vial for 24 h to remove any iodine adsorbed on the powder surface.

2.3.6. ITC experiments

For ITC titrations, aqueous iodine solutions containing potassium iodide were prepared by dissolving I_2 in a solution containing excess KI at concentrations such that the formation of I_3^- was virtually complete. The concentration of iodine was accurately determined by titration with a standard solution of sodium thiosulfate. ITC experiments were run in the overfilled mode; the reference cell was filled with ultrapure water. The reaction mixture in the sample cell was stirred at 250 rpm during the titration. All solutions were gently stirred and degassed under vacuum for about 15 min before each experiment. The calorimeter was calibrated chemically by a test HCl/TRIS reaction following the procedure described (Sgarlata et al., 2013) and further checked through an electrical calibration. ITC measurements were conducted by titrating a 10 mM aqueous solution of iodine (in 50 mM KI) into a 0.2–0.5 mM aqueous solution of either β-CD-Chol or β-CD (in 50 mM KI). Typically, 3–4 independent ITC measurements were run for each system. The heat of dilution was determined in separate "blank" experiments by titrating a 10 mM aqueous solution of iodine (in 50 mM KI) into a 50 mM KI aqueous solution. The power curve was integrated by NanoAnalyze (TA Instruments, USA) to obtain the gross heat released or adsorbed in the reaction. The net heats of reaction, obtained by subtracting the heat recorded in the blank experiments, were handled with HypCal, a software which enables the determination of both equilibrium constant and enthalpy change values for binding reactions in solution through a non-linear least-squares analysis of the calorimetric data (Arena et al., 2016). The program allows for the refinement of multiple titrations.

2.3.7. Determination of iodine in the β-CD-Chol/iodine solution

1 mL of β-CD-Chol/iodine complex solution was shaken with cyclohexane (4 × 1 mL) for an exhaustive iodine extraction. The same procedure was performed on the aqueous solution of iodine (0.3 mg/mL). The amount of iodine was determined in the organic phase by referring to a calibration curve.

2.3.8. Iodine release

The release of iodine from the β-CD-Chol/iodine complex (0.8 mL) aqueous solution was evaluated by depositing 2 mL of cyclohexane on the aqueous solution of each iodophor solution. The absorbance of iodine in cyclohexane was continuously recorded on a Jasco V-770 spectrophotometer at 37 °C for 24 h.

2.3.9. In vitro evaluation of β-CD-Chol cytotoxicity

Compound-dependent effects on cell viability were measured in confluent HaCaT keratinocyte monolayers using crystal violet staining. Treatments (24 h) were performed in 96-well plates, employing DMSO as the vehicle control. After incubation, cells were washed with phosphate-buffered saline (PBS), fixed (in 4 % paraformaldehyde) and stained with a crystal violet solution (1 %). Following other rounds of washing and crystal violet extraction with 10 % acetic acid at room temperature for 10 min, residual cell staining was quantified by measuring the absorbance at 590 nm with a microplate reader (Synergy HT, BioTek).

2.3.10. Animals

Seven-week-old, male Sprague Dawley (*Rattus Norvegicus*; n, 3) were obtained from ENVIGO (Milan, Italy) and housed in a controlled environment (22 ± 2 °C, 55 ± 15 % relative humidity, 12 h light/dark cycle). The animals were acclimatized to their environment for 1 week and had ad libitum access to tap water and rodent standard diet. Studies were approved by the Italian Ministry of Health (authorization n°306/2022-PR, released on 13 May 2022) and were in compliance with EU regulations (Directive 2010/63/EU).

2.3.11. Ex vivo evaluation of β-CD-Chol mucoadhesive properties

Assessment of mucoadhesive properties was performed ex vivo, on rat intestinal tissues. Fluorescent samples of β-CD-Chol and native β-CD were prepared by inclusion of coumarin-6 fluorophore into the cyclodextrin cavity. To this end, aqueous solutions of β-CD or β-CD-Chol (3.2 mg/mL) were prepared, and the pH of the solutions was adjusted to 6.5 with 0.1 M HCl. Then, 65 μL of a stock solution of fluorophore (0.33 mg in 1.65 mL ethanol) was added to each solution. After stirring for 24 h, the suspensions were centrifugated at 10,000 rpm for 5 min. The supernatants were recovered and freeze- dried. Tissues were obtained immediately after animal euthanasia and kept on ice. Intestinal tubes were cut longitudinally, divided into pieces of approximately 1 × 1 cm and placed onto 24-well plates. Then, aliquots (10 μl, at 10 mg/mL) of β-CD/coumarin or β-CD-Chol/coumarin complexes were placed onto mucosa surfaces of the intestinal specimens. After 90 min incubations, intestinal mucosae were washed (2 × 5 min) with phosphate-buffered saline (PBS), collected and homogenized. Finally, individual samples (100 μL) were transferred to a microplate reader (Synergy HT-BioTek) and fluorescence intensity was measured (λem 510 nm, λexc 445 nm). Mucoadhesion properties of the compounds were quantified as percentages of residual fluorescent complexes on intestinal mucosal surfaces by the formula:

$$\text{Substance adherent to the mucosa } [\%] = \text{Fluorescence of Sample} / \text{Fluorescence of Control} \times 100$$

where Control represents the respective unwashed sample condition.

2.3.12. Antibacterial activity

Antibacterial activity was investigated with the Time-Kill Assay. Bacterial suspensions were prepared from 16 h growth cultures diluted to ~1.5×10^8 CFU/mL, estimated by comparison with 0.5 McFarland turbidity standard employing an UV-Spectrophotometer (Synergy HT, BioTek). The final assay concentration of 6×10^5 CFU/mL of *Staphylococcus epidermidis* was achieved through intermediate microbial suspensions of ~7.5×10^6 CFU/mL. Then, 0.1 mL of bacterial suspensions were added to 1.9 mL of each compound solution and incubated for different times (10, 20, 40 s, and 1, 2, 4, 8, 60 min), before residual iodine neutralization with 0.5 % sodium-thiosulfate solution (1:10 dilution, *v*/v). After two additional rounds of dilutions (1:10, v/v) in sterile saline solutions, 0.1 mL of each tested condition was plated (by spreading) onto agar-enriched culture medium and incubated at 37 °C, for 24 h, before colony counting.

2.3.13. Statistical analysis

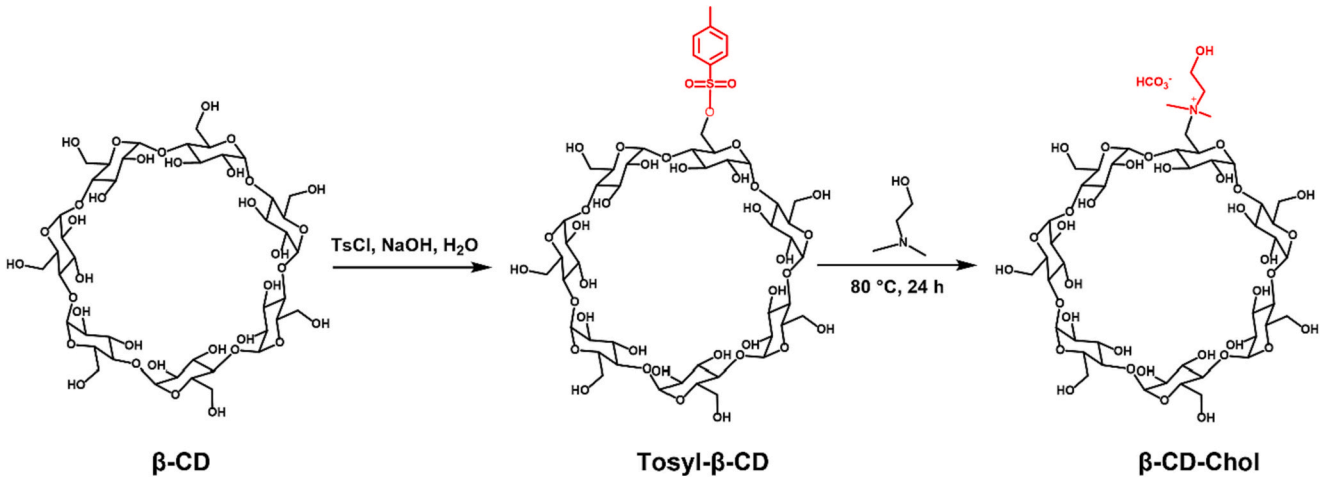
Results are means ± SEM of three independent experiments, performed in triplicate (unless otherwise indicated). Statistical comparisons were made with two-way ANOVA and Student's *t*-test. *P* values were considered significant at $\alpha \leq 0.05$. All analyses were done with GraphPad Prism 10 (GraphPad Software, Inc., San Diego, CA).

3. Results and discussion

3.1. Preparation and characterization of the mono-choline-β-CD (β-CD-Chol)

The designed β-CD-Chol was synthesized starting from native β-CD in which one CH_2OH group was activated by reaction with *p*-toluene-sulfonyl chloride (Pedotti et al., 2015; Tripodo et al., 2013). The obtained mono-tosylate β-CD was treated with *N,N*-dimethylethanolamine to give, after chromatography purification, pure β-CD-Chol (40 % yield) bearing a choline-like pendant (Scheme 1).

Structural characterization of β-CD-Chol was performed by 1D- and



Scheme 1. Synthetic route for the preparation of the mono-substituted β-CD-Chol.

2D-NMR spectroscopy (**Fig. S1-S5**) and HR-MAS spectrometry. ^{1}H and ^{13}C NMR spectra of β-CD-Chol showed proton signals at 3.11 and 3.12 ppm, 3.55 ppm, and 3.94 ppm, and carbon signals at 52.8 and 52.3 ppm, 55.3 ppm, and 65.9 ppm, related to the N-CH_3, N-CH_2 and CH_2O groups of the choline moiety. The monofunctionalization of the cyclodextrin skeleton was corroborated by changes in the chemical shift of H-5′ (downfield shift) and H-4′ (upfield shift) as well as C-5′ (upfield shift), C-4′ and C-6′ (downfield shift) signals of the functionalized glucose unit. The covalent linkage of a choline-like unit was confirmed by HRMS-spectrum showing the expected molecular ion peak at *m/z* 1206.456.

3.2. Water solubility of β-CD-Chol

CDs have two hydrophobic regions, located inside and outside the toroidal structure, connected by two hydrophilic edges. Dipole interactions between the hydrophilic outer edges and bulk water molecules allow for the dissolution of CDs in water. However, β-CD has been reported to have very low solubility (18.5 mg/mL at 25 °C) and a high tendency to self-aggregate in water (Dodziuk, 2006). Bonini et al. determined a critical aggregation concentration around 2–3 mM by dynamic and static light scattering measurements; moreover, by transmission electron microscopy at cryogenic temperature, they observed polydisperse nearly spherical objects with diameters of about 100 nm at lower concentrations and micrometer planar aggregates as predominant population at higher concentrations (Bonini et al., 2006).

β-CD-Chol showed a higher water solubility than native β-CD. A comparative study evidenced that the introduction of a quaternary ammonium group enhances the water solubility of the β-CD macrocycle from 18 mg/mL to at least 100 mg/mL. Dynamic light scattering (DLS) measurements confirmed the smaller tendency of the cationic β-CD-Chol to aggregate. In the view of pharmaceutical application, the samples were sterilized by filtration through a 0.2 μm filter. The filtered samples showed a population with mean hydrodynamic diameter around 255 nm (intensity, volume, and number % distribution mode) for native β-CD, whereas a population with mean hydrodynamic diameter around 0.7 nm (volume and number % distribution mode) was observed in the β-CD-Chol solution (Fig. 1). In this latter compound, an additional population with mean hydrodynamic diameter around 90 nm was observed in intensity % distribution mode (**Fig. S6**). The larger aggregates are a minority; in fact, it is known that the intensity distribution may detect even small amount of aggregates and when the relative peak disappears in transforming intensity to volume distribution its contribution is negligible (<0.001 %).

3.3. Preparation and characterization of β-CD-Chol/iodine complex

The capability of β-CD-Chol to complex iodine was investigated both in solution and in the solid state.

3.3.1. Preparation and characterization of β-CD-Chol/iodine complex in solution

For the preparation of the β-CD-Chol/iodine complex in aqueous solution, an excess of solid iodine (4:1 M ratio) was stirred in an aqueous colloidal solution of β-CD-Chol (2 mg/mL) and the undissolved iodine was removed by ultracentrifugation. As a reference, the same amount of iodine in the absence of β-CD-Chol was treated by the same procedure.

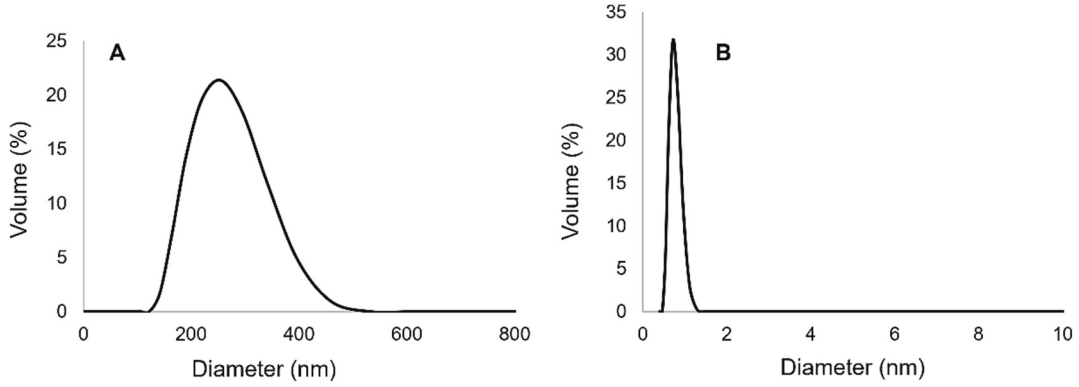


Fig. 1. Dynamic light scattering: volume weighted hydrodynamic diameter distribution of A) native β-CD (4.54 mg/mL, 4 mM) and B) β-CD-Chol (5 mg/mL, 4 mM) after filtration.

The formation of the β-CD-Chol/iodine complex was studied by NMR and UV–vis spectrometry. In the β-CD skeleton, H3 and H5 protons are located inside the conical cavity, with H3 situated next to the wider edge and H5 next to the narrower edge, while the remaining H1, H2, and H4 protons are located on the outer surface of the macrocycle. Thus, changes in the H3 and H5 chemical shift are generally indicative of inclusion complex formation (Duchêne, 2011). Comparison of the ^{1}H NMR spectra of β-CD-Chol (4.4 mM in D_2O) in the absence and presence of iodine clearly showed downfield shift of H3 ($\Delta\delta = 0.11$) and H5 ($\Delta\delta = 0.05$) resonances, and choline N-CH_3 ($\Delta\delta = 0.11$) and CH_2O ($\Delta\delta = 0.18$) signals. The observed chemical shift changes (Fig. 2A**, Fig. S7-8**, and **Table S1**) are attributable to the formation of an inclusion complex between iodine and β-CD-Chol and replacement of the bicarbonate counterion by iodide ion.

The formation of the β-CD-Chol/iodine inclusion complex was corroborated by UV–vis spectra. When iodine is dissolved in water, different species are formed including iodide (I^-) and triiodide ions (I_3^-). In the optical absorption spectrum, iodide, triiodide, and molecular iodine show absorption bands at 231 nm, 288 and 351 nm, and 410 nm, respectively (Fig. 2B), as described in the literature (Pursell & Pursell, 2016). Compared with the free iodine solution, the absorption spectra of the β-CD-Chol/iodine complex showed a redshift of the I_3^- absorption bands from 288 to 290 and from 351 to 354 nm as well as an isosbestic point at 434 nm (Fig. 2B) which rules out the presence of multiple equilibria in solution. As reported for other CD/iodine inclusion complexes (Pursell & Pursell, 2016), β-CD-Chol increases the amount of triiodide while decreasing the amount of molecular iodine.

3.3.2. *Preparation and characterization of the β-CD-Chol/iodine complex by solid-vapor reaction*

Due to the high volatility of iodine, having iodine/iodophor antiseptic complexes stable for long time storage is relevant. To this aim, the solid β-CD-Chol/iodine complex was prepared by exposing β-CD-Chol powder to iodine vapors. The color of the β-CD-Chol powder changed from white to brown (Fig. 2B, inset). The formation of the inclusion complex was confirmed by the UV–vis absorption spectra of the solid powder dissolved in pure water or in phosphate-citrate buffer: they both showed the same absorption profile of the complex prepared in solution (Fig. 2B). Amount of captured iodine vapor and loss of iodine over time were evaluated by the changes of the powder weight. Iodine capture resulted in a weight increase of 32.4 % (0.648 mg), that is higher than the 17 % value reported for native β-CD (Wang et al., 2011). The weight monitoring over time evidenced that the solid β-CD-Chol/iodine complex stored in a closed glass container is very stable. No significant weight loss was observed for months. A weight loss of only 8 % was found when the same sample was stored in an opened container at 25 °C for three months.

3.4. *β-CD-Chol increases iodine water solubility*

The dipole interaction between iodine and water is the main cause of iodine dissolution. Therefore, the degree of solubilization depends on the polarization of iodine and the subsequent modification of the water structure. This situation limits the iodine solubility in water to a small amount (0.3 mg/mL at 25 °C). To increase the concentration of iodine in antiseptic formulations, the conventional approaches base on addition of KI to iodine into ethyl alcohol, glycerol, mixtures of these solvents, or in water (Lugol's solution), where KI reacts with iodine to form water-soluble potassium triiodide (Gottardi, 1991). Nevertheless, the high content of free molecular iodine (e.g., 170 mg/L in Lugol's solution) generates staining and irritation of living tissues and provides an unstable formulation due to the high iodine vapor pressure.

The complexation of iodine in polymeric containers including CDs has shown to be a valid approach to increase iodine solubility and to control/limit the iodine side effects. Iodine is generally included in the CD cavity by host-guest interaction and possible replacement of water molecules which initially occupy the cavity.

The effect of CDs on elemental iodine water solubility can be regarded as a measure of the iodine loading capacity under laboratory test conditions. To evaluate the iodine solubilizing effect of β-CD-Chol, the amount of molecular iodine was quantified by extraction with an organic solvent that solubilizes iodine but not the water-soluble anionic species. Due to the well-known triiodide-iodine equilibrium, molecular iodine is regenerated from the triiodide reserve. Thus, repeated extractions with cyclohexane up to exhaustive discoloration of the aqueous solution can be a valid method to quantify molecular iodine in aqueous formulations (Migliore et al., 2023).

Fig. 3A shows that, as evident from the coloration of both organic (purple) and aqueous (yellow) phase (Fig. 3A, inset), a higher amount of iodine is solubilized in water in the presence of β-CD-Chol. By evaluation of the UV–vis absorbance of iodine at 525 nm in cyclohexane (Fig. 3A) and using a proper calibration curve, the amount of molecular iodine extract was quantified to be 0.72 mg/mL versus 0.29 mg/mL found for the aqueous solution of iodine only (this latter value is in line with the water solubility of iodine). Therefore, β-CD-Chol enhances the amount of solubilized iodine in water by 2.5 times. This value was corroborated by iodometric titration with thiosulfate. It is worth noting that the amount of extracted iodine in solution nicely compares with that obtained for the complex prepared by the solid-vapor reaction.

The exhaustive extraction of iodine from the β-CD-Chol/iodine

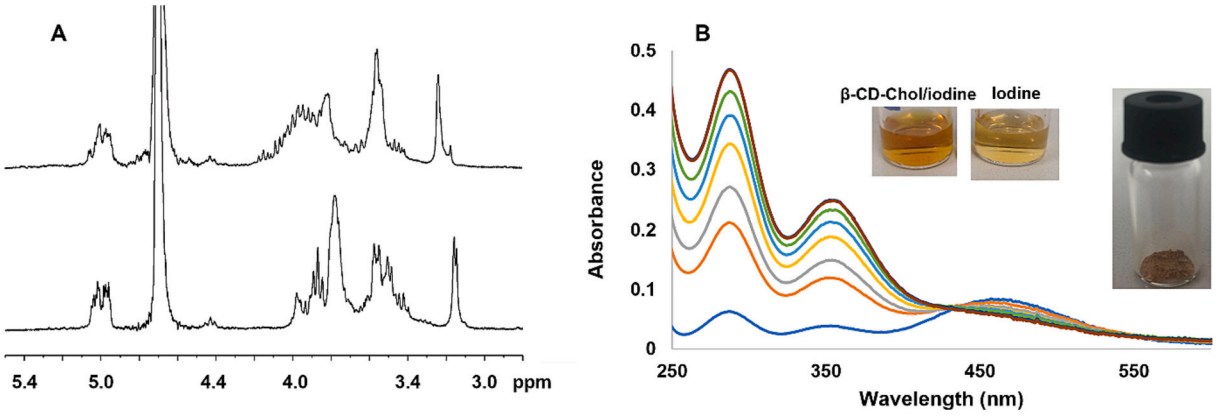


Fig. 2. A) ^{1}H NMR spectra (400.13 MHz, D_2O, 297 K) of β-CD-Chol (bottom, 2.8 mg/0.5 mL, 4.4 mM) and β-CD-Chol/iodine (top, 2.8 mg/0.5 mL and 1 mg/0.5 mL, respectively). B) UV–vis spectra of an aqueous solution of free iodine (0.16 mM, blu) and upon addition of increasing amounts of β-CD-Chol (iodine 0.16 mM, β-CD-Chol from 0.16 mM to 0.5 mM); inset: pictures of iodine and β-CD-Chol/iodine complex in aqueous solution and powder of β-CD-Chol/iodine complex from solid-vapor reaction.

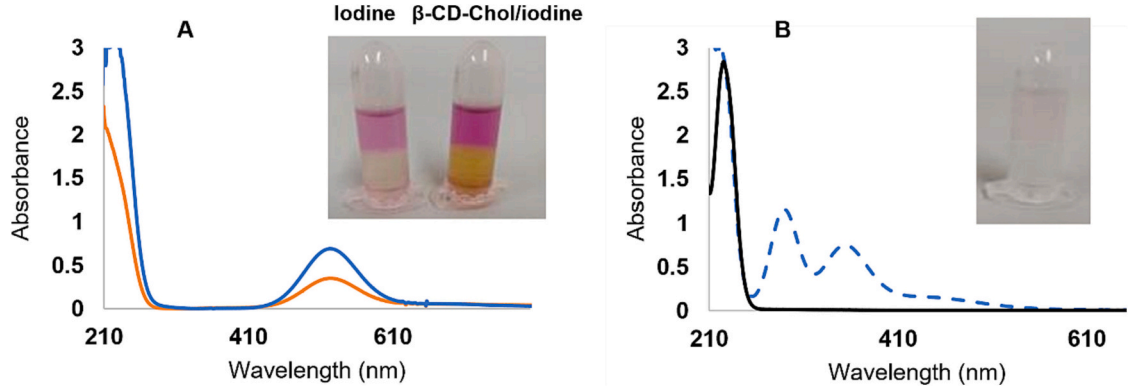


Fig. 3. UV–vis spectra of: A) cyclohexane solution (1 mL) of iodine partially extracted from iodine (0.3 mg/mL, orange) and β-CD-Chol/iodine complex (2 mg/mL and 0.72 mg/mL, blue) solutions; B) water solution of the β-CD-Chol/iodine complex before (dashed blue line) and after (solid black line) exhaustive extraction of iodine with cyclohexane. Insects: Pictures of A) partial and B) exhaustive extraction of iodine by cyclohexane from the β-CD-Chol-iodine water solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complex was confirmed by the optical absorption spectra of the aqueous solution that showed the disappearance of both the triiodide and iodine absorption bands (Fig. 3B).

It is well-documented that β-CD can form inclusion complexes with molecular iodine (I_2) and triiodide ions (I_3^-), which may establish interactions with the permanent β-CD dipole (Pursell & Pursell, 2016). As inferred by the NMR and UV–vis spectra discussed above, an arrangement encompassing iodide as the counterion of the choline ammonium group and triiodide included in the cyclodextrin cavity in equilibrium with iodine can be reasonably hypothesized for the β-CD-Chol/iodine complex (Fig. 4).

Noteworthy, differently than native β-CD, whose iodine inclusion complex formation results in a precipitation phenomenon (Szente et al., 1999), the β-CD-Chol/iodine complex was soluble in water. This is a fundamental property and a great advantage for our system as the low solubility in water has limited the wide practical use of iodine complexes with α and β-cyclodextrins in clinical applications.

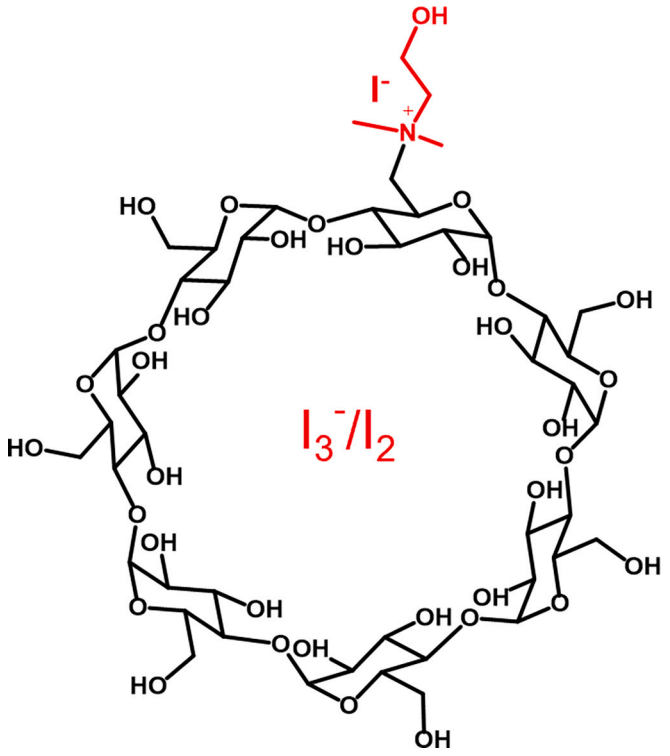


Fig. 4. Schematic representation of the hypothesized β-CD-Chol/iodine complex.

The pH of the β-CD-Chol/iodine complex in pure water was 4.5, that is compatible for ophthalmic and dermatologic applications. However, for a more biocompatible use, we proved that the β-CD-Chol/iodine complex can also be prepared in 10 mM phosphate/citrate buffer at pH 6, a solvent used in commercial iodine-based pharmaceutical formulations, maintaining unaltered all its stability and solubility features.

3.5. *Investigation of the β-CD-Chol/iodine complex by isothermal titration calorimetry (ITC)*

An ITC calorimetric study of the binding features of both β-CD-Chol and β-CD was carried out to determine the affinity, the driving forces as well as the influence of the structure of the receptors on the interaction with iodine in aqueous solution.

As previously described, molecular iodine is slightly soluble in water and is converted to triiodide in the presence of iodide. The binding of I_3^- by a host, such as the cyclodextrin, causes the conversion of iodine into triiodide thus increasing the overall concentration of I_3^- in solution. Consequently, two simultaneous equilibria must be considered for the proper depiction of the process:

$$I_2 + I^- \leftrightarrows I_3^- \quad (1)$$

$$I_3^- + \beta CD \leftrightarrows \beta CD/I_3^- \quad (2)$$

An appealing debate emerged in the literature (Pursell & Pursell, 2016) about the significance of accurately determining the iodine species forming in solution and thus available for the complexation by the receptor. We are interested in examining the binding features of the newly synthesized β-CD-Chol with iodine using a simple equilibria model and consequently we decided to set up our experimental conditions (10 mM of I_2 into a 50 mM KI solution) such that I_2 is fully converted to triiodide and no other iodine species are present in solution. A simple species distribution calculation using the equilibrium constant value (log K = 2.89) reported for the reaction (1) (Pursell & Pursell, 2016) reveals that, at our experimental conditions, I_3^- is basically the only existing species (I_2 is <3 %, **Fig. S9**). Furthermore, despite the large concentration of iodide in solution, the amount of I^- complexed by the cyclodextrin is negligible due to the small stability constant for the I^- + β-CD ⇆ β-CD/I^- binding equilibrium (Rekharsky & Inoue, 1998). Overall, the conditions suitably selected in this work allowed us to simplify the complicated (though well known) equilibria involving iodine thus enabling to ascribe the calorimetric fingerprint recorded as well as the binding parameters refined to the reaction equilibrium (2), with no additional species involved.

Typical ITC runs of triiodide into β-CD-Chol and β-CD at 25 °C are shown in Figs. 5 and 6 along with the corresponding binding curves (net heat values). A typical blank (dilution) experiment is shown in **Fig. S10**. Multiple ITC titrations were carried out and data were analyzed simultaneously by HypCal, which allows for the determination of the species formed in solution, their stability constants, and the values of ΔH^0 and ΔS^0 of formation. The curve fitting plots are shown in **Fig. S11-S12**. The binding thermodynamic parameters at 25 °C are reported in Table 1.

The binding process between small molecules and suitable receptors in solution may involve different interactions strictly related to their structural properties. Van der Waals forces, electrostatic and hydrophobic interactions are the main driving forces that occur in the recognition equilibria. In a complexation reaction, enthalpy changes reveal the formation/disruption of non-covalent interactions between the interacting molecules and/or the solvent while conformational or structural rearrangements as well as desolvation contribute to the entropy changes (Escobar & Ballester, 2012; Giglio et al., 2015). Both the investigated β-CDs form only a 1:1 complex with triiodide in aqueous solution; other possible stoichiometries or their combinations were tested in the model but were always rejected by the software. The affinity of β-CD-Chol for the target guest is larger than that of the bare β-CD, thus emphasizing the role of the positively charged choline functionality in the complexation of the triiodide anion.

The binding process is in all cases enthalpy favoured and driven: the interactions promoting the complexation of triiodide into the hydrophobic cavity of the cyclodextrin always result in an exothermic process (Kitamura et al., 1999). These negative ΔH^0 values are mainly attributable to hydrogen bonding, van der Waals forces and (in the case of β-CD-Chol only) electrostatic interactions between I_3^- and the host molecule. Conversely, all processes are entropy unfavoured due to the loss of degrees of freedom of the system as a result of the complex formation. The complex formed by β-CD-Chol, which has a larger stability, displays a smaller entropic loss/cost likely due to some structural rearrangement of the choline moiety and/or a desolvation process which favorably contribute to the Gibbs free energy value.

3.6. Iodine release

It was reported that the diffusion of iodine from an iodophor water solution to an organic solvent simulates the consume of iodine by the microorganisms and can be regarded as the release of iodine from the iodophor (Bhagwat et al., 1988). The diffusion of iodine to cyclohexane moves other molecular iodine from the triiodide reserve until an equilibrium is reached. To evaluate the diffusion of molecular iodine from the water solution of the β-CD-Chol/iodine complex to cyclohexane, the organic solvent was slowly placed on the aqueous solution and the amount of molecular iodine in cyclohexane was monitored at 37 °C over time by UV–vis spectra (λ 510 nm). The release curve in Fig. 7 shows that the amount of released iodine from the complex solution was around 20 % (0.14 mg/mL) in about 500 min. A linear release rate of about 0.23 μg/ mL min^{-1} was calculated from 10 min to 370 min.

3.7. In vitro evaluation of β-CD-Chol cytotoxicity and mucoadhesive properties

3.7.1. Cytotoxicity and mucoadhesion studies

Non-specific cytotoxicity by β-CD-Chol was checked employing human keratinocyte HaCaT cells. Compared to the β-CD parent scaffold, the addition of the choline moiety does not alter safety characteristics of this cyclodextrin derivative. In fact, similarly to native β-CD, β-CD-Chol does not cause over-cytotoxicity in HaCaT cells at concentrations ≤3 mg/mL (Fig. 8A).

Mucoadhesion is an important property for iodophor compounds. It ensures a prolonged residence time of iodine on host mucosae, a critical factor for effective eradication of microbial pathogens. To evaluate the mucoadhesion property of β-CD-Chol in comparison with the native β-CD, their fluorescent complexes (by inclusion of coumarin-6 in the cyclodextrin cavity) were prepared and incubated (90 min) on mucosal surfaces of rat intestines (Kali et al., 2023). After rinsing with PBS, percentages of residual cyclodextrin complexes adherent to rat mucosal surfaces were calculated. Results indicated that β-CD-Chol exhibited a larger mucoadhesivity (71.62 % of compound remaining adherent to mucosal surfaces after washing) than the precursor β-CD (36.58 %) (Fig. 8B). This behavior might be reasonably ascribed to additive charge-to-charge interactions of the cationic cyclodextrin with the negatively charged mucosa components.

These results also suggest the potential of β-CD-Chol as a novel nanocarrier system for targeted drug delivery to cells that expose or over-express choline transporter on their surfaces, including cancer cells (Hara et al., 2006; Nagashima et al., 2018) and Gram negative bacteria *E. coli* (Tøndervik & Strøm, 2007) and *P. aeruginosa* (Malek et al., 2011).

3.8. In vitro evaluation of β-CD-Chol/iodine antibacterial activity

To investigate the antiseptic activity of the β-CD-Chol/iodine complex and perform a comparison with PVP-I, the most used antiseptic, Time-Kill assays against a *Staphylococcus epidermidis* strain sensitive to PVP-I were carried out. The results demonstrated that the β-CD-Chol/Iodine complex has a rapid and complete bactericidal activity (Fig. 9). A 99.999 % inhibition of bacterial cell vitality (corresponding to a reduction >5 Log10) was observed for both PVP-I and β-CD-Chol/iodine as early as 10 s after the contact time (Fig. 9). In contrast, no differences were observed between the empty carrier and the vehicle control indicating absence of meaningful, rapid antibacterial activity by the β-CD-

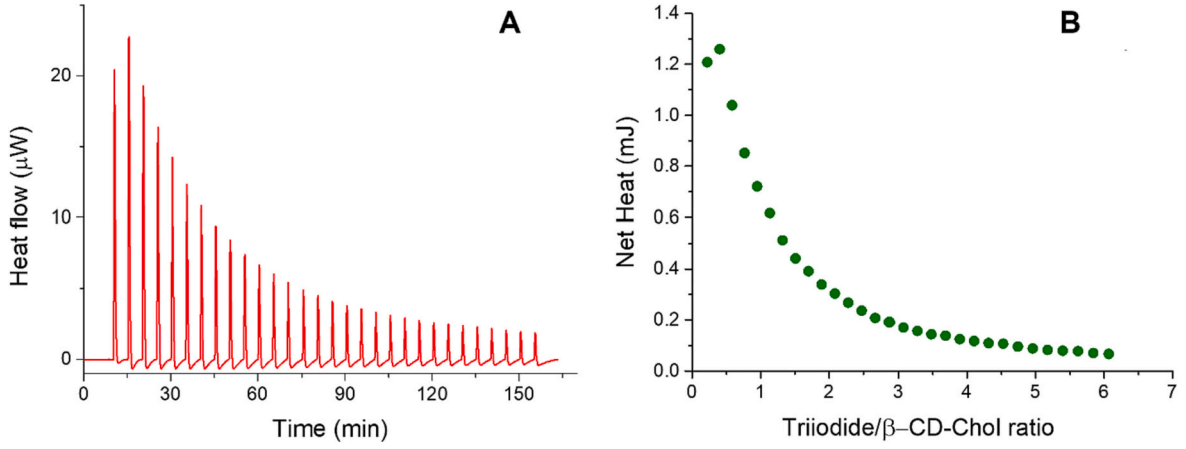


Fig. 5. A) ITC curve of 10 mM triiodide into 0.5 mM β-CD-Chol aqueous solutions (all in 50 mM KI) at 25 °C; B) integrated heat data.

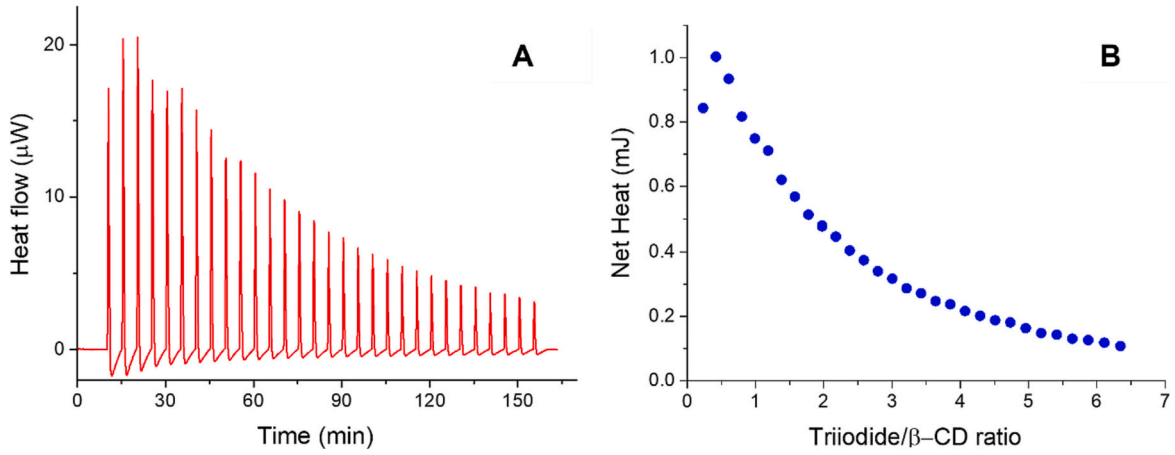


Fig. 6. A) ITC curve of 10 mM triiodide into 0.5 mM β-CD aqueous solutions (all in 50 mM KI) at 25 °C; B) integrated heat data.

Table 1
LogK values and thermodynamic parameters[a] for the binding of triiodide ion with β-CD-Chol or β-CD at 25 °C in 50 mM potassium iodide.

	log K	ΔH^0 (kJ mol^{-1})	ΔS^0 (J K^{-1} mol^{-1})
β-CD-Chol	3.60 (4)	−33.80 (1)	−45.5 (7)
β-CD	3.00 (2)	−46.87 (1)	−99.7 (4)

[a] Errors in the last significant digit are reported in parentheses.

Chol devoid of iodine (Fig. 9). In agreement, β-CD-Chol/iodine exhibited significantly superior antibacterial effects compared to the empty carrier β-CD-Chol, at all time-points investigated (Fig. 9). However, at the latest time (60 min), the empty β-CD-Chol exhibited an intrinsic antibacterial effect (CFU reduction from 10^{-6} to 10^{-3}). These results agree with the possibility that the cationic cyclodextrin may establish charge-to-charge interactions with the negatively charged bacterial membrane, as reported for other macrocyclic ammonium quaternary compounds (Melezhyk et al., 2015) including a choline-calixarene derivative (Consoli et al., 2022; Ferreri et al., 2022). They also suggest that β-CD-Chol could work as a nanocarrier for delivering iodine in proximity of the bacterial cell surface.

4. Conclusions

The functionalization of the β-CD skeleton with a choline-like substituent provided a novel cationic cyclodextrin with enhanced water solubility and increased iodine binding affinity if compared to the native β-CD. The β-CD-Chol/iodine complex showed higher solubility in aqueous solution and, when prepared at the solid state, yielded a very stable powder that is suitable for long time storage. β-CD-Chol offers the invaluable advantage of combining the drug complexing capability of the β-CD macrocycle with the bacterial cell targeting properties of the cationic choline pendant. As a proof of concept, the solid β-CD-Chol/iodine complex dissolved in buffer at neutral pH elicited a tremendous

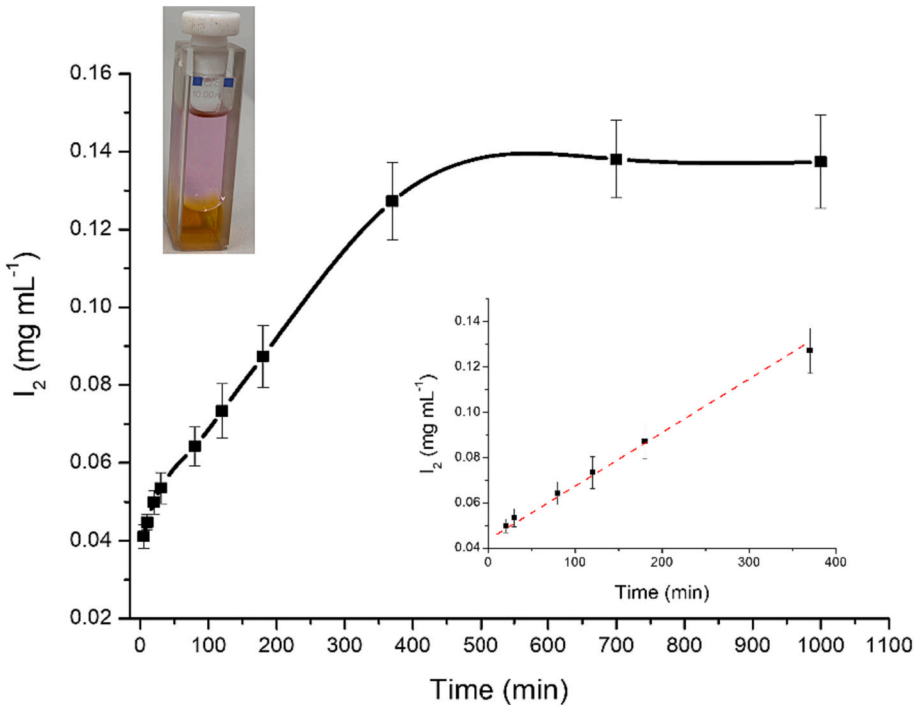


Fig. 7. Release of iodine (mg/mL) from an aqueous solution of β-CD-Chol/iodine to cyclohexane. Inset: the linear release rate in the range 10–370 min (Y = 0.043 + 2.3 × 10^{-4} X; R^2 = 0.9855).

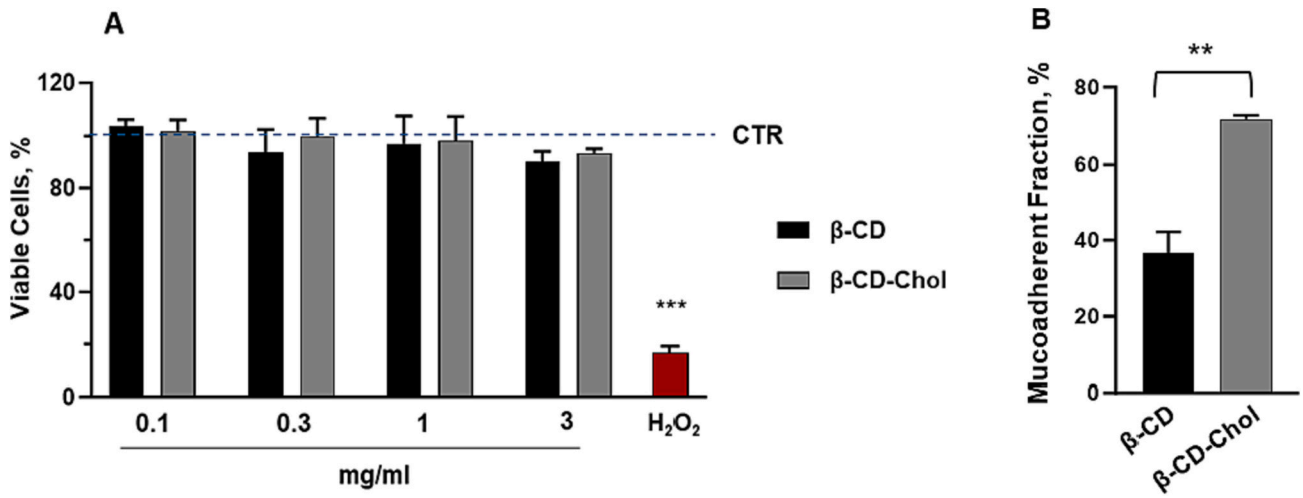


Fig. 8. A) Effects of β-CD-Chol on human keratinocyte viability. Cytotoxicity in confluent human keratinocyte HaCaT cells after 24 h treatment with β-CD-Chol or β-CD. Data shown are mean ± SEM from 3 independent experiments. CTR, vehicle; H_2O_2 (300 μM), positive control. ***, $p < 0.001$ vs CTR by two-way ANOVA. B) Mucoadhesivity of β-CD-Chol and β-CD. Mucoadherent fractions of β-CD-Chol and β-CD on rat intestinal mucosae (see Materials and Methods for details). Data shown are mean ± SEM from 3 independent experiments. **, $p < 0.01$ by unpaired Student's *t*-test.

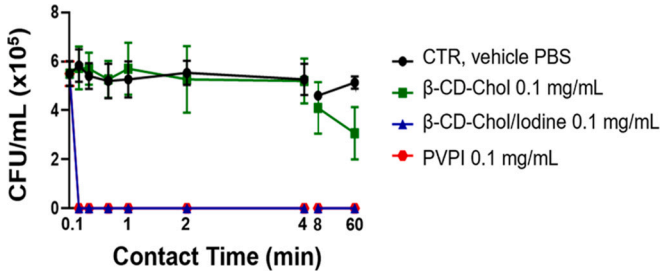


Fig. 9. Antibacterial effects of β-CD-Chol/iodine complex. CFU/mL of *Staphylococcus Epidermidis* from 3 independent Time-Kill experiments. CTR, vehicle control. Similarly to PVP-I, statistical analyses (two-way ANOVA) of results from all time-points (10, 20, 40 s, 1, 2, 4, 8, 60 min) indicate significant differences ($p < 0.0001$) between β-CD-Chol/iodine vs β-CD-Chol or CTR. No significant differences (by two-way ANOVA) were observed for β-CD-Chol vs CTR, with exception of the 60 min time-point ($p < 0.001$).

bactericidal effect on a *S. epidermidis* strain. These findings, along with the demonstrated biocompatibility and mucoadhesivity, which are fundamental for a prolonged residence time of iodine on mucosa, make the suitably designed β-CD-Chol macrocyclic receptor a promising and novel iodine-based antiseptic agent which may be employed for mucosa disinfection.

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CRediT authorship contribution statement

Sonia Pedotti: Conceptualization, Investigation, Validation, Writing – original draft. **Loredana Ferreri:** Investigation, Validation. **Rossella Migliore:** Investigation, Validation. **Claudia Giovanna Leotta:** Conceptualization, Investigation, Validation. **Giovanni Mario Pitari:** Conceptualization, Funding acquisition, Supervision. **Nicola D'Antona:** Conceptualization, Funding acquisition. **Salvatore Petralia:** Conceptualization, Investigation, Validation. **Danilo Aleo:** Conceptualization, Investigation. **Carmelo Sgarlata:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Grazia Maria Letizia Consoli:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The Authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

1D- and 2D-NMR spectra of β-CD-Chol and β-CD-Chol/iodine complex. DLS measurements of β-CD and β-CD-Chol. Species distribution diagram. ITC blank experiment. ITC curve fitting for the β-CD-Chol/iodine and β-CD/iodine complexes. Supplementary data to this article can be found online at: http://dx.doi:10.1016/j.carbpol.2023.121698.

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