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Polycationic calixarene micellar aggregate as a novel iodophor platform for on-demand iodine release to bacteria

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ABSTRACT

Iodine is the most potent antiseptic agent used in clinical applications, but its volatility and toxicity are serious drawbacks that are stimulating the search of new strategies to achieve formulations stable at low iodine concentration. Here, we report that polycationic micellar aggregates, formed by the self-assembling of the amphiphilic choline-calix[4]arene derivative (CholCalix) in aqueous medium, are able to complex triiodide anions (I_3^-) both in solution and in solid phase. This novel iodophor enhances the stability of 0.01 % iodine and 0.1 % povidone iodine (PVP-I) water solutions. The binding properties of the CholCalix aggregate towards iodine were investigated by UV–vis spectrophotometry, dynamic light scattering, and isothermal titration calorimetry. The bactericidal activity of the complexes was proved against a specimen of Gram-negative bacteria (*Escherichia coli* ATCC 10536) by time-killing assay. The molecular iodine release profile suggests that the calixarene-based micellar nanocarrier may succeed as a novel iodophor for on-demand I_2 release.

1. Introduction

Infectious diseases are spreading around the world faster than ever and are a serious threat to public health worldwide [1]. The mortality rate, caused by multidrug-resistant bacteria, has been estimated will increase to 10 million deaths by 2050 and therefore finding new antiseptic formulations is a challenge of socio-economic relevance. Molecular iodine (I_2) is one of the most potent and broad-spectrum antiseptic agents, effective against viruses, bacteria, fungi, and protozoa. Iodine can penetrate the bacterial cell wall, oxidize biological molecules and impair the synthesis of bacterial cell components. Noteworthy, in the fight against pathogens the iodine multimodal mechanism of action offers the advantage of rare onset of resistance phenomena.

Early uses of iodine as antiseptic involved hydroalcoholic preparations affected by low stability, caused by iodine volatility, and unpleasant side effects including pain, irritation, and tissue staining. To overcome these drawbacks, compounds able to bind iodine and control its release (iodophors) have been developed [2,3].

Traditional iodophor systems have primarily relied on polymeric carriers or macrocyclic receptors able to include and stabilize molecular iodine in aqueous solutions [4]. Poly(N-vinylpyrrolidone) (PVP) is the most widely employed iodophor as it can complex molecular iodine within the polymeric matrix by establishing interactions involving triiodide anions (I_3^-) and forming PVP-I complexes [5,6]. PVP-I can act as iodine reservoir, allowing the release of the active antimicrobial species, the free iodine [7]. Despite its clinical utility, especially in ophthalmology, PVP-I faces challenges including low free-iodine content, stability issues upon dilution, rare cases of povidone hypersensitivity and environmental concerns [8]. Other natural polymers, including starch, hemicellulose and cellulose derivatives, may also form iodine complexes often exhibiting chain length dependent antimicrobial activity, whilst acylated gum arabic provides a iodophor with high iodine content and antimicrobial effect [3,9–11].

Cyclodextrin-iodine complexes represent a promising alternative as

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their hydrophobic cavities can encapsulate molecular iodine, enhance its solubility and reduce its volatility [12,13]. For example, β-cyclodextrins have been explored for enhanced mucoadhesion and wound-healing applications [14], while solid γ-cyclodextrin-iodine complexes have shown potential for inhalation therapies [15].

Among synthetic macrocyclic hosts, calix[*n*]arenes resulted attractive molecular scaffolds in biomedical applications [16], including drug discovery [17] and delivery [18,19], due to their ability to complex a variety of guests and easy chemical modification [20]. Calixarene derivatives embedded in polymeric matrices have been proposed as iodine sequestering agents [21]. Recently, our group demonstrated that a polyanionic *p*-sulfonate-calix[4]arene micellar aggregate improved the stability of an antibacterial 0.1 % povidone-iodine water solution by slowing down the iodine release [22].

However, the iodophor formulations still face limitations such as long-term stability and instability under storage conditions (especially in plastic containers) as well as the need for relatively high iodine loading to maintain the antimicrobial efficacy. Longer time storage formulations can be prepared by increasing the iodine concentration, but the associated enhanced toxicity precludes their use in delicate tissues (e.g. ocular tissues) and in routine applications, such as hand disinfection. Consequently, there is a significant interest in finding new strategies for achieving stable formulations at low iodine concentration. The nanotechnological approach can be particularly advantageous as the entrapment into nanocarriers can increase the stability and reduce the toxicity of iodine-based formulations by a controlled and prolonged iodine release. Furthermore, nanoparticles can enhance the iodine efficacy by favoring the penetration through biological barriers [23]. As examples, nanoformulated iodine resulted more effective than free iodine in reducing bacterial load and severity of corneal ulcers in rabbits [24], iodine-loaded nanoparticles increased survival in mice cancer models with low degree of side effects [25], and proper nanoformulation allowed the concentration of commercial PVP-I solutions to be reduced from 10 % to 0.6 % for more tolerable and safer patient's treatments [26].

In the search for novel iodophors, several papers described the complexation of triiodide anion (I_3^-) by quaternary ammonium-based compounds [27], and data back to 1957 the first patent reporting germicidal compositions wherein iodine is complexed by carriers exposing quaternary ammonium groups [28]. These findings prompted us to introduce a polycationic micellar aggregate, generated by the self-assembling of an amphiphilic choline-calix[4]arene derivative (CholCalix, Fig. 1) and exposing multiple quaternary ammonium groups, as a novel iodophor system. The amphiphilic nanocarrier differs from the traditional iodophors as it combines the presence of ammonium groups at the upper rim with the capability of establishing electrostatic interactions with triiodide species; furthermore, it contains an hydrophobic aromatic cavity enabling halogen-π interactions with molecular iodine as well as an intrinsic ability to deliver iodine to bacterial cells by interacting with the negatively charged bacteria surface or by binding choline transporters expressed on the surface of Gram-negative bacteria [29,30]. The previously demonstrated biocompatibility [31,32], intrinsic antibacterial and antibiofilm activity [33], and effective drug delivery capability [34] are significant additional features supporting the application of the CholCalix nanosystem in the development of novel antiseptic formulations.

PVP-I formulations at 10 % and 0.6 % concentration are currently used to disinfect skin and eye, respectively. Reducing the PVP-I concentration to achieve less cytotoxic antiseptic formulations that are stable especially in plastic containers is still a challenge. Therefore, we examined the CholCalix nanoaggregate as a potential novel and effective iodophor and as a stabilizer of a 0.1 % povidone iodine (PVP-I) solution. The binding interactions of CholCalix with iodine and PVP-I in aqueous solution were investigated by UV–vis spectrophotometry, isothermal titration calorimetry (ITC), and dynamic light scattering (DLS). The stability over time of the colloidal solutions as well as their bactericidal effect against a specimen of Gram-negative bacteria (*Escherichia coli* ATCC 10536) were proved.

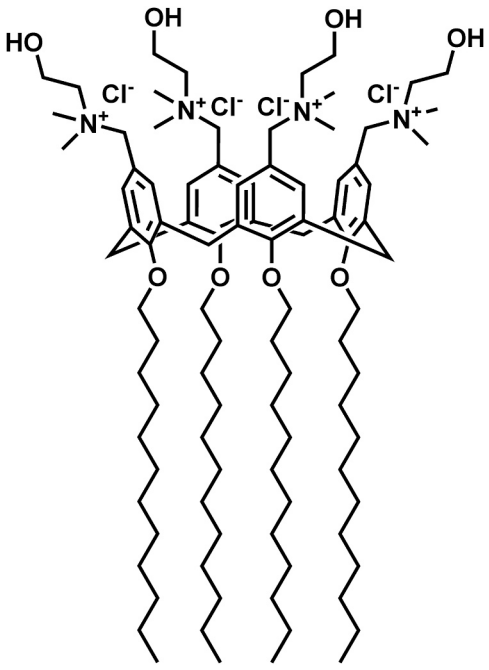

Fig. 1. Molecular structure of the CholCalix receptor.

2. Experimental section

2.1. Materials and methods

All reagents from Merck (Milan, Italy) were used without any further purification. CholCalix was synthesized as previously reported [31,32]. High-purity water (Millipore, Milli-Q Element A 10 ultrapure water) and A-grade glassware were employed throughout.

2.2. Instrumentation

UV–vis spectra were recorded on an Agilent Technologies 8453 UV−vis spectrophotometer. Size measurements were performed on a ZetaSizer NanoZS90 (Malvern Instrument, UK), equipped with a 633 nm laser, at the scattering angle of 90° and at 25 °C. The calorimetric measurements were run on an actively controlled (power compensated) Nano-ITC calorimeter from TA Instruments (USA).

2.3. Preparation of CholCalix/iodine solution

Solid iodine at different amounts (0.4 and 0.1 mg mL^{-1}) was dissolved in pure water or in an aqueous solution of CholCalix (1 mg mL^{-1}), and the samples were shaken at room temperature for 6 hrs.

2.4. Preparation of CholCalix/PVP-I solution

Solid PVP-I (1 mg mL^{-1}) was dissolved in an aqueous solution of CholCalix (0.4 or 1 mg mL^{-1}), and the samples were shaken at room temperature for 6 hrs.

2.5. ITC titrations

ITC experiments were performed at 25 °C with a Nano-ITC calorimeter having an active cell volume of 988 μL and a 250 μL injection syringe. The calorimeter was chemically calibrated (HCl/TRIS reaction) and checked with an electrical calibration. Measurements were run as

reported elsewhere [35].

- *I_3^-/CholCalix system*: the interactions of iodine with the micellar aggregates were studied by titrating a solution of I_3^- (2.40 mM) into CholCalix (0.10–0.12 mM), well above its critical micellar concentration. Experiments were also performed by titrating a solution of I_3^- (2.40 mM) into a solution of CholCalix in its monomeric form (2.5 μM).

- *PVP-I/CholCalix system*: experiments were performed by titrating a solution of the micellar CholCalix aggregate (0.10 mM) with a solution of PVP-I (2.50–5.00 mM).

- *PVP/CholCalix system*: experiments were conducted by titrating a solution of PVP (5.00–10.0 mM) into a solution of micellar CholCalix (0.10 mM).

The concentration of the polymers (PVP-I and PVP) was expressed per monomer unit of vinylpyrrolidone. All solutions were prepared in KI 15 mM. At least three independent experiments were run for each system. Heats of dilution were determined through "blank" experiments by titrating proper titrant solutions (triiodide, PVP-I or PVP) into a 15 mM KI solution. The power curve was integrated using the NanoAnalyze software (TA Instruments, USA) to obtain the gross heat from the reaction. The net heats of the reaction obtained from different titrations were analyzed simultaneously by the HypCal software [36].

2.6. Estimation of I_2 amount diffusing to cyclohexane

Cyclohexane (1 mL) was added to a CholCalix/iodine aqueous solution (1 mL) and the mixture was stirred at room temperature for 20 min and then centrifuged at 3500 × *g* for 3 min. The organic phase was collected, and absorbance was measured to calculate the amount of diffused iodine by using a calibration curve.

2.7. Time-killing assay

Overnight culture of *E. coli* ATCC 10536 grown in Mueller Hinton Broth was washed twice and suspended in PBS at a concentration of 1×10^7 CFU/mL, approximately. Subsequently, standardized inoculum was added to each sample solution to achieve a final concentration of 1×10^6 CFU/mL and incubated for different times (5, 10, 15, 40 min). The control sample was also prepared in PBS. The inoculum was counted by decimal dilutions (time t_0). After incubation, the samples were neutralized with a 0.5 % sodium thiosulfate solution (1:10 dilution, v/v), serially diluted in PBS and plated onto Mueller Hinton agar to determine total cell number. The plates were then incubated at 37 °C for 24–48 up to 120 hrs, CFU were counted and time kill plots were constructed. All determinations were performed in triplicate including the growth control. The percentage (%) of bactericidal activity was calculated by the following equation:

$$\text{Bactericidal activity \%} = [1 - (\text{CFU } t_x / \text{CFU } t_0)] \times 100$$

where t_x is CFU at different time x (x = 5, 10, 15, 40 min) and t_0 is CFU at time zero.

The results are expressed as means ± standard deviation of three experiments. Statistical analyses were performed using ANOVA test. A *p*-value < 0.05 was considered significant.

3. Results and discussion

3.1. Formation of CholCalix/iodine complex in aqueous solution

By acting as a Lewis acid, molecular iodine (I_2) can react readily with electron-rich molecules via a charge transfer mechanism whereas, in the anionic form (I_3^-), it can establish charge-to-charge interactions with ammonium compounds. Therefore, the polycationic macrocyclic receptor CholCalix (Fig. 1) might complex iodine by exploiting both aromatic moieties and multiple quaternary ammonium groups decorating the cavity.

The solubilization of iodine in water depends on its polarization and subsequent capability to establish dipole interactions with water molecules; the process implies a large energetic cost that justifies the low iodine water-solubility, reported to be around 0.3 mg mL^{-1}.

To investigate the effect of CholCalix on iodine solubility in water, solid iodine (0.4 mg mL^{-1}, above its maximum solubility) was added to an aqueous solution of CholCalix (1 mg mL^{-1}, 0.6 mM). The presence of CholCalix sped up iodine dissolution and a complete iodine solubilization was evident to the naked eye when compared to the same amount of iodine dissolved in pure water, (Fig. 2**A**). A faster solubilization was also observed when 0.1 mg mL^{-1} of iodine was dissolved in an aqueous solution of CholCalix (Fig. 2**B**) or in 1 mM phosphate buffer solution (Fig. 2**C**) which provided a clear yellow solution (pH 6.6).

When the phosphate buffer concentration was increased from 1 to 10 mM or CholCalix was dissolved in phosphate buffered saline (phosphate 10 mM, pH 7.4), the addition of iodine resulted in precipitation phenomena ascribable to coordination events involving the tridentate iodide anions (I_3^-). The pH of the water solution of CholCalix (1 mg mL^{-1}) and iodine (0.4 mg mL^{-1}) was measured to be 3.3, whereas the pH of the soluble iodine alone (0.3 mg mL^{-1}) was 4.5. The more acidic pH of the complex agreed with the formation of HI after iodination of the surfactant. A less acidic solution was obtained reducing the iodine concentration to 0.1 mg mL^{-1}. A pH value of 6.0 was found for iodine alone and 4.3 for the colloidal solution of CholCalix and iodine. Remarkably, a pH value of 4.3 is suitable for topical applications.

The formation of the CholCalix/iodine complex was corroborated by UV–vis spectra (Fig. 3).

The absorption spectrum of iodine in water showed distinct peaks for iodide ion I^- (225 nm), triiodide anion I_3^- (287 and 351 nm) and molecular iodine I_2 (460 nm), according to the known reactions of iodine in water. In the presence of CholCalix, the absorption bands of the triiodide anions at 287 and 351 nm red-shifted respectively to 294 and 367 nm (Fig. 3).

The absorption spectrum of the complex showed a clear hyperchromic effect. The increase of the triiodide anion absorption bands and decrease of the molecular iodine one (Fig. 3) evidenced that CholCalix complexes iodine mainly in I_3^- form, analogously to what observed for quaternary ammonium [37], polymer [38], and cyclodextrin [39] iodophors.

3.2. Loading of iodine into CholCalix by solid-vapor method

CholCalix also complexed iodine via solid-vapor method. Under iodine vapors, the color of the CholCalix powder changed from white to brown (Fig. 4). The absorption spectrum of the powder dissolved in water showed a profile very similar to that of the commercial PVP-I (Fig. 4), that is known to complex iodine mainly in I_3^- form.

3.3. Solution thermodynamics of CholCalix complexes with iodine and PVP-I

Calorimetric measurements were conducted to thoroughly characterize the interactions between iodine and the amphiphilic CholCalix derivative in both its aggregated and monomeric forms. These measurements enabled the determination of binding affinities and driving forces for the molecular recognition processes occurring in aqueous solution.

We have previously discussed the critical importance of accurately identifying the iodine species present in solution as crucial step for evaluating the availability of iodine to be complexed by the calixarene receptor [22]. Consequently, experimental conditions (2.40 mM I_2 into a 15 mM KI solution) were carefully selected to ensure that iodine exists primarily in the form of triiodide ions (I_3^-). According to the species distribution diagrams (Fig. S1), I_3^- is the main species in solution (about 90 %) with I_2 being present in negligible amount. This careful selection of the conditions enabled us to simplify the intricate equilibria involving

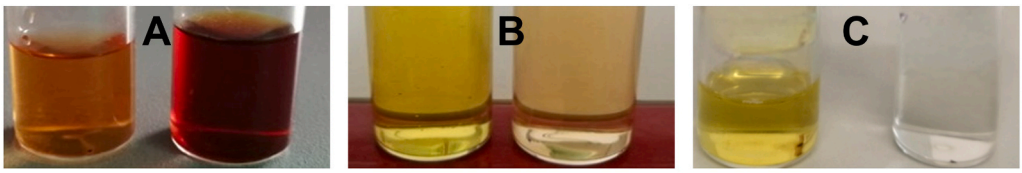

Fig. 2. Pictures of: A) iodine in water (0.4 mg mL^{-1}) without (left) and with CholCalix (1 mg mL^{-1}, 0.6 mM) (right); B) iodine in water (0.1 mg mL^{-1}) without (right) and with CholCalix (0.6 mM) (left); C) iodine (0.1 mg mL^{-1}) in phosphate buffer (1 mM) with (left) and without CholCalix (right) after 30 min at room temperature with no stirring.

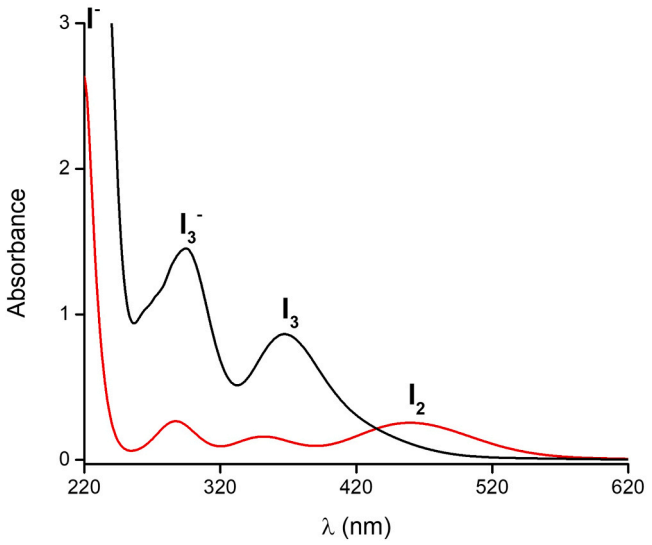

Fig. 3. UV–vis spectra in water of iodine (0.1 mg mL^{-1}, 0.39 mM, red line) and iodine at the same concentration in the presence of CholCalix (0.6 mM), 5-fold dilution (black line).

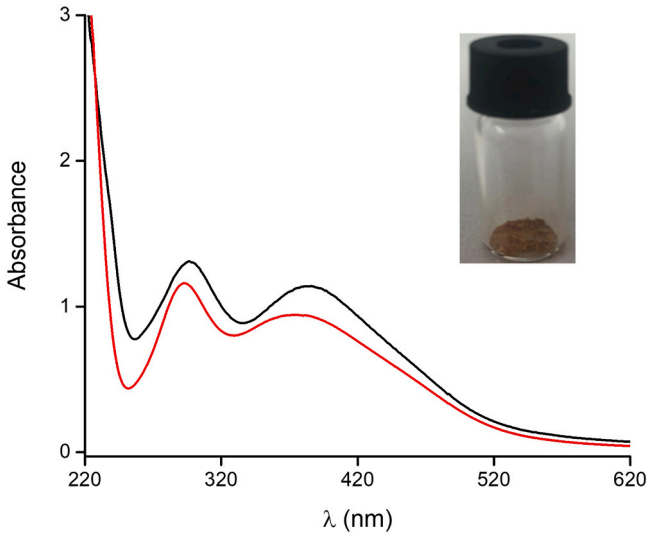

Fig. 4. UV–vis spectra (3-fold dilution) of CholCalix/iodine powder (1 mg, black line) and commercial PVP-I powder (1 mg, red line) dissolved in pure water (1 mL) and picture of CholCalix/iodine powder obtained by solid-vapor method after 24 hrs in an opened container (inset).

iodine in solution. As a result, the calorimetric data and the refined binding parameters pertain solely to the equilibrium (1), which involves interactions between CholCalix and triiodide ion (I_3^-) without interference from other iodine species.

$$I_3^- + \text{CholCalix} \rightleftarrows (I_3^-\text{- CholCalix}) \quad (1)$$

Furthermore, the nearly complete conversion of I_2 into I_3^- when working in excess KI prevented dismutation reactions commonly associated to iodine in aqueous solution, thereby avoiding acidification and subsequent significant pH variations that would otherwise occur.

Calorimetric experiments were conducted by titrating solutions of I_3^- into CholCalix solutions (0.10 mM) at conditions that guarantee the presence of the micellar aggregate in the cell. A typical ITC curve and the corresponding integrated net heat data are shown in Fig. 5**a** and **5b**, both indicating that the complexation of I_3^- by the calixarene-based micelle is an exothermic process.

To evaluate the role played by the micellar nanocarrier in the triiodide binding process, ITC measurements were also performed using a CholCalix solution at a concentration below CMC (2.5 μM), where the amphiphilic calixarene exists in its monomeric form. The calorimetric curves (Fig. S2a) show that the gross reaction heat released when I_3^- is titrated into the monomer CholCalix solution basically overlaps with the heat released from the blank experiment. This observation, together with the negligible net heat values recorded (Fig. S2b), clearly demonstrates that the non-aggregated CholCalix is not able to establish any significant interaction with I_3^- in solution. It may be concluded that the cooperative effect caused by multiple calixarene moieties assembled in a micellar architecture provides the requested boost for the efficient binding of triiodide. The same evidence was observed with non-ionic amphiphilic molecules, which cannot interact with iodine at concentrations below their CMC [40].

Further calorimetric titrations were carried out to examine the observed stabilizing effect of the CholCalix micelles on a PVP-I solution (Fig. 6**a** and **6b**). Also in this case, the interaction between the aggregate and the polymer resulted in an exothermic process though the heat released is smaller than that recorded for the binding of triiodide by the micellar nanocarrier.

ITC titrations of free PVP into CholCalix were also conducted at the same experimental conditions (Fig. S3) to ascertain the mechanism of interaction and the possible role of the polyvinylpyrrolidone scaffold towards the micellar aggregate in solution. The large concentration of iodide in solution does not interfere with the process studied since the amount of I^- that would be complexed by PVP is negligible, due to the very small stability constant for the PVP $+ I^-$ ⇆ PVP-I^- equilibrium [41]. The overlap of blank and titration experiments along with the insignificant net heat values recorded (Fig. S3) indicate that free PVP may not interact with the micellar system. Accordingly, the interaction process observed in the presence of PVP-I is ascribable exclusively to the release of triiodide from the polymeric iodophor and the simultaneous binding of these anions to the micellar system, as per the following competition equilibrium:

$$\text{PVP} - \text{I} + \text{CholCalix} \leftrightarrows (I_3^-\text{- CholCalix}) + \text{PVP} \quad (2)$$

This finding allowed us to elucidate the mechanism responsible for the smaller amount of iodine released from PVP-I solutions in the presence of the calixarene micellar system thus emphasizing the fundamental stabilizing effect of this supramolecular aggregate in solution.

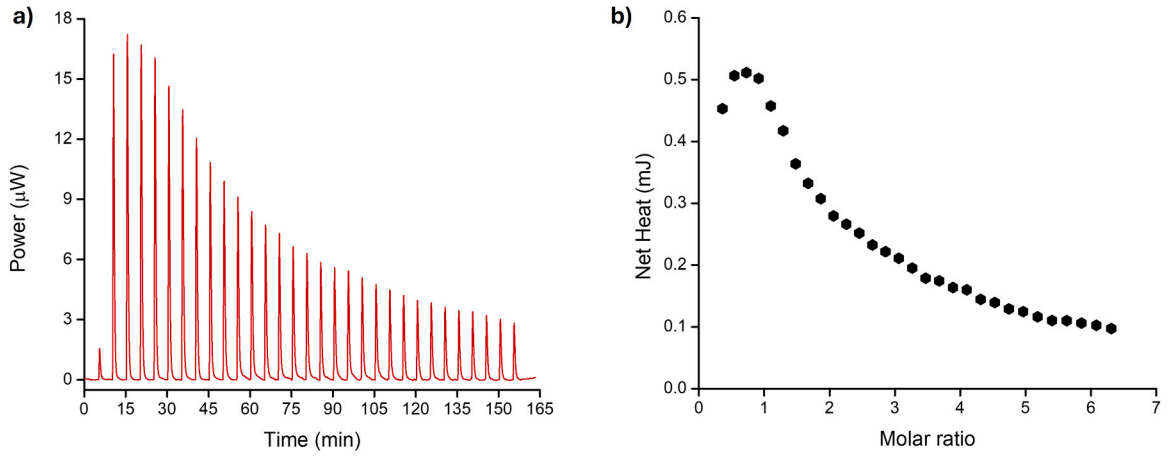

Fig. 5. a) ITC titration of I_3^- (2.40 mM) into CholCalix (0.10 mM) at 25 °C and KI 15 mM; b) integrated heat data.

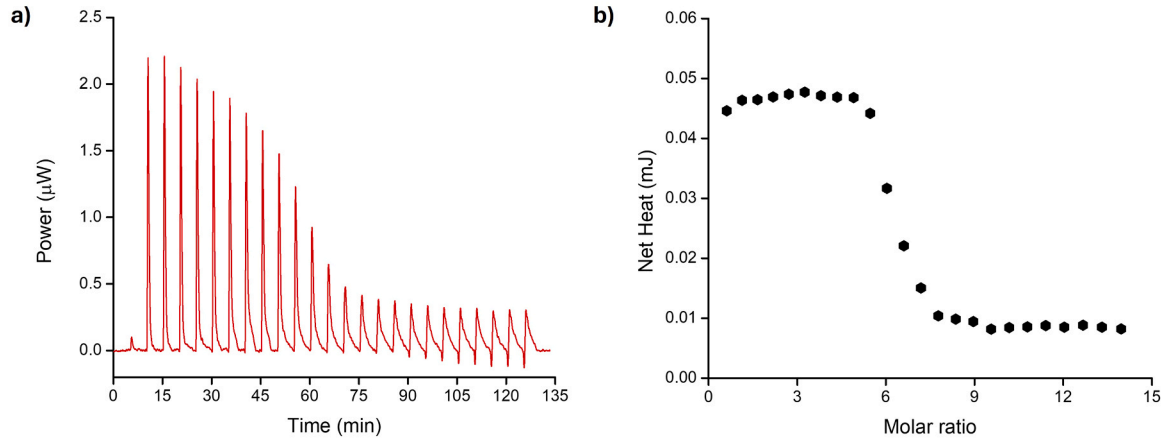

Fig. 6. a) ITC titration of PVP-I (5.00 mM) into CholCalix (0.10 mM) at 25 °C and KI 15 mM; b) integrated heat data.

Calorimetric data were analyzed by assuming the formation of a 1:1 complex between I_3^- or PVP-I and the CholCalix micellar aggregate in line with the "one site binding model" commonly used for refining thermodynamic parameters for similar systems [20,22,42]. Different stoichiometries and chemical models have been tested but they were always rejected by the program. This outcome, along with a close inspection of the "shape" of the thermograms in Fig. 5 and 6, strongly indicates that the binding process occurs through a single-phase interaction and does not involve multiple or sequential binding events/sites, unlike reported for some polymeric iodophors, like poly (2-ethyl-2-oxazoline), PVP or other more sophisticated systems [10].

The log K value for the I_3^-/CholCalix system was found to be 3.53 (6). The binding process resulted enthalpically favored and driven with an unfavorable entropic contribution. An enthalpic drive for the binding process has been also found for the complexation of iodine by large-ring cyclodextrins which can act as suitable iodophors in aqueous solution [43]. The log K values for the PVP-I/CholCalix system is 3.1(1) and, also in this case, the binding process is enthalpically favored and driven with a small favorable entropic contribution (thermodynamic parameters are shown in Fig. 7 and Table S1). The smaller affinity of CholCalix towards triiodide ions released by PVP-I indicated that the binding is favored when the anions are free/uncomplexed in solution.

To unveil the forces and factors that influence the molecular recognition processes in solution and have a comprehensive understanding of the thermodynamics of the complexation process, it is crucial to split the Gibbs free energy into its enthalpic and entropic components. Generally, anion binding based on host-guest interactions is governed by hydrogen

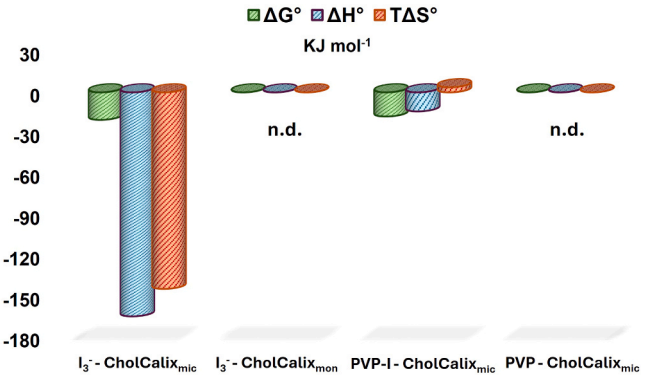

Fig. 7. Thermodynamic parameters for the complex formation of CholCalix (micellar or monomer) with I_3^-, PVP-I and PVP at 25 °C and KI 15 mM.

bonds, hydrophobic effects, electrostatic and anion−π interactions [44–47]. The highly negative ΔH^0 values found for the systems examined are mainly attributable to electrostatic interactions between the triiodide anions and the positively charged ammonium groups of the CholCalix aggregate. Hydrogen bonds and van der Waals forces may also contribute to the enthalpy gain. The same evidence was observed for the complexation of triiodide by a β-cyclodextrin properly functionalized with a positively charged choline moiety [39]. The entropic penalty for the I_3^-/CholCalix binding is attributed to the loss of degrees of freedom

of the interacting molecules upon complex formation, which outweighs the (typically entropy favored) desolvation process [48,49].

A different scenario appears when dealing with the interaction of CholCalix with the PVP-I polymer. The small favorable entropy term may be attributed to a structural rearrangement of the polymer backbone as well as desolvation of both the polymer and the external layer of the micelle surface upon interaction [50]. The negative ΔH^0 value results from the favorable attractive interactions between the positively charged micellar aggregate and the triiodide anions released from the PVP-I, which ultimately ensure the driving force for the binding process. A similar thermodynamic profile, where both ΔH^0 and $T\Delta S^0$ favorably contribute to the Gibbs free energy of reaction, has been reported for the second-phase binding of iodine to PVP [10].

3.4. Determination of the CholCalix/iodine complex size

CholCalix spontaneously self-assembles into nanoaggregates in aqueous medium due to its amphiphilic structure [32]. Dynamic light scattering measurements showed that the CholCalix/iodine complex in pure water forms two populations of nanoaggregates with hydrodynamic diameter centered at 158 nm (89 %) and 28 nm (11 %), when observing the sample in intensity % distribution mode, whereas only one population of 24 nm was observed in volume % distribution mode. The polydispersity index was 0.39.

3.5. CholCalix retains iodine in water solution

Due to the iodine volatility and toxicity, having long time stable formulations at low iodine concentration is challenging and stimulates interest in novel iodophors able to control iodine release. The absorption spectra in Fig. S4 indicate that the colloidal solution of CholCalix (1mg mL^{-1}) and 0.01 % iodine (0.1 mg mL^{-1}) in water, stored in glass at room temperature in the dark, maintains almost unchanged its iodine concentration after 18 months from the preparation. The diffusion of iodine from aqueous solutions to cyclohexane is a simple but useful method to monitor the release of iodine from an iodophor and to mimic the consumption of iodine by bacteria [51]. To evaluate the diffusion of I_2 from the CholCalix/iodine water solution, the aqueous phase (1 mL) was shaken with cyclohexane (1 mL) (Fig. 8) and the amount of diffusing iodine was detected by measuring the absorbance of I_2 in the organic medium at 510 nm.

Unlike the water iodine solution (0.3 mg mL^{-1}) that gave a violet-colored organic phase containing around 0.27 mg mL^{-1} of I_2, the amount of I_2 diffusing from the CholCalix/iodine aqueous solution to cyclohexane was calculated to be around 0.010 mg mL^{-1}. Noteworthy, no significant diffusion of I_2 was observed by further shaking the CholCalix/iodine solution with pure cyclohexane, thus suggesting that the micellar CholCalix strongly retains iodine in the I_3^- form, which is insoluble in the organic solvent.

The retention of I_2 by CholCalix was higher than that observed from a 0.1 % PVP-I aqueous solution containing the same amount of iodine (0.01 % available iodine, that is 1 mg mL^{-1}). The amount of I_2 diffusing to cyclohexane from a water solution of 0.1 % PVP-I was calculated to be around 0.080 mg mL^{-1}, eight-fold larger than the amount released from the CholCalix/iodine complex. The different iodine retention agrees with the diverse structure of the two iodophors.

3.6. CholCalix enhances the stability of a 0.1 % PVP-I aqueous solution

PVP-I is one of the most efficient antiseptics used in clinical applications [52] and is present on the market at concentration ranging from 10 to 0.6 %. Reducing the concentration of PVP-I solutions is desirable to achieve not only safer but also faster working formulations. It is known that, unexpectedly, PVP-I exhibits a more rapid biocide effect at lower concentrations (paradoxical effect). At 10 % PVP-I concentration, iodine is more efficiently entrapped in the aggregated polymer and the amount of available molecular iodine has been determined to be around 1 ppm, whereas at 0.1 % PVP-I concentration the less aggregated polymer yields around 20 ppm of bioactive iodine [53]. Due to iodine volatility, producing stable antiseptic formulations based on PVP-I at low concentration (so to be less toxic) is still challenging.

Different amounts of CholCalix were then added to a 0.1 % PVP-I aqueous solution to investigate the effect of the micellar aggregate. Interestingly, CholCalix slowed down the diffusion of I_2 to cyclohexane and the amount of diffusing I_2 resulted to be dependent on the micelle concentration (Table 1). The amount of diffusing iodine reduced from 0.080 mg mL^{-1} in the absence of CholCalix to 0.024 and 0.001 mg mL^{-1} in the presence of 0.2 and 0.4 mg mL^{-1} of micelle, respectively. No detectable amount of I_2 in cyclohexane was observed when increasing the CholCalix concentration to 1 mg mL^{-1}. In the latter case, it is plausible to assume that non-covalent interactions involving the cationic micelles and PVP-I, as evidenced by ITC measurements, can be responsible for the iodine retention.

The addition of CholCalix (1 mg mL^{-1}) to 0.1 % PVP-I aqueous solution stored in both glass and plastic dropper containers showed no significant reduction of the triiodide anion absorbance at 369 nm after

Table 1
Amount of I_2 diffusing to cyclohexane from a 0.1 % PVP-I water solution containing different amounts of CholCalix.

CholCalix	PVP-I	Iodine in cyclohexane
-	1 mg mL^{-1}	0.080 mg mL^{-1}
0.2 mg mL^{-1}	1 mg mL^{-1}	0.024 mg mL^{-1}
0.4 mg mL^{-1}	1 mg mL^{-1}	0.001 mg mL^{-1}
1 mg mL^{-1}	1 mg mL^{-1}	ND

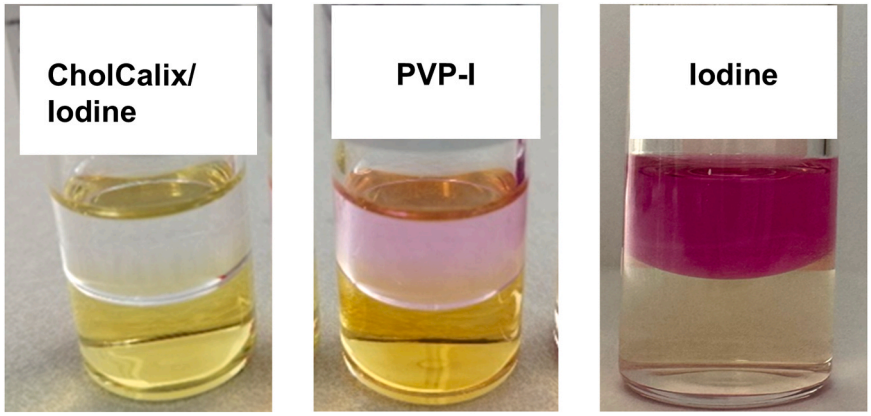

Fig. 8. A) Pictures of aqueous solutions of CholCalix/iodine (1 mg/0.1 mg mL^{-1}), 0.1 % PVP-I and iodine (0.3 mg mL^{-1}) shacked with cyclohexane (1 mL).

at least one year from the preparation. Conversely, the absorbance reduced by 60 % and 40 % in solutions containing 0.2 and 0.4 mg mL^{-1} of CholCalix, respectively, after one-year storage at 4 °C in plastic dropper containers. At the same storage conditions and within a few weeks, the 0.1 % PVP-I solution in plastic containers discolored and the absorbance at 369 nm reduced to less than 1 % (Fig. S5). These results unambiguously revealed that CholCalix can be an effective additive to improve the stability of a 0.1 % PVP-I solution.

3.7. Time-killing assay

To evaluate the bactericidal effect of the prepared formulations, time-killing assays were performed on *E. coli* ATCC 10536, selected as a specimen of Gram-negative bacteria. The samples containing CholCalix (1 mg mL^{-1}) and 0.01 % iodine or 0.1 % PVP-I showed marked cell killing activity (reduction >4 log in CFU/mL, 99.99 % reduction) (Fig. 9). A much weaker effect (50–70 % CFU/mL reduction) of the polycationic CholCalix micelle, whose antibacterial activity was previously demonstrated by our group [33], confirmed that the bactericidal effect of the CholCalix/iodine and CholCalix/PVP-I colloidal solutions is related to the presence of iodine. Noteworthy, the CholCalix/iodine solution (1 mg and 0.1 mg mL^{-1}, respectively) treated with cyclohexane to remove the readily available free I_2 also showed bacterial killing effect (reduction in the range between 3.63 and >4 in CFU/mL, 99.97–99.99 % CFU/mL reduction).

The triiodide anion is easily polarized and, in some conditions, one I–I bond can become shorter than the other. The production of I_2 by changing the structure of I_3^- from the symmetric $[I\text{-}I\text{-}I]^-$ to the asymmetric $[I\text{-}I\cdots I]^-$ has been described for quaternary ammonium ligands/I_3^- complexes when they come into contact with the surface of bacteria [54]. If this were the case, given the negligible bactericidal properties of I_3^- compared to I_2 [55] and the already reported capability of the micellar nanocarrier to interact with the surface of bacterial cells [33], we suggest that the CholCalix/I_3^- complex could act as a potential novel nanosystem for bacteria-triggered on-demand release of I_2. The microbiological assays performed in PBS medium further support the conversion of I_3^- to I_2 in the presence of bacteria. Indeed, the precipitation phenomena that occurred in PBS for the CholCalix assembly and other polyammonia ligands (that are ascribable to coordination events of the tridentate I_3^- anions in the presence of Na^+ ions) were not observed in bacterial cultures.

4. Conclusions

We demonstrated that a polycationic nanoarchitecture formed by the self-assembling of an amphiphilic calix[4]arene derivative exposing quaternary ammonium groups (CholCalix) is a promising novel iodophor. It improves the stability of 0.01 % iodine and 0.1 % PVP-I aqueous solutions by exploiting host-guest interactions. The molecular recognition process is driven by enthalpy favorable attractive forces and occurs efficiently only when the CholCalix receptor is in its aggregated form. Time-killing assays evidenced that the proposed formulations elicit fast and effective antibacterial activity. Preliminary results suggested that the production of bioactive molecular iodine (I_2) from the complexed triiodide ions may be triggered by the contact with the bacterial cell surface enabling "on-demand" release of the species of interest. Overall, these findings indicate that the polycationic CholCalix nanoaggregate could work as promising novel iodophor for the development of more stable and safer iodine-based antiseptic formulations for applications in hand and wound disinfection other than environmental sanitation. It is worth pointing out that the proposed formulation contains only 0.01 % iodine. This value is markedly lower than those typically employed in commercially available disinfectants, such as povidone-iodine solutions, which are routinely used at concentrations of "available iodine" ranging from 0.75–1 % (for general skin and hand hygiene) to 0.025 % (for more sensitive applications like ophthalmic or mucosa). On this basis, it is plausible to consider our CholCalix/iodine formulation as safe and biocompatible under similar usage conditions.

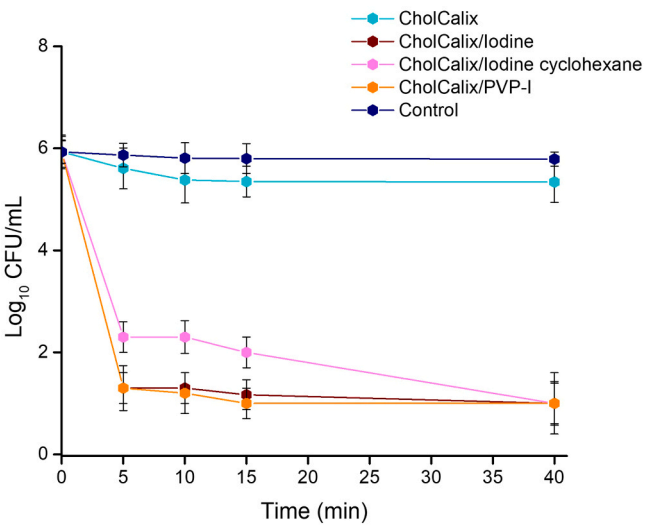

Fig. 9. Time-killing experiments for *E. coli*: bactericidal activity of CholCalix (1 mg mL^{-1}), CholCalix/PVP-I (1 mg/1 mg mL^{-1}), CholCalix/iodine (1 mg/0.1 mg mL^{-1}), treated and not treated with cyclohexane, and control. The experiments were performed in triplicate. All data are presented as the mean ± standard deviation of three experiments. Significant difference ($p < 0.05$) was detected for CholCalix/Iodine, CholCalix/PVP-I, and CholCalix/Iodine cyclohexane (brown, orange, and pink lines, respectively) compared to CholCalix (sky blue line).

CRediT authorship contribution statement

Giovanna Ginestra: Methodology, Investigation. **Giuseppina D. G. Santonoceta:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Nicola D'Antona:** Methodology, Funding acquisition. **Giuseppe Granata:** Methodology, Investigation. **Loredana Ferreri:** Visualization, Methodology, Investigation, Data curation. **Carmelo Sgarlata:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Antonia Nostro:** Writing – original draft, Data curation, Conceptualization. **Salvatore Petralia:** Methodology, Investigation. **Grazia M. L. Consoli:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2025.115071

Data availability

Data will be made available on request.

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