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## From beta-cyclodextrin polyelectrolyte to layer-by-layer self-assembly microcapsules: from inhibition of bacterial growth to bactericidal effect

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**Abstract.** Pathogenic bacteria resistance to antimicrobial agents has emerged as a major health issue. Developing new systems enabling inhibitory and bactericidal effects is crucial. Herein, layer-by-layer technique (LbL) was used to fabricate microcapsules composed of biopolymers, cationic polycyclodextrin (P(CD<sup>+</sup>)) and anionic alginate (alg<sup>-</sup>). This was achieved through successive adsorption of these polyelectrolytes onto calcium carbonate microparticles (CaCO<sub>3</sub>) used as template. Then, a chelating agent was added for removing the CaCO<sub>3</sub> core, leading to microcapsules. Quartz crystal microbalance and zeta potential measurements evidenced the successful alternate adsorption of polyelectrolytes and related charge reversal, respectively, upon the LbL deposition. The shape of the capsules was characterized by Scanning Electron Microscopy (SEM) and fluorescence microscopy. The antibacterial activity of P(CD<sup>+</sup>) was first evidenced on two well-known bacteria: *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) using well diffusion method on Plate Count Agar (PCA) medium. Then, an growth inhibitory effect on the bacteria growth has been observed for the P(CD<sup>+</sup>)-based microcapsules during bacterial culture in Luria-Bertani (LB) broth medium. Our results pointed out towards a strong dependence of the extent of inhibition on the number of self-assembled polyelectrolytes' layers and capsules' concentration. The effect increased with both concentration and number

of layers. Acridine orange staining evidenced the bactericidal effect of P(CD<sup>+</sup>)-based microcapsules. This contribution demonstrates unambiguously that: *i*) aqueous solutions of P(CD<sup>+</sup>) exhibit antibacterial activity at physiological pH suggesting that P(CD<sup>+</sup>) can act as a versatile alternative to the widely studied chitosan whose antimicrobial activity is limited to acidic pH and *ii*) (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>n</sub> hollow capsules can be exploited as an antibacterial agents with additional host properties.

**Keywords:** particle-templated layer-by-layer assembly, cationic polycyclodextrin, capsule, pathogenic bacteria, antimicrobial activity, polysaccharide

## **1. Introduction**

Human beings are often impacted by microorganisms such as bacteria, fungi and viruses.(Rosenberg, Koren, Reshef, Efrony, & Zilber-Rosenberg, 2007) Furthermore, contamination by microorganisms leads to severe problems in food and pharmaceutical industries where microbes may contaminate raw materials, products and equipments. (Marambio-Jones & Hoek, 2010)

Antimicrobial agents can be regarded as materials that kill or simply inhibit the growth of microorganisms with minimal or no harm to the host. Various mechanisms of microorganism' inhibition have been discussed to date, of which are intimately related to the chemical composition and structure (organic, inorganic, charged...) of the antimicrobials, each exhibiting advantages and limitations.(Batoni, Maisetta, Lisa Brancatisano, Esin, & Campa, 2011)

The antimicrobial activity of cationic polymers is generally assigned to the interaction of the positive charge moiety of the polymer with the charged surface of bacteria cell walls. Natural and synthetic nanomaterials have also been widely employed due to their strong antimicrobial properties inherent to their unique properties in terms of adsorption and surface area (Chirkov, 2002; Wei, et al., 1994). On this basis, the self-assembly of polymers including cationic polyelectrolytes (Kiss, et al., 2012) or peptides (H. Wang, et al., 2010) into nano-antimicrobial agents has been proved efficient for infections treatment (Liu, et al., 2009; H. Wang, et al., 2010).

Polyelectrolyte capsules are fabricated by successive adsorption of negatively and positively charged polymers onto particulate substrates which serve as sacrificial templates for polyelectrolytes shell that can be easily removed by dissolution providing hollow particles. This approach offers the possibility to easily introduce multiple functionalities, leading to structured materials with unprecedented properties. For instance, these hollow particles have a

great potential for biological applications. (Peyratout & Dähne, 2004; Shchukina & Shchukin, 2011) The loading and release properties can be easily adjusted by controlling the capsules composition, wall thickness or even and in the case of smart capsules, upon application of an external stimulus including temperature, pH, redox, light... (Sabrina Belbekhouche, Charaabi, Picton, Le Cerf, & Carbonnier, 2018; S Belbekhouche, Mansour, & Carbonnier, 2018; Donath, et al., 2002).

Because of their low toxicity toward mammalian cells, intrinsic environmentally-friendly nature and effective antimicrobial properties, biopolymers could be commonly used in consumer products.(Cha & Chinnan, 2004) Among biopolymers, chitosan has been widely used as a bioactive polysaccharide taking advantage of its inhibitory and bactericidal effect while exhibiting minimal toxicity.(Sudarshan, Hoover, & Knorr, 1992) However, the antibacterial activity of chitosan is promoted only under its cationic form limiting its application to acidic medium (Divya, Vijayan, George, & Jisha, 2017). The mode of action of chitosan-based antimicrobial agents has been explained through different mechanisms. The first one is based on the electrostatic attraction between polycationic chitosan and the negatively charged membrane of the bacterial cell, ultimately leading to intracellular components disruption. (Qi, Xu, Jiang, Hu, & Zou, 2004) The second one implies inhibition of the enzymatic activity and bacterial growth through chelation of trace metals by chitosan (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Antimicrobial efficiency of chitosan and chitosan-based nanostructures is highly influenced by pH. Indeed, because chitosan is soluble in acid pH, its use is restricted to a limited range of pH and application.

In this context, we investigate herein the possibility to use a water soluble cationic polycyclodextrin (PCD<sup>+</sup>) bearing permanent quaternary ammonium charges, as a new antimicrobial agent. PCD<sup>+</sup> will then be applied to the elaboration of microcapsules as a new approach to prevent the growth of two models of pathogenic bacteria, *Escherichia coli* and

*Staphylococcus aureus*. (Goy, Britto, & Assis, 2009) In the literature, two strategies are described to obtain ~~CD~~ cyclodextrin-containing LbL assemblies. The first one involves the use of charged ~~CDs~~ cyclodextrins (CD), and the LbL assemblies are prepared through electrostatic interactions (Sato, Suzuki, & Anzai, 2003, 2004; Yang, Johnson, Shi, Holesinger, & Swanson, 1997). The second strategy for constructing CD-containing LbL assemblies relies on host–guest complexation between guest molecules and CD acting as the driving force for the assembly build-up. (Dubacheva, et al., 2010; Kaftan, et al., 2011; Suzuki, Egawa, Mizukawa, Hoshi, & Anzai, 2002; Van der Heyden, Wilczewski, Labbe, & Auzely, 2006) It deserves noting that ionic polymers of cyclodextrin have already successfully been used for elaborating multilayer films on flat supports.(Jatupol, et al., 2016; Junthip, Tabary, Leclercq, & Martel, 2015)·(Jatupol, et al., 2016)·(Martin, Tabary, Chai, et al., 2013; Martin, Tabary, Leclercq, et al., 2013)·(Martin, Tabary, Leclercq, et al., 2013)·(Pérez-Anes, et al., 2015)

In the literature, relevant works discussed ~~through~~ elegant systems based on cyclodextrin loaded with antimicrobial agents. ~~Host-guest inclusion complex was involved in these works.~~ (Duan, Zhao, Össurardóttir, Thorsteinsson, & Loftsson, 2005; J.-h. Wang & Cai, 2008)

~~Herein~~, the dual objective ~~of this work~~ is (1) to demonstrate the possibility of using a cationic polycyclodextrin as an antimicrobial agent instead of the well-known chitosan, and (2) to explore the preparation of capsules based on cationic polycyclodextrin and ~~alginate as well as~~ their antibacterial activity. Both aspects have not yet been investigated in the literature. Indeed, the synthesis of cationic polyclodextrin has been previously reported but its use has been limited to separation sciences (Pédehontaa-Hiaa, Guerrouache, Carbonnier, Derf, & Morin, 2015) and no study considered its potential use as an antimicrobial agent by itself. ~~Herein~~ In the present study, the selected microtemplate is calcium carbonate. This choice is motivated because CaCO<sub>3</sub> microparticles can be easily synthesized in a reproducible and size-

controlled way. ~~This material~~CaCO<sub>3</sub> ~~is they are~~ non-toxic and can be easily dissolved under slightly acidic conditions or by complexation with ethylenediaminetetraacetic acid (EDTA) (~~Sabrina~~Belbekhouche, et al., 2018).

The effect of number of polyelectrolyte layers and the concentration of capsules on the pathogenic bacterial growth and viability is investigated. ~~Finally, we~~ ~~We~~ evidence that CD cavities within the LbL assemblies retain their inclusion properties towards tocopherol acetate, an antioxidant selected as a model hydrophobic compound. ~~“Tocopherol acetate” is then used as model of antioxidant, as this class of chemicals may be used to retard oxidation of compounds in foods. This then provides providing~~ evidence for potential dual functionality of the multilayered capsules: ~~antioxidation and antimicrobial~~.

## 2. Experimental section

### 2.1. Materials

These chemicals were purchased ~~and used as received~~: chitosan (chi<sup>+</sup>, Sigma-aldrich), alginate (alg<sup>-</sup>, Sigma-aldrich), acetic acid (Alfa aesar), tocopherol acetate (Sigma Aldrich), calcium chloride (CaCl<sub>2</sub>, rectapur, 99%), sodium nitrate (rectapur, 99%), sodium 2-mercaptoethanesulfonate (Sigma-Aldrich) and sodium acetate (Normapur).

### 2.2. Preparation of cationic cyclodextrin (P(CD<sup>+</sup>))

The cationic cyclodextrin (P(CD<sup>+</sup>)) was prepared by using the procedure developed by Renard *et al.* (Renard, Deratani, Volet, & Seville, 1997) and Thuaud *et al.* (Thuaud, Seville, Deratani, & Lelievre, 1991) The cationic cyclodextrin polymer was synthesized through the crosslinking of β-cyclodextrin (β-CD) with epichlorohydrin (Renard, et al., 1997) followed by reacting the obtained copolymer with 2,3 epoxypropyltrimethylammonium chloride. (Thuaud, et al., 1991) Briefly, β-CD in NaOH solution (33 %) was allowed to react with

epichlorohydrin for 210 min at 50°C. After neutralizing the pH of the reactive mixture, the polymer was concentrated by ultrafiltration to remove salt and further low molecular compounds and further recovered through freeze-drying. The resulting  $\beta$ -CD polymer was chemically modified with quaternary ammonium side groups through reaction with 2,3-epoxypropyltrimethylammonium chloride in aqueous basic solution. The resulting modified polymer was purified by a dialysis step using a cellulose dialysis membrane (1 kDa cut off). The product was isolated via freeze-drying to yield a white solid. The characteristics of the involved polyelectrolytes are given in Table 1. ~~Average molar masses and molar masses distributions were determined by size exclusion chromatography (SEC) coupled online with multi angle light scattering (MALS) and differential refractive index (DRI) detectors. The MALS apparatus is the DAWN Heleos II from Wyatt Technology (Ca, USA) filled with a K5 cell and a Ga As laser ( $\lambda = 690$  nm). The DRI detector is a Shimadzu RID 10A (Japan). Columns [Shodex SB OHpak 804 and 806 HQ for alginate, Eprogen CATSEC 100 and 1000 for chitosan, PL aquagel OH 40+30 for the cationic polycyclodextrin] were eluted with  $0.1 \text{ mol} \cdot \text{L}^{-1}$   $\text{LiNO}_3$  or acetate buffer 0.1M, respectively at  $0.5 \text{ mL} \cdot \text{min}^{-1}$ . The flow rate was fixed at  $1 \text{ mL} \cdot \text{min}^{-1}$ . The samples were filtered on  $0.45 \mu\text{m}$  unit filter before injection through a  $100 \mu\text{L}$  full loop.~~

~~**Table 1:** Average molar masses of the polyelectrolytes used (determined by size exclusion chromatography SEC/MALS/DRI analysis).~~

Samples	Abbreviation	$M_n$ ( $\text{g} \cdot \text{mol}^{-1}$ )	$M_w$ ( $\text{g} \cdot \text{mol}^{-1}$ )	$D^*$	$DS^{**}$
Chitosan	$\text{chi}^+$	$8.5 \times 10^4$	$2.0 \times 10^5$	2.3	$0.14 \pm 0.02$ a
Cationic polycyclodextrin	$\text{P}(\text{CD})^+$	$6.5 \times 10^3$	$2.4 \times 10^4$	3.6	$0.40 \pm 0.05$ <sup>b</sup>



Alginate	alg <sup>-</sup>	2.7 x 10 <sup>5</sup>	3.8 x 10 <sup>5</sup>	1.4	-
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\* Polydispersity index (Đ) Đ = Mw/Mn

\*\* Degree of substitution (DS): <sup>a</sup> Degree of acetylation of chitosan, <sup>b</sup> Degree of substitution of P(CD<sup>+</sup>) (estimated from <sup>1</sup>H NMR (Sabrina Belbekhouche, et al., 2018; Renard, et al., 1997))

### **2.2. 2.3. Elaboration of cationic-polycyclodextrin-based multilayered films**

The multilayer buildup was monitored by the QCM (Quartz crystal microbalance) technique (~~Q-Sense E1, Q-Sense, Sweden~~). Gold coated QCM Sensor Crystal was first flushed with two solvents, water and ethanol respectively. It was then placed into a UV/ozone chamber for a 20 min treatment and rinsed again with ethanol. This first step allows cleaning the gold coated QCM Sensor. Then, the surface was pretreated with a sulfonated thiol solution (5 g.L<sup>-1</sup> of a sodium 2-mercaptoethanesulfonate solution prepared in water, during 24 h). This sulfonated thiol derivative is used as a negatively charged anchoring layer enabling the further adsorption of the polycation. After introducing in the QCM flow chamber, the surface was flushed with the buffer for 10 min. The self-assembly of P(CD)<sup>+</sup> / alg<sup>-</sup> was performed onto flat gold substrate via alternative contact with the polyelectrolyte solutions for 15 min. A 10 min rinsing step was performed with water between each polyelectrolyte adsorption. Technically, polymer solutions (1 mg.mL<sup>-1</sup>) were injected into the cell with a flow rate of 250 μL.min<sup>-1</sup>. All measurements were performed at 25 °C. This procedure describes the assembly of (P(CD)<sup>+</sup> / alg<sup>-</sup>)<sub>n</sub> (*n* representing the number of bilayers).

### **2.3. 2.4. Elaboration of biopolymer microcapsules based on cationic-polycyclodextrin**

The elaboration of the cationic-polycyclodextrin/alginate (P(CD)<sup>+</sup>/alg<sup>-</sup>) bilayered capsules consists in the successive deposition of this polyelectrolytes pair onto calcium carbonate microparticles followed by the template removal. More precisely, CaCO<sub>3</sub> microparticles were ~~elaborated~~-fabricated according to Volodkin *et al.* (Volodkin, Larionova, & Sukhorukov, 2004; Volodkin, Petrov, Prevot, & Sukhorukov, 2004) Equal volume of 0.33 M calcium

chloride ( $\text{CaCl}_2$ ) and sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solutions were mixed under a vigorous stirring for 3 min. This method led to a rapid precipitation of the  $\text{CaCO}_3$  microparticles. A centrifugation process of the suspension during 10 min allows removal of the residual ions. Lastly, the resulting particles were rinsed several times with water. Acetone was used to remove the water. Particles were dried under a stream of filtered air. The LbL technique was used to prepare the multilayer films from  $1 \text{ mg.mL}^{-1}$  polycation and  $1 \text{ mg.mL}^{-1}$  alginate solutions. 2 mg of  $\text{CaCO}_3$  particles were incubated in 1 mL of water. The first layer of  $\text{P}(\text{CD}^+)$  was deposited onto the surface of the calcium carbonate particles by adding 500  $\mu\text{L}$  of this polycation stock solution to the particles suspension. A stirring time of 15 min is sufficient to allow the polymer adsorption to the particles surface as deduced from the QCM investigations. The final volume was always kept at 1 mL. The particles were then centrifuged (5 min, 10 000 rpm) and washed three times. The particles were suspended in water and 500  $\mu\text{L}$  of alginate was added to the particles suspension and the mixture was stirred for 15 min for alginate deposition on the previous  $\text{P}(\text{CD}^+)$  layer. **Note that the centrifugation method is used to separate the free polyelectrolyte from polyelectrolyte-coated microparticles, then after each polyelectrolyte adsorption step.** The particles were then rinsed three times. This overall process describes the assembly of a single bilayer: (cationic-polycyclodextrin /alginate)<sub>1</sub> and was repeated to obtain different numbers of assembled bilayers. Finally, the  $\text{CaCO}_3$  microparticles were removed with 0.2 M EDTA solution. To do this, the coated  $\text{CaCO}_3$  microparticles were shaken for 1h with the EDTA solution. A dialysis method was applied to purify the capsules for three days using a cellulose dialysis bag (3.5 kDa cut off) against distilled water. Water was refreshed every hour. The procedure is slightly adapted when using chitosan instead of cationic-polycyclodextrin. Indeed, chitosan is solubilized in 10 mM acetate buffer pH 4. ~~Zeta potential measurement was performed on a Zetasizer Nano-ZS (Malvern Instrument) to monitor the adsorption of the polyelectrolytes onto the  $\text{CaCO}_3$  core~~

~~(Zetasizer 4700 Malvern Instruments, Brookhaven Instruments Corporation, USA). Scanning Electron Microscopy (SEM) enables to study the shape of the resulting particles (Leo Elektronenmikroskopie, Oberkoche in Germany). Prior to analysis, the calcium carbonate particles and capsules were covered with palladium. Images of microcapsules were obtained with an optical fluorescence ZEISS microscope equipped with a CCD camera and a fluorescence illuminator and various mirror units. Images were recorded with ZEN software. A 5  $\mu$ L drop of capsule suspension was deposited on a cover slip to observe the microcapsules.~~

## 2.5. Microbial studies

*Staphylococcus aureus* ATCC6538 (Gram-positive) and *Escherichia coli* ATCC25922 (Gram-negative) were used. Prior to experiment, bacteria were grown aerobically at 37°C overnight in agitated (150 rpm) liquid Luria-Bertani broth medium (LB, Sigma Aldrich).

### 2.5.1 Antibacterial activity of polymers and well diffusion method

Antibacterial activities of the studied polymers were evaluated using well diffusion method in Petri dish on solid Plate Count Agar (PCA)). The diameter of inhibition zones (including the diameter of the well) were measured in centimeter (cm). Samples from overnight cultures of each bacteria was diluted in physiological solution (9 g.L<sup>-1</sup> NaCl) to obtain a bacterial suspension of the two different strains (OD<sub>620nm</sub> = 0.5). Note that for enterobacteria as *E. coli* (*Escherichia coli*) and *S. aureus* (*Staphylococcus aureus*), 1 OD at 620 nm is equivalent to 8 x 10<sup>8</sup> CFU/mL.(Willey, et al., 2008) 100  $\mu$ L of this suspension were inoculated onto the surface of a PCA Petri dish. Four wells (diameter = 6 mm) were done **after** in the agar surface and were filled with 20  $\mu$ L of antimicrobial samples (1 mg.mL<sup>-1</sup>). The **studied** antimicrobial samples **were diluted from stock solution of each polymer. The stock solutions** are: P(CD) (10

mg.mL<sup>-1</sup>), P(CD<sup>+</sup>) (10 mg.mL<sup>-1</sup>), chi<sup>+</sup>(7 mg.mL<sup>-1</sup>) and alg<sup>-</sup> (10 mg.mL<sup>-1</sup>). A total of four tests (three replicates of each sample and one filled with physiological solution as reference (9 g.L<sup>-1</sup> NaCl in distilled water)) ~~were was~~ done into each plate. ~~This was used as referenced, i.e. physiological medium without polymer to clearly evidenced the influence of each of them. Physiological medium (without polymer) was used as reference to clearly evidence the influence of P(CD), P(CD+), chi<sup>+</sup> and alg<sup>-</sup>.~~ After 24 h of incubation at 37 °C in aerobic conditions, the diameters of inhibition around the well were measured.

### **2.5.2. Antibacterial effect of (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>n</sub> bilayer-based microcapsules: Inhibition of bacterial growth method**

Inhibitory impact of microcapsules on growth of *E. coli* and *S. aureus* was studied during kinetic growth under shaking in liquid Luria-Bertani broth medium (LB) at 37°C. For the assay, eight parallel wells of a 96-well microplate were used (eight replicates). Microcapsules suspension of each compound described above (section 2.3 4) at various concentrations of 0.02 g.L<sup>-1</sup>, 0.06 g.L<sup>-1</sup> and 0.1 g.L<sup>-1</sup> was added to a same bacterial suspension and then incubated at 37°C. The final volume is 200 µL for each well. The initial optical density of bacterial suspension was 0.05 at 620 nm. Sterile physiological distilled water was mixed with a bacterial suspension as a control bacterial growth test. Bacterial growth was followed periodically by reading the optical density of bacterial suspensions at 620 nm ~~using UV-Visible spectrophotometer micro plate reader~~. To confirm the results obtained through optical density measurement, bacteria were counted with a Mallassez cell.

### **2.5.3. Bacterial viability assay: microscopic observation**

The effect of microcapsules on bacterial viability was ~~performed~~ ~~assessed~~ using an orange acridine test followed by fluorescence microscopy observation. 10 µL of acridine orange (0.1

% wt) was added to 100  $\mu\text{L}$  of bacterial suspension taken at the end of the antibacterial assay (described in section 2.5.2). A 10  $\mu\text{L}$  drop was then introduced on a cover slip to observe the bacteria.(Boulos, Prevost, Barbeau, Coallier, & Desjardins, 1999) ~~An optical fluorescence ZEISS microscope equipped with a CCD camera and a fluorescence illuminator and various mirror units were used after staining of bacteria using alcidine orange.~~

## 2.6. Loading of hydrophobic agent

A solution of tocopherol acetate (5 mg/ml) was added to a suspension of  $(\text{P}(\text{CD}^+)/\text{alg}^-)_n$  microcapsules ( $n = 1$  to 4). Then, the suspensions were stirred for 24 h to ensure effective tocopherol acetate complexation. The samples were introduced in a dialysis bag (3.5 kDa cut off) to remove unloaded tocopherol acetate. The loaded microcapsules were subjected to absorbance measurement at 292 nm (~~Varian, Cary 100~~).

## 2.7. Methods

### 2.7. Size exclusion chromatography (SEC)

Average molar masses and molar masses distributions were determined by size exclusion chromatography (SEC) coupled online with multi-angle light scattering (MALS) and differential refractive index (DRI) detectors. The MALS apparatus is the DAWN Heleos-II from Wyatt Technology (Ca, USA) filled with a K5 cell and a Ga-As laser ( $\lambda = 690$  nm). The DRI detector is a Shimadzu RID-10A (Japan). Columns [Shodex SB OHpak 804 and 806 HQ for alginate, Eprogen CATSEC 100 and 1000 for chitosan, PL aquagel OH 40+30 for the cationic polycyclodextrin] were eluted with  $0.1\text{mol.L}^{-1}$   $\text{LiNO}_3$  or acetate buffer  $0.1\text{M}$ , respectively at  $0.5\text{ mL min}^{-1}$ . The flow rate was fixed at  $1\text{ mL.min}^{-1}$ . The samples were filtered on  $0.45\text{ }\mu\text{m}$  unit filter before injection through a  $100\text{ }\mu\text{L}$  full loop.

### 2.8. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

A Q-Sense E1 (Q-Sense, Sweden) system employing ATcut gold-coated quartz crystal sensors (Lot-Oriel, France) with a nominal frequency of about 5 MHz was used to monitor *in situ* formation of the  $P(CD)^+ / alg^-$  self-assembly.

## **2.9. Zeta -Potential Measurements**

Zeta potential measurement was performed on a Zetasizer Nano-ZS (Malvern Instrument) to monitor the adsorption of the polyelectrolytes onto the  $CaCO_3$  core (Zetasizer 4700 Malvern Instruments, Brookhaven Instruments Corporation, USA).

## **2.10. Scanning Electron Microscopy (SEM)**

This technique enables to study the shape of the resulting particles (Leo Elektronenmikroskopie, Oberkoche in Germany). Prior to analysis, the calcium carbonate particles and capsules were covered with palladium.

## **2.11. Fluorescence microscope**

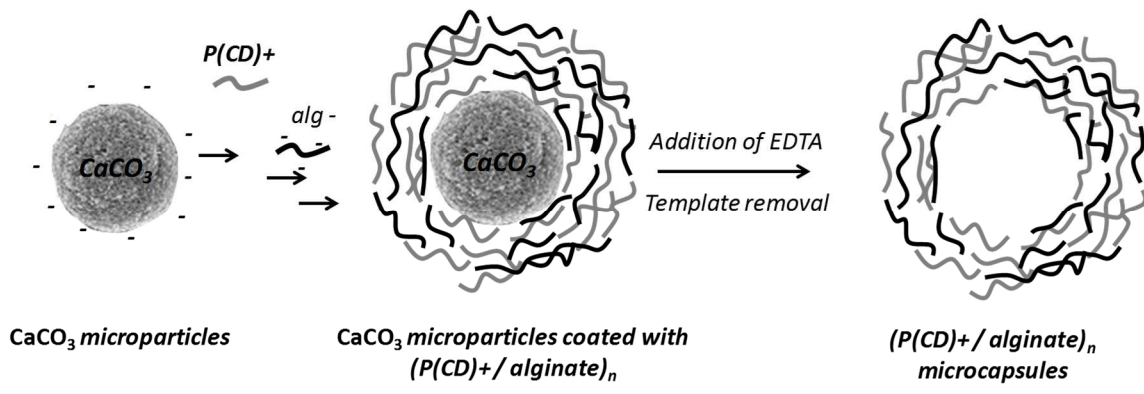
Images of microcapsules were obtained with an optical fluorescence ZEISS microscope equipped with a CCD camera and a fluorescence illuminator and various mirror units. Images were recorded with ZEN software. A 5  $\mu$ L drop of capsule suspension was deposited on a cover slip to observe the microcapsules. This technique was used after staining of bacteria using alcidine orange.

## **2.12. Absorbance measurement.**

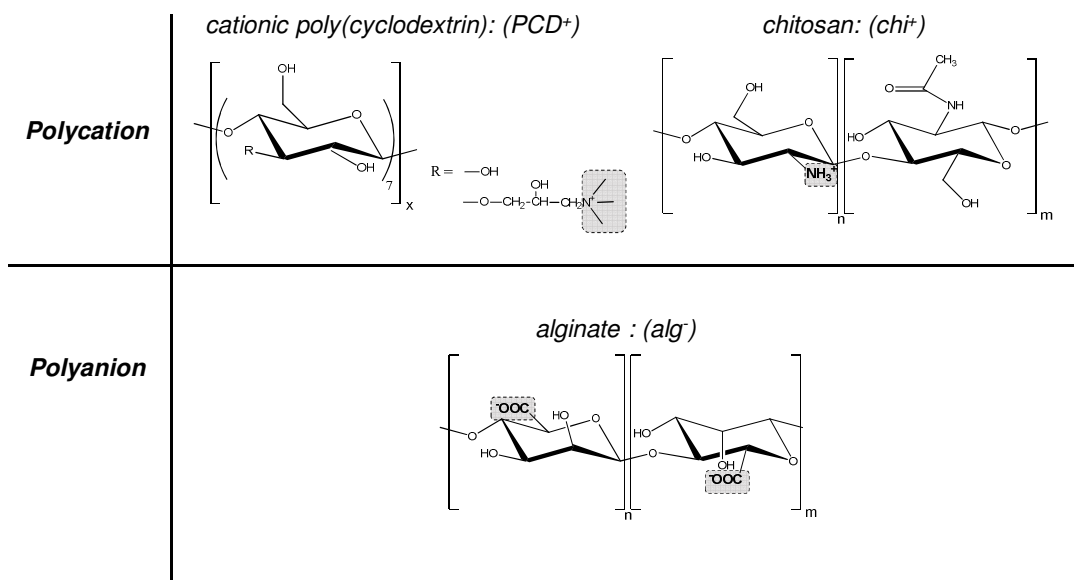
Absorbance spectra were recorded with a UV-Visible spectrophotometer micro plate reader (bacterial assay) and a Varian Cary 100 (tocophorol acetate)

### 3. Results and discussion

Figure 1 illustrates the approach used to ~~elaborate~~ prepare CD-containing microcapsules. The surface of CaCO<sub>3</sub> microparticles was modified through alternate deposition of the positively charged cationic polycyclodextrin and a layer of negatively charged alginate. The chemical structures of the P(CD<sup>+</sup>), chitosan and alginate are presented in Figure 2. ~~Their characteristics are reported in Table 1. For the alginate, the  $\overline{Mn}$  value is  $2.7 \cdot 10^5 \text{ g}\cdot\text{mol}^{-1}$ , the  $\overline{Mw}$  value is  $3.8 \cdot 10^5 \text{ g}\cdot\text{mol}^{-1}$ . For the cationic poly(cyclodextrin), the  $\overline{Mn}$  value is  $6.5 \cdot 10^3 \text{ g}\cdot\text{mol}^{-1}$ , the  $\overline{Mw}$  value is  $2.4 \cdot 10^4 \text{ g}\cdot\text{mol}^{-1}$  and the degree of substitution is  $0.40 \pm 0.05$ . For the chitosan, the  $\overline{Mn}$  value is  $8.5 \cdot 10^4 \text{ g}\cdot\text{mol}^{-1}$ , the  $\overline{Mw}$  value is  $2.0 \cdot 10^5 \text{ g}\cdot\text{mol}^{-1}$ . The degree of acetylation of chitosan is  $0.14 \pm 0.02$ . After the LbL process, the calcium carbonate template is selectively removed to ensure the capsule formation.~~ After each coating step, free or unbound polyelectrolytes are removed by centrifugation to prevent the formation of inter-polyelectrolyte complexes. ~~After the LbL process, the calcium carbonate template is selectively removed to ensure the capsule formation.~~ Herein, we compare the antimicrobial agent performances of the cationic polycyclodextrin to the well-known chitosan ~~as antimicrobial agent~~. Thus, the antibacterial activities of P(CD<sup>+</sup>) and chitosan, under its cationic form, are initially tested in aqueous solutions, to provide comparative data for the intrinsic antibacterial activity of the said polymers. Then, P(CD<sup>+</sup>) and chitosan were layer-by-layer assembled with alginate onto CaCO<sub>3</sub> core, and the resulting multi-layered capsules were subjected to antibacterial assay.



**Figure 1.** Schematic illustration of the polyelectrolytes adsorption process applied to the elaboration of cationic-polycyclodextrin (P(CD<sup>+</sup>)) /alginate (alg<sup>-</sup>) capsules.



**Figure 2.** Polyelectrolytes **implied** involved in the present study

**Table 1:** Average molar masses of the polyelectrolytes used (determined by size exclusion chromatography SEC/MALS/DRI analysis).

Samples	Abbreviation	Mn (g·mol <sup>-1</sup> )	Mw (g·mol <sup>-1</sup> )	Đ*	DS**
Chitosan	chi <sup>+</sup>	8.5 x 10 <sup>4</sup>	2.0 x 10 <sup>5</sup>	2.3	0.14 ±0.02 a



Cationic polyclodextrin	P(CD) <sup>+</sup>	6.5 x 10 <sup>3</sup>	2.4 x 10 <sup>4</sup>	3.6	0.40 ± 0.05 <sup>b</sup>
Alginate	alg <sup>-</sup>	2.7 x 10 <sup>5</sup>	3.8 x 10 <sup>5</sup>	1.4	-

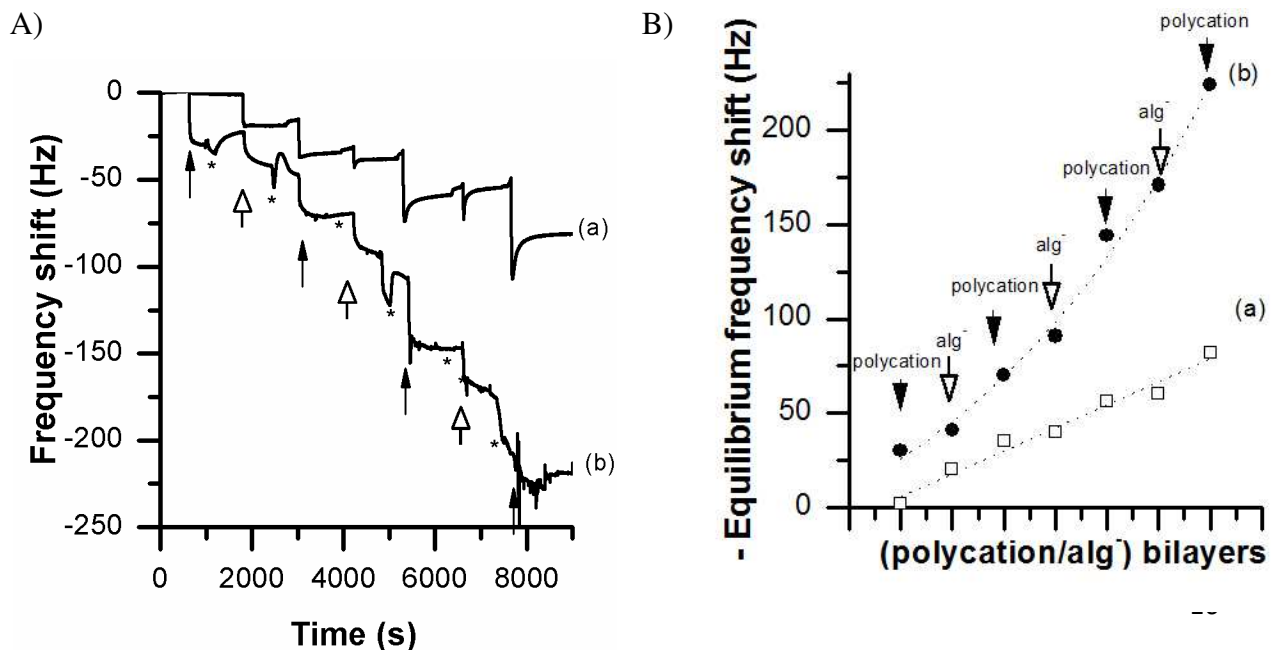
\* Polydispersity index (Đ) Đ = Mw/Mn

Mn = number average molecular mass and Mw weight average molecular mass

\*\* Degree of substitution (DS): <sup>a</sup> Degree of acetylation of chitosan, <sup>b</sup> Degree of substitution of P(CD<sup>+</sup>) (estimated from <sup>1</sup>H NMR (Sabrina Belbekhouche, et al., 2018; Renard, et al., 1997))

### 3.1. Characterization of microcapsules based on cationic polyclodextrin

The assembly process was first monitored on QCM resonators to evidence the mass increase after each deposition step of each polyelectrolyte (figure 3). (P(CD<sup>+</sup>)/alg<sup>-</sup>) and (chi<sup>+</sup>/alg<sup>-</sup>) have been studied as polyelectrolytes couples. From the QCM frequency shift plots, we observe a decrease of the frequency shift which ~~could be correlated to is in accordance with~~ an increase of mass due to the successive polyelectrolytes adsorption. ~~This result evidences the formation of the corresponding multilayer films.~~



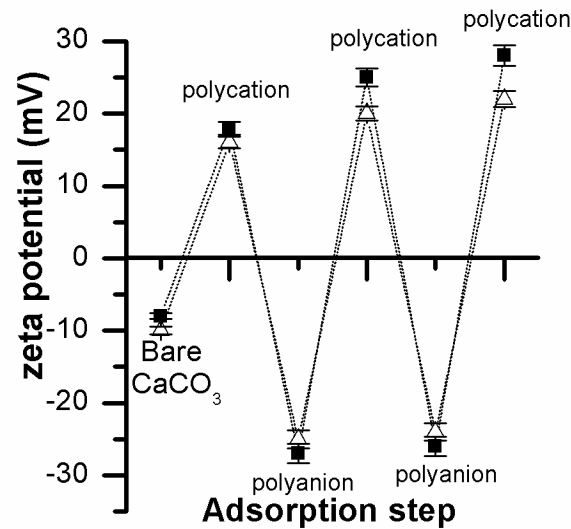
**Figure 3.** A) QCM result, alternative adsorption of polycation and alginate ((a) P(CD<sup>+</sup>)/alg<sup>-</sup> and (b) chi<sup>+</sup>/alg<sup>-</sup>) (filled arrow: addition of polycation and unfilled arrow, “\*” rinsing with water). B) Dependence of the equilibrium frequency according to the number of (polycation/alg<sup>-</sup>) bilayer ((a) □ P(CD<sup>+</sup>)/alg<sup>-</sup> and (b) • chi<sup>+</sup>/alg<sup>-</sup>).

The final frequency as measured after the adsorption of 7 layers is around -75 Hz when using P(CD<sup>+</sup>) as polycation. This value reaches -225 Hz with chi<sup>+</sup> as polycation.

Figure 3 B presents the equilibrium frequency shift as measured after each polyelectrolyte adsorption. Two growth regimes are evidenced from these plots, an exponential growth and a linear one. The exponential behavior is observed **by employing with** chitosan as polycation. This result suggests the ability of the chitosan to diffuse “in” and “out” of multilayer films. (Lavalle, et al., 2004) This is in agreement with the work of Richert *et al.* (Richert, et al., 2004) who studied chitosan/hyaluronan films. In contrast, the multilayer film **elaborated with based on** the cationic polycyclodextrin shows a linear build-up process. Using this polycation instead of chitosan may lead to a decrease in the diffusion phenomena and consequently to a linear variation of the thickness with the layers number.

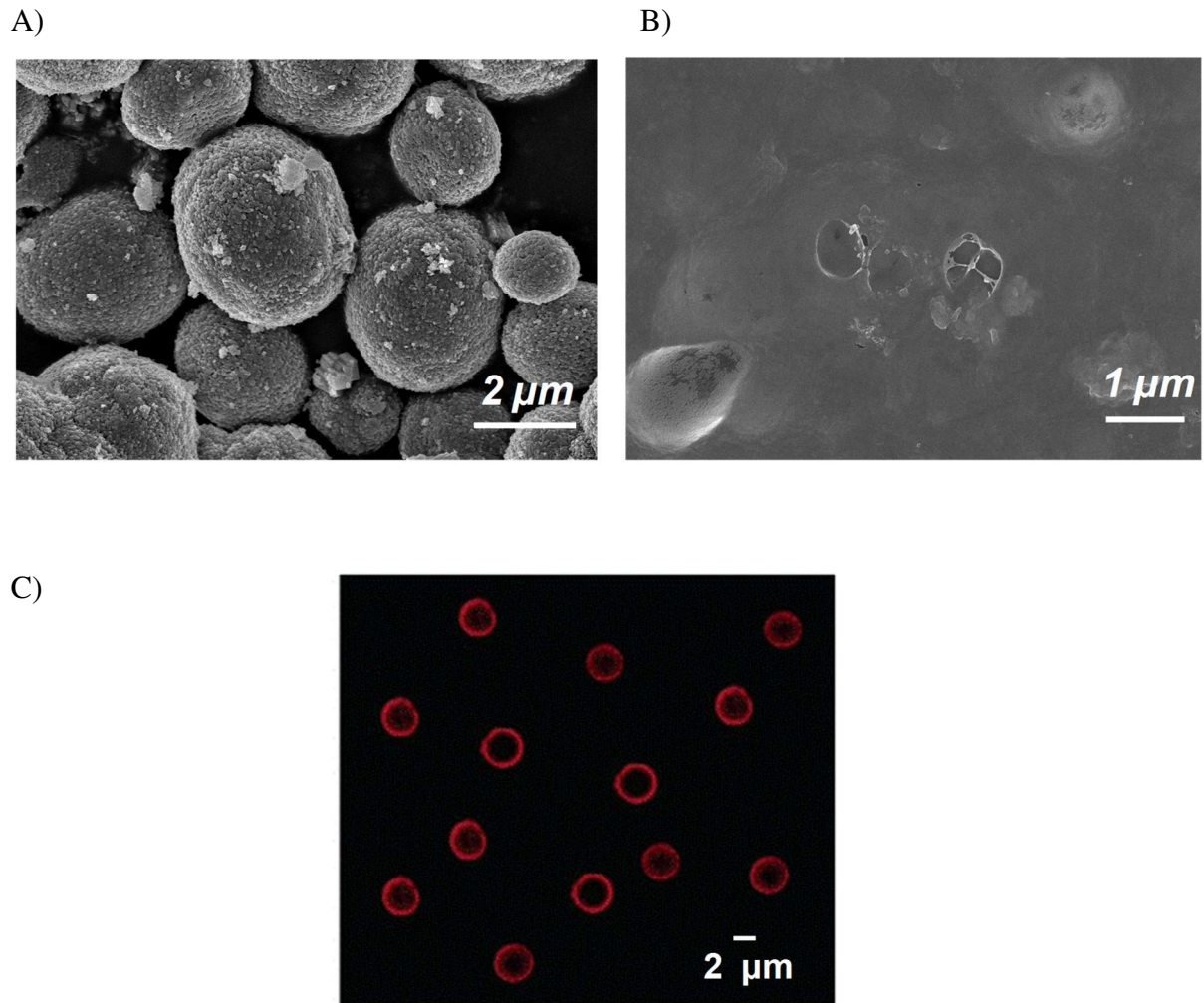
Zeta measurement is one of the most widely used techniques for following the adsorption of polyelectrolytes onto particles. (Kirby & Hasselbrink, 2004) The zeta potential of the modified calcium carbonate microparticles was determined after deposition of each polyelectrolyte (figure 4). The initial surface potential measured for native CaCO<sub>3</sub> particles is - 10 mV. **That is in accordance with a negative surface charge.** This offers the possibility to adsorb positively charged polyelectrolyte. An alternation of surface potential from +20 mV or +25 mV for P(CD<sup>+</sup>) and chi<sup>+</sup> respectively, to -25 mV for alginate is observed. This confirms the successful

formation of the multilayer films on  $\text{CaCO}_3$  particles (Caruso, 2000) through electrostatic interactions.



**Figure 4.** Zeta potential values for ■ chitosan/alginate or  $\Delta$  cationic polycyclodextrin/alginate onto calcium carbonate microparticles.

After elaboration of the multilayered shells, the template cores were removed to obtain microcapsules. SEM micrographs of  $(\text{P}(\text{CD}^+)/\text{alg}^-)_4\text{-P}(\text{CD}^+)$ -coated microparticles and the corresponding microcapsules demonstrate ~~that these particles have~~ an actual size in the micrometer range. These also indicate uniform spherical morphology ~~of the materials~~ (figure 5 A and B). Successful design of microcapsules was also clearly demonstrated by confocal microscopy when using rhodamine-labelled alginate during the multilayer build-up (Figure 5 C). The same tendency is observed if using  $(\text{chi}^+/\text{alg}^-)_4$  as polyelectrolytes pair. (Sabrina Belbekhouche, et al., 2018)



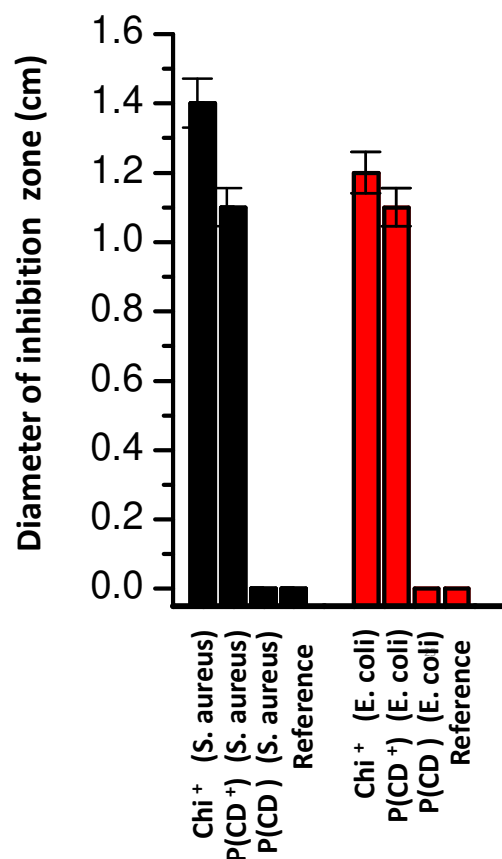
**Figure 5.** SEM pictures of  $(P(CD^+)/alg^-)_4-P(CD^+)$  (A) microparticles (magnification of 10KX) and (B) microcapsules (magnification of 15KX), C) Confocal microscopy image of microcapsules consisting of  $(P(CD^+)/alg^-)_4-P(CD^+)$  where the alginate was fluorescently tagged with rhodamine derivative.

### 3.2. Effect of cationic polyclodextrin particles on pathogenic bacterial growth

Chitosan is known to show excellent bactericidal effects, however it suffers from poor solubility at neutral pH or close to neutrality (pKa of chitosan ~ 6.2 - 7.0). It is then crucial to investigate alternative biopolymers being soluble under physiological conditions (pH ~ 7.4). The studied cationic polycyclodextrin ~~is proposed herein as~~ could then be an excellent candidate.

The impact of cationic polycyclodextrin on the two pathogenic environmental bacteria *E. coli* and *S. aureus*, was investigated in agar plates and then in liquid LB medium. *E. coli* is a Gram-negative bacterium which could be found in gastrointestinal tract normal flora. *S. aureus* is a Gram-positive bacterium that can induce skin infection and pus.

Results from antibacterial tests ~~performed on agar plates and using well diffusion method~~ showed no inhibition zone for the unmodified native polycyclodextrin. Interestingly, the modification of the polycyclodextrin (incorporation of cationic charge) leads to an inhibition zone for both bacteria. This could directly be correlated to the incorporation of the quaternary ammoniums ~~which are described as being able to~~ known to be responsible of damage of the microbial membranes and ~~to denature~~ of denaturation of the soluble proteins. This denaturation can disrupt cell functions and induce bacterial death. (Kügler, Bouloussa, & Rondelez, 2005) Diameter of inhibition zones were around 1.2 and 1.1 cm ± 0.1 for *E. coli* and 1.4 and 1.1 cm ± 0.1 for *S. aureus* with chitosan and cationic polycyclodextrin respectively (Figure 6)



**Figure. 6.** Diameters of inhibition zones against *E. coli* and *S. aureus* when using chitosan (chi<sup>+</sup>), the cationic poly(cyclodextrin) (P(CD<sup>+</sup>)), the poly(cyclodextrin) (P(CD)) and the reference (physiological solution).

The results obtained with chitosan, are in agreement with those reported by Li *et al.* (Li, et al., 2007) and Du *et al.* (Du, Zhao, Dai, & Yang, 2009) These authors suggested that this polysaccharide usually presents higher antibacterial activities against *S. aureus* than *E. coli*. Compared to chitosan, P(CD<sup>+</sup>) shows a lower efficiency on *S. aureus* (Figure 6) and a same inhibitory effect against *E. coli*. P(CD<sup>+</sup>) provided the same inhibition toward *E. coli* and *S. aureus*. This similar effect on these two bacteria suggests that the structure of the bacterial wall is not involved in inhibitory mechanisms. Like quaternary ammonium-containing

materials, P(CD<sup>+</sup>) ~~would~~ may cause the denaturation of bacterial cytoplasmic proteins (Willey, et al., 2008). It is worth noting that no antimicrobial effect is observed with the alginate.

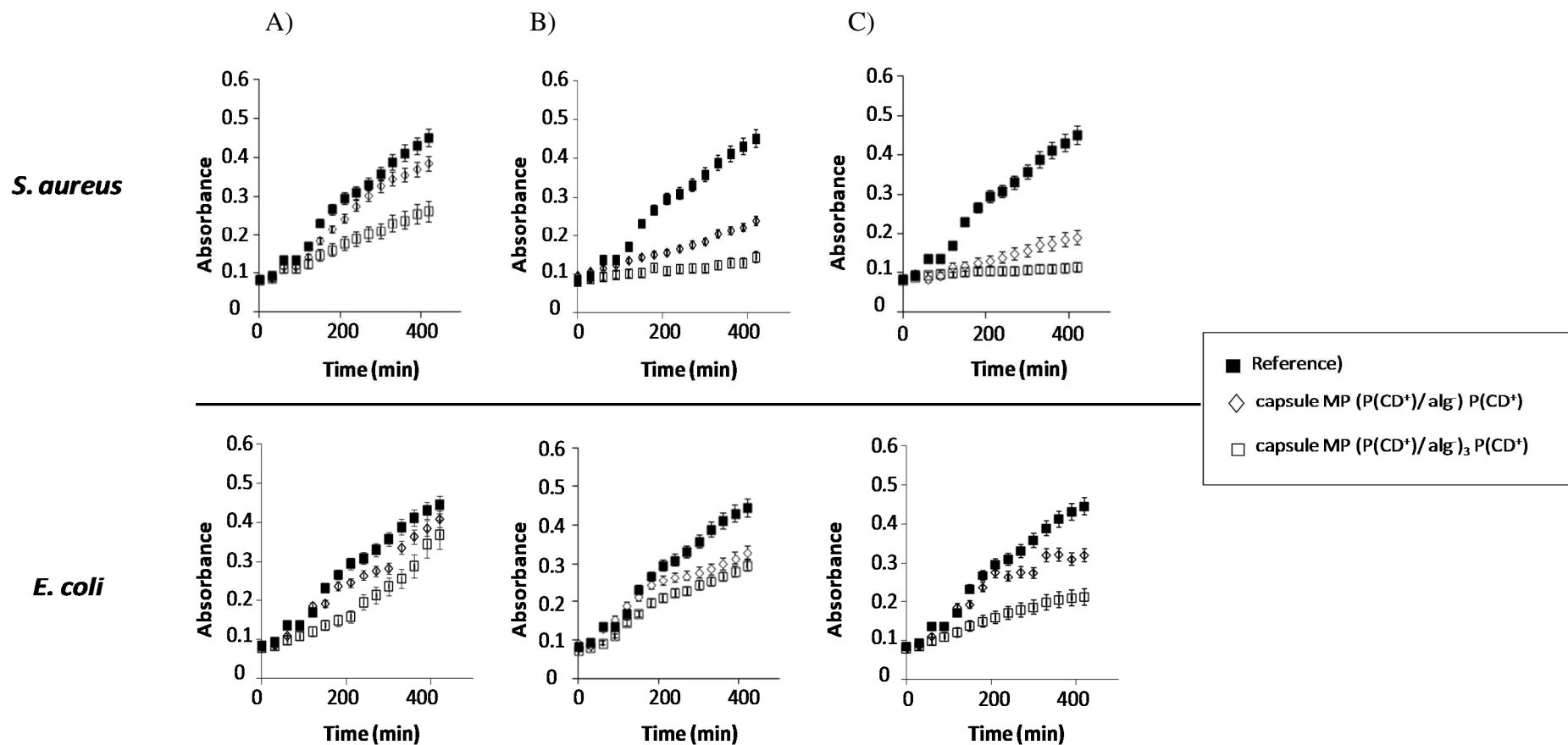
The bacterial growth kinetics was followed in LB medium (initial bacterial concentration  $6.4 \times 10^7$  cell/mL) in presence of different composition and concentration of microcapsules. Each experiment was performed in six replicates (Figure 7). Note that no inhibition was observed with bare calcium carbonate particles at the studied particle concentration. The 7 hour (420 min) experimental time for monitoring the growth curve has been selected as it is assumed that the physicochemical conditions of the medium, such as ~~for instance~~ pH, does not significantly change during this period. Therefore, ~~it is expected that the presence of microcapsules is~~ the sole parameter which can impact the ~~behavior of~~ bacteria growth ~~could only be the presence or not of microcapsules~~.

To investigate the influence of several particles parameters (numbers of layers, concentration of particles...) on the bacteria growth, the percentage of bacterial inhibition at the end of the experiment was estimated (see table 2) ~~through the difference of the number of bacteria per millimeter at the end of the experiment for the particles and the reference essay where bacteria is in presence of sterile physiological distilled water only~~. Whatever the strain studied, (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>) particles inhibit the growth rate more than single-layer particles (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>). For instance, at 0.02 g.L<sup>-1</sup> the percentage of inhibition for *E. coli* is 8.9 % and 28.1 % for (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>) and (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>) respectively. The same trend is observed for *S. aureus*, i.e. 15.6 % and 42.2 % for (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>) and (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>) respectively (table 2). The bacterial density reached at the end of the experiment is lower in the presence of capsules. For *E. coli* the following OD values are measured at the end of the experiment 0.44 ( $3.6 \times 10^8$  bacteria per mL), 0.40 ( $3.2 \times 10^8$  bacteria per mL) and 0.32 ( $2.6 \times 10^8$  bacteria per mL) for the reference, (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>)

and  $(P(CD^+)/alg^-)_3P(CD^+)$  respectively. The same observation is made for *S. aureus*. The obtained OD values at the end of bacterial incubations are 0.45 ( $3.6 \times 10^8$  bacteria per mL), 0.38 ( $3.0 \times 10^8$  bacteria per mL) and 0.26 ( $2.1 \times 10^8$  bacteria per mL) for the reference,  $(P(CD^+)/alg^-)P(CD^+)$  and  $(P(CD^+)/alg^-)_3P(CD^+)$  respectively. The comparison of the results obtained for the two strains shows a greater inhibitory impact on *S. aureus* than on *E. coli*. The results ~~also~~ clearly evidence that the increase in the concentration ~~particles~~ (0.02 g.L<sup>-1</sup>, 0.06 g.L<sup>-1</sup> and 0.1 g.L<sup>-1</sup>) of  $(P(CD^+)/alg^-)P(CD^+)$  and  $(P(CD^+)/alg^-)_3P(CD^+)$  ~~particles~~ significantly leads to an enhancement on the inhibition of bacteria growth. For *E. coli*, the inhibition level increases from 8.9 % to 32.6 % and from 28.1 to 53.3 % for  $(P(CD^+)/alg^-)P(CD^+)$  particles and  $(P(CD^+)/alg^-)_3P(CD^+)$  particles respectively, ~~due to the increase of the particles concentration (0.02 g.L<sup>-1</sup>, 0.06 g.L<sup>-1</sup> and 0.1 g.L<sup>-1</sup>)~~. For *S. aureus*, the inhibition level increases from 15.6 % to 57.8 % and from 42.2 to 57.8 % for  $(P(CD^+)/alg^-)P(CD^+)$  particles and  $(P(CD^+)/alg^-)_3P(CD^+)$  particles respectively, ~~due to the increase of the particles concentration~~.

The difference can be explained by the interaction mechanisms of  $P(CD^+)$ -particles with the bacterial membrane. Like chitosan, the ~~elaborated~~  $P(CD^+)$ -microparticles may induce membrane damage, enzyme inhibition and other protein denaturation. (Nair, et al., 2009) In the future, biochemical analysis and electron microscopy would allow us to know which the dominant inhibition mechanism is.





**Figure 7.** Growth study of *S. aureus* and *E. coli* in LB medium inoculated with  $6.4 \times 10^7$  /mL of bacteria in the presence of different concentrations of capsules: *i.e.* A)  $0.02 \text{ g.L}^{-1}$ , B)  $0.06 \text{ g.L}^{-1}$  and C)  $0.1 \text{ g.L}^{-1}$

**Table 2.** Impact on *E. coli* (EC) and *S. aureus* (SA) growth due to microcapsules based on cationic poly(cyclodextrin) at different concentrations

SA	R	Ac			Bc		
C (g/L)	OD <sub>final</sub>	OD <sub>final</sub>	Bacteria per mL	I (%)	OD <sub>final</sub>	Bacteria per mL	I (%)
0.02	0.45	0.38	3.0 x 10 <sup>8</sup>	15.6	0.26	2.1 x 10 <sup>8</sup>	42.2
0.06	0.45	0.24	1.9 x 10 <sup>8</sup>	46.9	0.14	1.1 x 10 <sup>8</sup>	68.2
0.1	0.45	0.19	1.5 x 10 <sup>8</sup>	57.8	0.11	9.1 x 10 <sup>7</sup>	57.8

EC	R	Ac			Bc		
C (g/L)	OD <sub>final</sub>	OD <sub>final</sub>	Bacteria per mL	I (%)	OD <sub>final</sub>	Bacteria per mL	I (%)
0.02	0.44	0.40	3.2 x 10 <sup>8</sup>	8.9	0.32	2.6 x 10 <sup>8</sup>	28.1
0.06	0.44	0.33	2.6 x 10 <sup>8</sup>	26.1	0.29	2.4 x 10 <sup>8</sup>	33.5
0.1	0.44	0.30	2.4 x 10 <sup>8</sup>	32.6	0.19	1.5 x 10 <sup>8</sup>	57.3

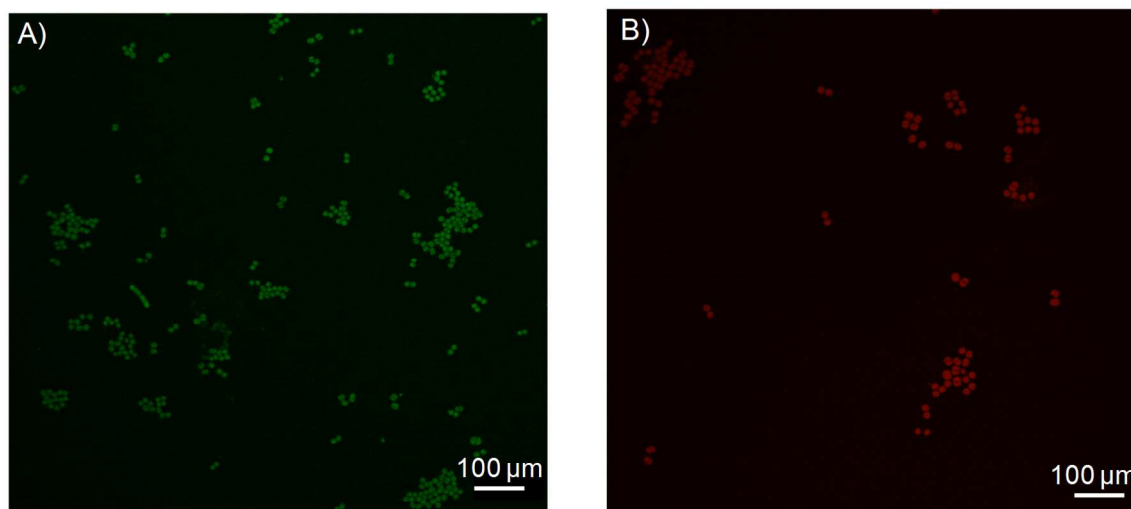
I (%) : percentage of inhibition ( $I = (100 - \frac{\text{number of bacteria per mL at the end of the experiment for the sample}}{\text{number of bacteria per mL at the end of the experiment for the reference}}) \times 100$ )

R : reference (sterile physiological distilled water), Ac : capsule (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>), Bc: capsule (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>)

Note that for enterobacteria as *E. coli* and *S. aureus*, 1 OD at 620nm is equivalent to 8 x 10<sup>8</sup> CFU/mL(Willey, et al., 2008)

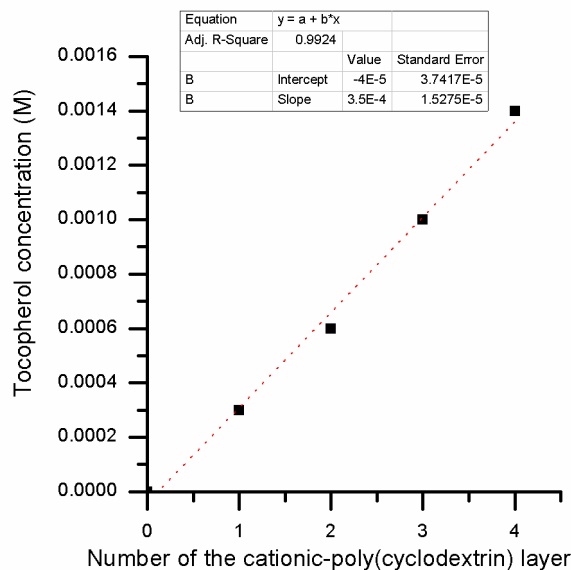
From the above results, we have demonstrated that the microcapsules inhibit significantly the growth of both bacteria. The following question is then raised, are the bacteria alive or not? Several research works have demonstrated that the color of bacteria stained with acridine orange (AO) allows discriminating between living bacteria and dead bacteria. (Francisco, Mah, & Rabin, 1973) This relationship between their activity and the color of acridine orange-stained bacteria is based upon the *in vitro* spectrofluorescence of nucleic acids (DNA) with AO. (Rigler, 1969) Indeed, acridine orange is a cell-permeant nucleic acid binding dye and is able to differentially double-stranded (ds) and single-stranded (ss) nucleic acids. AO emits red fluorescence when bound to ssDNA denatured DNA found in dead bacteria and green fluorescence when bound to ds DNA characterizing living bacteria. Herein, a red fluorescence is observed after the bacteria staining (figure 8) evidencing that exposition to capsule (P(CD<sup>+</sup>)/alg<sup>-</sup>)P(CD<sup>+</sup>) or capsule (P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>) induce the death of bacterial cells. It deserves noting that a green fluorescence is observed for the *S. aureus* without capsule (negative control, figure 8 A), pointing that the bacteria are alive with no capsule. ~~Furthermore, the Note that the test implying using~~ the acridine orange is a qualitative test of observations and not a quantitative one. ~~The number of living bacteria in a suspension was then confirmed- determined by counting them with a Mallassez cell.(Willey, et al., 2008) It should be noted here that no No~~ changes in shape of bacteria are observed suggesting that the effect of the microcapsules is not exerted ~~on the level of~~ the bacterial ~~cellular~~ structure. ~~Thus, herein the As~~ quaternary ammonium-containing microparticles ~~in our case~~ would rather induce inhibition and denaturation of bacterial proteins (Willey, et al., 2008). ~~The number of bacteria in a suspension was confirmed by counting them with a Mallassez cell.~~ Furthermore, the death of bacteria cells was qualitatively checked. At the end of the experiment, we have inoculated bacterial suspension on PCA solid medium for growth overnight in aerobic

conditions at 37 °C. No bacterial colonies were observed which confirms the death of the bacteria due to their contact with microcapsules.



**Figure 8:** *S. aureus* stained with acridine orange after the antibacterial assay, A) without capsules (negative control) and B) in contact with the capsules ((P(CD<sup>+</sup>)/alg<sup>-</sup>)<sub>3</sub>P(CD<sup>+</sup>)) (magnification: x200)

After clearly evidencing the interest in modifying polymer based on cyclodextrin to bring antibacterial activity, we have investigated the possibility to still exploit the host-guest complexation in the particle's shell. The studied hydrophobic compound is the tocopherol acetate, an antioxidant molecule. Interestingly, in water, the solubility of tocopherol acetate increased linearly (from 0 to 0.014 M) as the number of the cationic-poly(cyclodextrin) layers increased (from 1 to 4) for a concentration of microcapsules of 2 mg.mL<sup>-1</sup> (Figure 9).



**Figure 9 :** Variation of the solubility of tocopherol acetate due to the increase of the number cationic-poly(cyclodextrin) layers in the microcapsule. Note that the tocopherol acetate is insoluble in water.

#### 4. Conclusion

For antibacterial purposes, we first evidenced the possibility to use a water soluble cationic polycyclodextrin bearing permanently charged quaternary ammonium groups rather than the cationic chitosan. This latter has low solubility in water as well as in physiological conditions. Then, P(CD<sup>+</sup>)-microcapsules were ~~elaborated~~ prepared through an easy and efficient approach based on template-assisted self-assembly of polyelectrolytes in aqueous solutions without employing neither organic solvents or surfactants. This process, followed by zeta measurements, relied on the capacity of the cationic-polycyclodextrin to be layer-by-layer assembled with alginate onto sacrificial carbonate microparticles. ~~The LbL assembly is based on the electrostatic interactions occurring in aqueous solution as evidenced by zeta measurements.~~ The ~~MEB~~ SEM and fluorescence microscopy results disclosed capsules formation. These polyelectrolytes-coated particles have then been evidenced to inhibit growth and eliminate pathogenic bacteria.

The following conclusions were drawn from this work: (i) cationic polycyclodextrin presents an antibacterial activity ~~and this result which~~ could only be correlated to the incorporation of positive charges in the polycyclodextrin chain, (ii) microcapsules based on the cationic polycyclodextrin inhibited the *E. coli* and *S. aureus* growth ~~and~~ (iii) CD cavities within the LbL assemblies retain their inclusion properties towards tocopherol acetate. However, antibacterial activity towards both *E. coli* (Gram negative ~~bacteria~~ bacterium) ~~and was less than that for~~ *S. aureus* (Gram positive ~~bacteria~~ bacterium) was similar.

~~Note that after clearly evidencing the benefit of modifying polymer based on cyclodextrin to bring antibacterial activity, we have also investigated the possibility to still exploit the host-guest complexation in the particle's shell. The studied hydrophobic compound is the tocopherol acetate, an antioxidant molecule. Interestingly, in water, the solubility of the tocopherol acetate increased (from 0 to 0.014 M) linearly as the number of the cationic poly(cyclodextrin) increased (from 1 to 4) for a concentration of microcapsules of 2 mg·mL<sup>-1</sup>~~

This work, following previous researches, offers the possibility to formulate new types of bactericides, *i.e.* (i) based on cationic poly(cyclodextrin) by itself or ~~implied~~ in self-assembly and (ii) capsules (hollow particles).

In a forthcoming study, we will investigate the impact of the degree of substitution of the cationic-polycyclodextrin and take advantage of the cyclodextrin property by considering host-guest interactions for carrying one or multiple hydrophobic antimicrobial, as a penicillin derivative, inside the cyclodextrin cavity. Furthermore, the study will be extended to other bacteria.

## 6. References

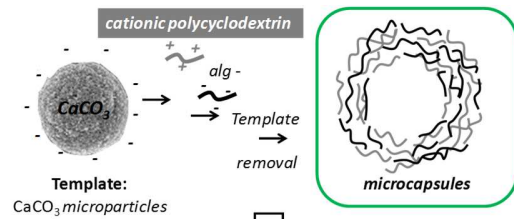
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