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## Porous CaCO<sub>3</sub> Microparticles for Antibiotic Loading and the Development of Antibacterial Capsules by using Porous CaCO<sub>3</sub> Microparticles as Starting Material

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### Abstract.

Porous calcium carbonate (CaCO<sub>3</sub>) particles have been shown to be highly advantageous for biological applications, mainly due to their large surface area and their stability in physiological media. Also, developing appropriate antibacterial materials presenting the benefits of non-formation of harmful compounds is of major interest. Two characteristics of CaCO<sub>3</sub> particles were investigated herein: (i) antibiotic-loading capacity and (ii) the possibility of using CaCO<sub>3</sub> particles as a template for the fabrication of biocapsules presenting inherent antibacterial capacity. The particles were tested against two representative pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*). On one hand, a method for antibiotic (namely penicillin, ampicillin and ciprofloxacin) loading inside calcium carbonate particles was developed and antibacterial activity was investigated. Encapsulation efficiency and loading content were 95% and 5%, respectively. We showed that antibiotics prevented bacterial growth within 2 h, with no evidence of bacterial regrowth within 16 h; bactericidal effects were also observed. On the other hand, the self-assembly of charged polysaccharides, namely chitosan (chi<sup>+</sup>) and dextran sulphate (dex<sup>-</sup>), were assessed on calcium carbonate microparticles used as a sacrificial matrix. During bacterial growth in a liquid medium, an inhibitory effect of these particles was observed, i.e. *Staphylococcus aureus* (Gram-positive) (from 16.3% to 48.8% for (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> coated CaCO<sub>3</sub> materials and from 41.9% to 93.0% for (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> capsules) and *Escherichia coli* (Gram-negative) (from 18.2% to 45.5% for (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> coated CaCO<sub>3</sub> materials and from 40.0% to 89.1% for (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> capsules). Staining with acridine orange highlighted the bactericidal effect of the designed particles. These findings demonstrate the excellent potential of using calcium carbonate particles in antibiotic therapy as a starting point for the development of smart materials.

**Keywords:** calcium carbonate-based particles; porous particle, capsule; antibiotic loading; antibacterial agent; polysaccharide

## 1. Introduction

The interest in using porous materials for biological purposes is mainly due to the possibility of controlling both their physico-chemical properties and characteristics <sup>1</sup>. Porous particles are highly appropriate carriers for drug delivery purposes. Among them, we may cite silica, calcium carbonate and calcium phosphate particles <sup>2</sup>. For more insight into the fabrication of porous materials, the reader may refer to the recent published review articles <sup>3-4</sup>. Most of these particles are biodegradable, can be safely excreted by the human body through the kidney and are able to transport a high amount of therapeutic bioactive chemicals with high efficiency and few side effects <sup>5</sup>. Interestingly, the surface of porous carriers such as silica or calcium carbonate particles can be easily functionalised by a variety of ligands for improved in vivo stability in the bloodstream <sup>6</sup>. Among the different porous carriers, in our studies we focus on porous calcium carbonate (CaCO<sub>3</sub>) particles which have the advantages of being biocompatible, non-toxicity and easily produced by using standard methods. Among the different porous carriers, calcium carbonate (CaCO<sub>3</sub>) particles have the advantages of being biocompatible, non-toxicity and easily produced by using standard methods<sup>7</sup>.

CaCO<sub>3</sub> particles exist in three anhydrous crystalline polymorphs forms, i.e. calcite, aragonite and vaterite <sup>8-9</sup>. Studies have reported the possibility of fabricating spherical mono-dispersed CaCO<sub>3</sub> particles in the nano- to micrometre range <sup>9-10</sup> and loading drugs inside these porous materials <sup>11-12</sup>. The cytotoxicity, influence on cell viability and efficient cellular uptake of substance-loaded CaCO<sub>3</sub> containers have been investigated <sup>13</sup>. In our study, we propose exploiting the excellent versatility of these materials for antibacterial purposes.

Indeed, antibacterial resistance is a growing problem impacting the entire world <sup>14</sup>. The emergence of intracellular bacterial infections and acquired resistance in pathogenic microbes has been a challenging issue in human medicine <sup>15</sup>. The use of antibiotic is required for these infections, but due to their poor stability or solubility in biological media, a step of vectorisation is often required for their efficient administration. <sup>16</sup>

Using safe and efficient antibiotic delivery systems is thus needed that as to be able are required to deliver therapeutic agents at an adequate concentration within the intracellular medium to overcome antibacterial resistance <sup>17-18</sup>. Developing an antibacterial carrier (loaded with an antibiotic or having inherent antibacterial capacity) could avoid/prevent antibiotic efflux and thus increase the intracellular retention of drugs. Indeed, as the antibacterial carriers are not substrates of efflux pump proteins <sup>19</sup>. Interestingly; several studies have reported on the increased antibacterial activity of antibiotic-conjugated particles <sup>20-22</sup>.

In this context, we have implemented two approaches based on calcium carbonate microparticles for developing an efficient antibacterial material. The first one involved the loading of antibiotics inside the pores of the particles. The second approach consisted in exploiting CaCO<sub>3</sub> particles to develop new tailor-made antibacterial particles.

We took advantage of the porosity of CaCO<sub>3</sub> particles for loading antibiotics, namely penicillin, ampicillin and ciprofloxacin. Penicillin and ampicillin are widely employed to treat many different types of infections caused by bacteria such as *E. coli* or salmonella. Ciprofloxacin is a fluoroquinolone commonly used in cystic fibrosis to treat lung infections<sup>23</sup>. However, at high concentrations, these antibiotics present toxicity in the blood. In contrast, at low concentrations, the resistance of bacteria is promoted at the site of infection<sup>24</sup>. The concentration problem is also mediated by solubility limitations in aqueous solution. The selected antibiotics present a wide solubility range, i.e. highly soluble (solubility about 25 mg/ml in water for penicillin G sodium), slightly soluble (solubility about 13 mg/ml in water for ampicillin) and practically insoluble in water (solubility less than 1 mg/ml in water for ciprofloxacin). Thus, their vectorisations are of major interest for both the achievement of high concentrations of these antibiotics at the site of infection while preventing bacterial resistance.

As alternative to antibiotics, some polyelectrolytes with inherent bactericidal components exist as quaternary ammonium or phosphonium groups. These charged polymers are able to interact with the negatively charged bacterial cell walls, resulting in compromised integrity of the bacterial cell envelope<sup>25</sup>. This is the reason why these polymers have been naturally involved in self-assembly processes (e.g. the layer-by-layer,<sup>26-27</sup> (LbL) approach) for developing materials with antibacterial activity. Polyelectrolyte multilayers can be used to obtain ultrathin films through the alternating deposition of oppositely charged polyelectrolytes. This offers well-defined compositions and physical properties. This process presents the following advantages: (i) no specific equipment is required, (ii) various chemical and biological compounds can be used and (iii) nanometre-ordered functional layers can be easily achieved. Moreover, the LbL process is an efficient method for fabricating carriers (namely capsules<sup>28-29</sup>) combining both templating particles and self-assembly methods. Particles provide a compelling alternative to antibiotic because they rely on entirely different mechanisms of antibacterial activity than antibiotics. The mode of action of particles is based on direct contact with the cell wall of the bacteria, leading to damage to of the bacterial cell<sup>25</sup>. Herein, we propose to self-assemble biopolymers naturally charged onto the surface of

calcium carbonate particles as a sacrificial template<sup>30-33</sup>; its selective removal provides hollow particle also called capsules.

One of the concerns about the current antimicrobial material is related to their highly toxic and irritant action in humans. Implementing new routes for the production of cost-effective and safe biocide systems is thus of major interest. Biopolymers like polysaccharides are a promising solution, mainly because of their weak toxicity toward mammalian cells and their effective antibacterial properties<sup>34</sup>. Among such biopolymers, chitosan is a natural biopolymer that is both biocompatible and possesses antibacterial activity. The bactericidal mechanism of action of chitosan is mediated by (i) an electrostatic attraction between the negatively charged wall of the bacterial cell and polycationic chitosan, leading to the disruption of intracellular materials<sup>35</sup> and (ii) the inhibition of bacterial enzymatic activity<sup>36</sup>. The preparation of chitosan-based particles has been successfully reported for antibacterial purposes<sup>37-40</sup>. For instance, chitosan can be easily formed through self-aggregation<sup>41</sup>. Furthermore, chitosan tripolyphosphate nanoparticles have been reported to present antibacterial activity<sup>42</sup>.

Thus, In the present study, we then aimed to investigate the use of calcium carbonate microparticles for the development of versatile antimicrobial agents. To this end, we developed two parts using the calcium carbonate microparticles as a basic material to design antibacterial agents. To accomplish this aim, we first First, we investigated the possibility of to load antibiotics (presenting a wide range of solubility) within the space available in the pores of porous calcium carbonate. Second, we were interested in exploring the use of calcium carbonate as a template for the production of capsules presenting inherent antibacterial activity. This last approach is particularly interesting and little studied in the literature because here the objective is not to see an effect of particle charge or concentration but to compare solid particles (considered as rigid) to particles with an aqueous liquid cores and multilayer shell materials (considered as flexible). For these two parts approaches, particles were tested to evaluate their effects on the growth of model pathogenic bacteria, namely *Escherichia coli* and *Staphylococcus aureus*.

## **2. Experimental section**

### **2.1. Materials**

These following chemicals were purchased and used as received: chitosan (chi<sup>+</sup>, low molecular weight Sigma-aldrich), dextran sulfate (dex<sup>-</sup>, 40 000 g/mol, Sigma-aldrich),

ethylenediaminetetraacetic acid (EDTA), and calcium chloride (CaCl<sub>2</sub>, rechapur, 99%) and sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>, rechapur, 99%). Penicillin, ampicillin and ciprofloxacin are bought from Sigma-Aldrich.

## **2.2. Preparation of the porous calcium carbonate microparticles**

The calcium carbonate microparticles were fabricated by mixing equal volume of 0.33 M calcium chloride (CaCl<sub>2</sub>) and sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solutions under a vigorous stirring for 3 min. This results in a rapid precipitation of the CaCO<sub>3</sub> microparticles. They were centrifuged 10 min to remove all the residual ions. The particles were then rinsed several times with water and acetone to remove the water. Particles were dried at 80 °C. Pore size and distributions were recorded using the Brunauer, Emmett and Teller (BET).

## **2.3. Antibiotic-loaded hybrid particles.**

500 µL of a solution of antibiotic (penicillin or ampicillin or ciprofloxacin) at 1g/L were mixed to 500 µL of a suspension of calcium carbonate particles and stirred for 24 h. The finale concentration of the particle is 1g/L. The particles were then centrifuged and washed with water to remove free drug. The entrapment efficiency is estimated as follows (equation 1).

$$\text{entrapment efficiency} = \frac{\text{amount of entrapped probe}}{\text{initial amount of probe}} \times 100\% \quad \text{equation 1}$$

The antibiotic release rate studies were investigated by introducing each of loaded microparticles (10 mg) into a vial with 10 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4) with constant shaking (100 rpm) and at 37°C. The solution was replaced with fresh buffer solution and antibiotic concentration was determined by using a UV-visible spectrophotometer at the maximum absorbance.

## **2.4. Preparation of capsules by using calcium carbonate microparticles as template**

Surface modification of the negatively charged calcium carbonate microparticles was performed by the LbL process. First, to 1000 µL of the negatively charged calcium carbonate microparticle, 100 µL of a solution of chitosan (1 g.L<sup>-1</sup> prepared in 10 mM of buffer acetate, pH 4) was added and stirred during at least 15 min (1 g.L<sup>-1</sup>). Then, the particles were

centrifuged (5 min, 7 000 rpm) to remove the free polycation and 900  $\mu\text{L}$  of water was added. To this coated calcium microparticles, 100  $\mu\text{L}$  of a solution of dextran sulfate was added similarly (starting from a solution of  $1 \text{ g}\cdot\text{L}^{-1}$  and mixed at least 15 min). Free polyanion was removed further by implying via a centrifugation technique (5 min, 7 000 rpm). The assembly of a single bilayer is then written as: (chitosan/dextran sulfate)<sub>n</sub> (with n representing the number of bilayers). Zeta potential measurements enable to monitor the adsorption of each polyelectrolyte onto the calcium carbonate microparticles (Zetasizer Nano-ZS, Malvern Instruments, Brookheaven Instruments Corporation USA and Varian, Cary 100). Zeta potential measurement was performed on a Zetasizer Nano-ZS (Malvern Instrument) to monitor the adsorption of each polyelectrolyte onto the  $\text{CaCO}_3$  core (Zetasizer 4700 Malvern Instruments, Brookheaven Instruments Corporation, USA).

Dissolution of the calcium microparticle template enabled the elaboration of capsules. This was performed *via* addition of a solution of EDTA (0.2 M) to the coated calcium carbonate microparticles, whilst stirring at room temperature. A centrifugation process was applied to remove the complex.

Scanning Electron Microscopy (SEM) enables to study the shape of the materials (Leo Elektronenmikroskopie, Oberkoche in Germany). Prior to analysis, the particles were covered with palladium. Structural characterization was performed by X-ray diffraction (XRD) using a D8 advance Bruker diffractometer (Cu  $K\alpha$  radiation). Data were recorded over a  $2\theta$  range from  $5$  to  $90^\circ$  by step of  $0.02^\circ$  at an incident wavelength  $\lambda$  of  $1.54056 \text{ \AA}$ . The Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analyses ( $\text{Ca}^{2+}$  wt.%) were carried out with a ICP-OES spectrometer (Simultaneous Varian Vista Axial).

## **2.5. Assay for antibacterial activity:**

*Microbial cultures.* *Staphylococcus aureus* ATCC6538 (Gram-positive) and *Escherichia coli* ATCC25922 (Gram-negative) were used as the model bacteria. They were grown aerobically at  $37^\circ\text{C}$  overnight during 24h in Luria-Bertani lysogeny broth medium (LB, Sigma Aldrich). To estimate the inhibitory impact of the sample on heterotrophic bacterial growth, *Escherichia coli* and *Staphylococcus aureus* were grown under shaking in Luria lysogeny broth medium (LB) at  $37^\circ\text{C}$ . For the assay, eight parallel wells of a 96-well microplates received 180  $\mu\text{L}$  of bacterial suspension (eight replicates). 20  $\mu\text{L}$  (1 mg/mL) of the suspension of materials were added to each well and then incubated at  $37^\circ\text{C}$ . The final volume is 200  $\mu\text{L}$ . The initial optical density of the bacteria medium was 0.05 at 620 nm. A physiological

solution (9 g.L<sup>-1</sup> NaCl in distilled water) was used as reference. Bacterial growth was followed periodically by reading the optical density of bacterial suspensions at 620 nm using a UV-Visible UV-visible spectrophotometer from multiscan FC.

The percentage of inhibition was estimated using equation 2:

$$I (\%) : \text{percentage of inhibition } (I = 100 - ((OD_{\text{sample}} / OD_{\text{ref}}) \times 100)) \quad \text{equation 2}$$

OD<sub>ref</sub> and OD<sub>sample</sub> correspond to the optical density (OD) of the reference (suspension of bacteria containing water) and sample (suspension of bacteria containing a suspension of sample), respectively.

To confirm results obtained through the optical density measurement, bacteria were counted with a Mallassez Cell.

The studied samples are (i) calcium carbonate microparticles loaded with antibiotics (prepared in section 2.3) and (ii) coated CaCO<sub>3</sub> particles and capsules (prepared in section section 2.4).

Absorbance spectra measurements were recorded with a UV-Visible UV-visible spectrophotometer microplate reader (bacterial assay).

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

The effect of the particles on bacterial viability was assessed using an orange acridine test followed by fluorescence microscopy observation. 10 µL of acridine orange (0.1 % wt) was added to 100 µL of bacterial suspension taken at the end of the antibacterial assay (described in section 2.5). A 10 µL drop was then introduced on a cover slip to observe the bacteria.<sup>43</sup> Images were obtained with an optical fluorescence ZEISS microscope equipped with a CCD camera and a fluorescence illuminator and various mirror units (ZEN software).

## **2.7. Zeta -Potential Measurements**

Zeta potential measurement was performed on a Zetasizer Nano-ZS (Malvern Instrument) to monitor the adsorption of each polyelectrolytes onto the CaCO<sub>3</sub> core (Zetasizer 4700 Malvern Instruments, Brookheaven Instruments Corporation, USA).

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

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

## **2.9. X-ray diffraction (XRD)**



Structural characterization was performed by X-ray diffraction (XRD) using a D8 advance Bruker diffractometer (Cu K $\alpha$  radiation). Data were recorded over a  $2\theta$  range from 5 to 90° by step of 0.02° at an incident wavelength  $\lambda$  of 1.54056 Å.

### **2.10. Fluorescence microscope**

Images were obtained with an optical fluorescence ZEISS microscope equipped with a CCD camera and a fluorescence illuminator and various mirror units (ZEN software).

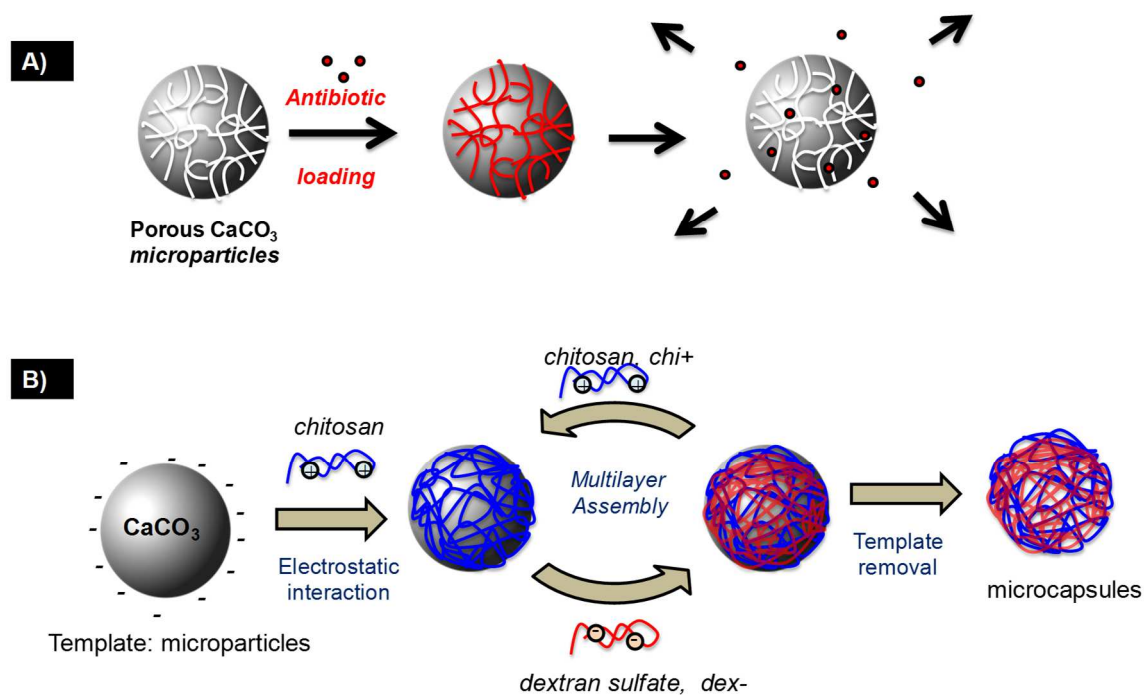
### **2.11. Absorbance measurement**

Absorbance were recorded with a UV-Visible spectrophotometer micro plate reader (bacterial assay).

## **3. Results and discussion**

As presented in Figure 1, our overall strategy relied on the use of calcium carbonate microparticles for antibiotics loading (strategy A, Figure 1) and as the template for the production of capsules presenting inherent antibacterial property (strategy B, Figure 2).

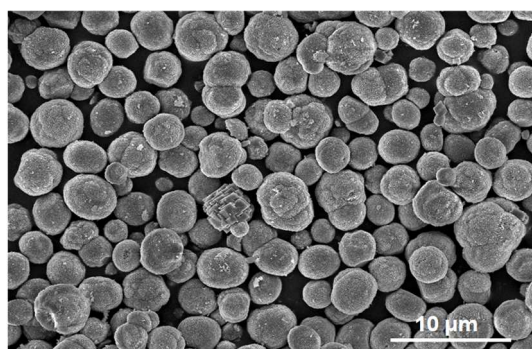
The antibacterial activity of the fabricated materials in strategies A and B were investigated on two environmental bacteria, namely *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) in liquid LB medium. *E. coli* is a Gram-negative bacterium and an important component of the normal intestinal microflora of humans, but it can be a highly versatile and frequently deadly pathogen. *S. aureus* is a Gram-positive bacterium and is virulent pathogen responsible for infections in hospitalised patients.



**Figure 1.** Schematic illustration of A) the antibiotic-loaded calcium carbonate microparticles and B) the fabrication of the capsules coated with polysaccharides using calcium carbonate microparticles as template.

### 3.1. Antibiotic loading inside the pores of calcium carbonate particles

Calcium carbonate microparticles were selected due to their non-toxic nature, low price and easy preparation. Herein, we prepared them via the direct mixing of soluble salts containing both of the ions, Ca<sup>2+</sup> and CO<sub>3</sub><sup>2-</sup>. This immediately led to the fabrication of CaCO<sub>3</sub> microparticles. SEM measurements (Figure 2) showed that such particles were uniform, homogeneously sized (around 4-6 μm average size 5±1 μm) and well-dispersed.



**Figure 2.** SEM images of calcium carbonate microparticles

Herein, the studying of the their porosity was a key point because we aimed at taking advantage of these pores for antibiotic loading. The Brunauer-Emmett-Teller (BET) method of nitrogen adsorption/desorption was used to estimate the surface area of the fabricated CaCO<sub>3</sub> microparticles and the effective pore size distribution. From these nitrogen adsorption-desorption measurements, the surface area was found to be above 9 m<sup>2</sup>.g<sup>-1</sup>, and the average pore size was 40 nm and the average pore volume was 0.2 cm<sup>3</sup>.g<sup>-1</sup>. These values are in accordance with the values determined by Sukhorukov *et al.* who reported on the production of CaCO<sub>3</sub> microparticles (35 nm for the average pore size) <sup>44</sup>. To demonstrate the high porosity of the produced microparticles, we then theoretically calculated the surface area of non-porous microparticles presenting the same diameter; we found a surface area value of 0.9 m<sup>2</sup>.g<sup>-1</sup>. Thus, the fabricated CaCO<sub>3</sub> microparticles were found to be highly porous, as the effective surface area was about 10 times greater than that of the compact particles (i.e. non-porous particles).

Penicillin, ampicillin and ciprofloxacin have a short active half-life in the human body. There is therefore a need to administer consecutive doses of antibiotic to cure patients. However, the use of excessive amounts of antibiotics may induce bacterial resistance and toxicity. Furthermore, one commonly encountered problem is solubility in aqueous media. The selected antibiotics present a broad range of solubility, i.e. highly soluble (solubility about 25 mg/ml in water for penicillin G sodium), slightly soluble (solubility about 13 mg/ml in water for ampicillin) and practically insoluble in water (solubility less than or equal to 1 mg/ml in water for ciprofloxacin). The vectorisation of antibiotics is therefore crucial to deliver them to the site of infection a controlled amount of drug to achieve a dose above the antibiotic's MIC (Minimum Inhibitory Concentration) over a prolonged period. Moreover, it is important to take into account antibiotic resistance in bacteria. In the search for more efficient antibacterial treatments, we evaluated the use of biodegradable CaCO<sub>3</sub> microparticles as they are highly porous for the loading and subsequent release of selected antibiotics. <sup>44</sup>

Loading was accomplished by soaking the calcium carbonate material into antibiotic solutions. These molecules have an average size of above 3-4 nm; thus, they can easily permeate into the internal volume of CaCO<sub>3</sub> microparticles through the pores (40 nm). This is supported by the fact that both the pore volume value and the surface area decreased, i.e. 0.15 cm<sup>3</sup>.g<sup>-1</sup> and 5 m<sup>2</sup>.g<sup>-1</sup>, respectively. Images of the particle obtained via SEM revealed the unaltered spherical shape of the particles and uniformly distributed. The size of bare particles was found to be the same as that of antibiotic-loaded particles. This result is in agreement

with the work of Mu *et al.*, who showed that loading of a drug into microparticles does not affect the size of the particle <sup>45</sup>.

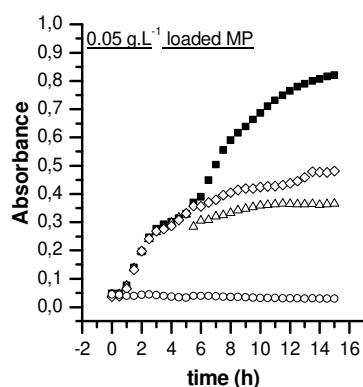
The percentage loading content (LC) was found to be above 5% and up to 95% for the encapsulation efficiency (EE). Our results indicated that the fabricated calcium carbonate microparticles provided high drug loading and entrapment efficiency for the studied antibiotics. This result is in accordance with those of Isa. *et al.* <sup>46</sup> who loaded ciprofloxacin inside calcium particles (LC 5.9% and EE was 99.5%). The loading process enables minimal loss of the antibiotic during the loading process inside the pores of the calcium carbonate particles. This finding is also in accordance with the results reported in the literature <sup>47-48</sup>.

The growth curves of both *S. aureus* and *E. coli* were determined based on bacterial cell optical density (OD). It is worth noting that bacterial growth in the presence of the bare calcium carbonate microparticles was normal, indicating the non-impact of such particles on bacterial growth. However, bacterial growth was strongly impacted in the presence of calcium carbonate microparticles loaded with antibiotics. Figure 3 shows the bacterial growth response (OD) according to exposure to the antibiotic-loaded materials over time. For the particles loaded with penicillin or ampicillin, bacterial growth was inhibited within two hours and bacterial growth was immediately prevented when using loaded particles with ciprofloxacin. An initial burst release of the antibiotic from the microparticles was observed within 2 h. The observed burst release could be correlated to the fact the pores fill with the medium, leading to this initial surge. After that, the subsequent readings showed no significant change in the optical density of the bacteria throughout the rest of the assay, which was performed for a total of 15 h. After antibiotic release from the surface, it reached an equilibrium; the antibiotics stayed trapped in the pores of the particles and were then slowly released over time. Increasing the concentration of the antibiotic loaded nanoparticles (Figure 3 A to C or Figure 3 D to E) led to a faster effect and more efficient inhibition of bacterial growth. Indeed, at the end of 14h, the absorbance decreases with the increase of the concentration of the loaded particles. The antibiotics were released at a sufficient amount for inhibiting the growth of both *S. aureus* and *E. coli*. For all the studied concentrations of loaded microparticles, using ciprofloxacin led to the direct inhibition of bacterial growth. This is a very interesting point because ciprofloxacin is almost insoluble in water ( $\leq 1 \text{ mg.mL}^{-1}$ ) and bacterial resistance to this antibiotic has emerged <sup>49</sup>. Indeed, Fantin *et al.* reported that a higher dose of this fluoroquinolone has a better bactericidal effect. However, increasing the

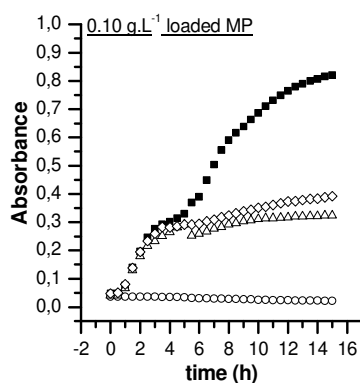
concentration of this antibiotic was found to induce resistance to ciprofloxacin in human commensal bacteria <sup>49</sup>.

The MICs of penicillin and ampicillin are reported to be above 1-32  $\mu\text{g/ml}$  against *S. aureus* and *E. coli* <sup>50</sup>. As for the MIC value of ciprofloxacin, it is reported to be above 0.15-4  $\mu\text{g/ml}$  against *S. aureus* and *E. coli* <sup>50-51</sup>. Thus, at least 2 h was required to release the necessary amount of ampicillin and penicillin to reach their MIC values; however, the required concentration was directly released when using ciprofloxacin, which is effective at a lower concentration (lower MIC against these bacteria compared to penicillin and ampicillin). This result is comfort by investigating the release of these antibiotics. As seen from Figure 4, the amount of released ciprofloxacin is immediately near the MIC value of ciprofloxacin, i.e., 2  $\mu\text{g/ml}$  is release within 10 minutes, whereas it takes at least 2 hours to release the necessary concentration of penicillin and ampicillin to reach the MICs of penicillin and ampicillin (above 30  $\mu\text{g/ml}$  in 2 hours). This enabled the inhibition of bacterial growth. Our study also indicates that the present system continues to deliver antibiotics for an extended period of time (i.e. 15 h).

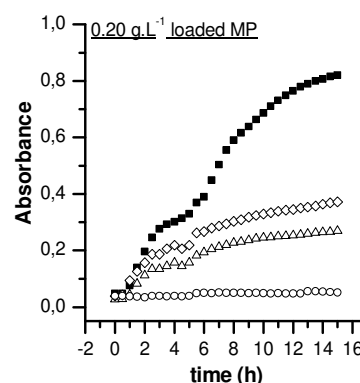
A) *E.coli*

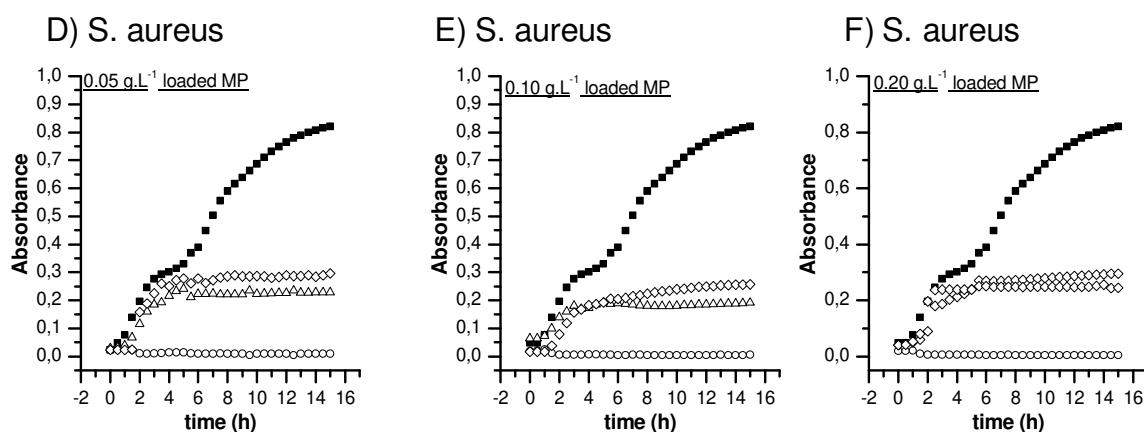


B) *E.coli*

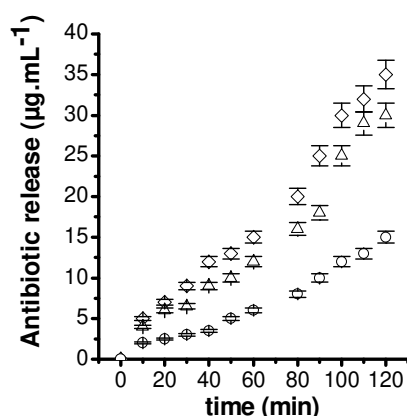


C) *E.coli*





**Figure 3.** Growth study of *E. coli* (A to C) and *S. aureus* (D to E) in LB medium inoculated with bacteria in the presence of CaCO<sub>3</sub> particles loaded with antibiotics (■ bare calcium carbonate, ◇ ampicillin, Δ penicillin G and ○ ciprofloxacin).



**Figure 4.** Release profile of ◇ ampicillin, Δ penicillin G and ○ ciprofloxacin in phosphate-buffered saline

Ciprofloxacin is a member of the antimicrobial class of fluoroquinolones. They are DNA-targeting agents, and interact with their target type II topoisomerases to increase the generation of single- and double-strand DNA breaks associated with stalled or collapsed replication forks<sup>52-53</sup> Penicillin and ampicillin inhibit the transpeptidase catalysing the final step in cell wall biosynthesis and the cross-linking of peptidoglycan<sup>54</sup>. The CaCO<sub>3</sub> microparticles loaded with these antibiotics may first interact with bacterial surfaces. Then, the antibiotic is released slowly, which leads to bacterial cell wall damage such that each of the above discussed bactericidal mechanisms can occur. This phenomenon enhances the

permeability of cell membranes by antibiotics, preventing bacterial growth and even leading to the death of bacteria.

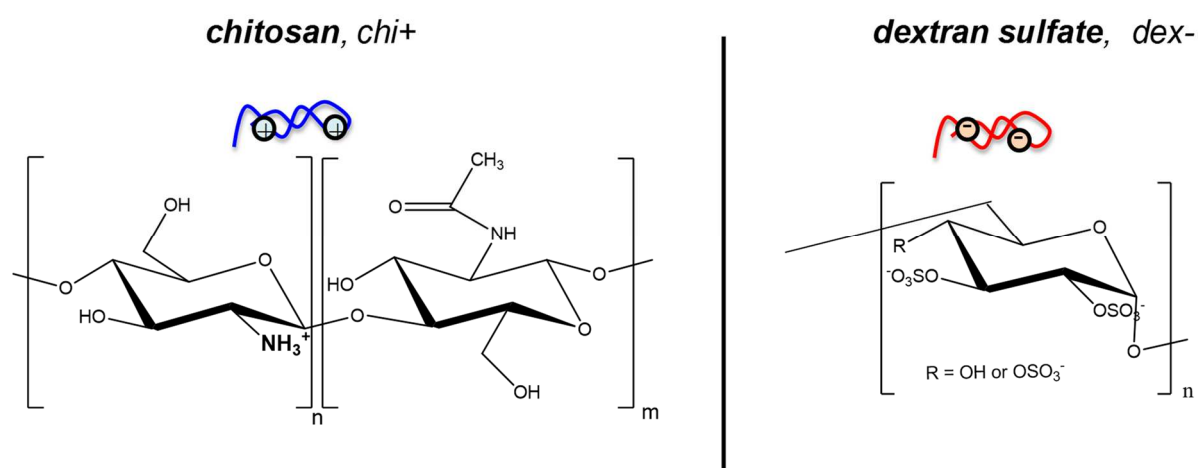
In this first part of our study, we clearly evidence the interest of using the porous calcium carbonate particles for the antibiotic loading presenting a wide range of solubility. In a second part, we will investigate the interest of using calcium carbonate particles as template to prepare capsule with well controlled size and surface chemistry. More specifically, the aim is to study the possibility to use the capsule as antibacterial agent by itself without any antibiotic loading.

### **3.2. Design of antibacterial particles based on calcium carbonate particles as the template**

One of the concerns about the current antimicrobial material is related to their highly toxic and irritant action in humans. Implementing new routes for the production of cost-effective and safe biocide systems is thus of major interest. Biopolymers like polysaccharides are a promising solution, mainly because of their weak toxicity toward mammalian cells and their effective antibacterial properties<sup>34</sup> Among such biopolymers, chitosan is a natural biopolymer that is both biocompatible and possesses antibacterial activity. The bactericidal mechanism of action of chitosan is mediated by (i) an electrostatic attraction between the negatively charged wall of the bacterial cell and polycationic chitosan, leading to the disruption of intracellular materials<sup>35</sup> and (ii) the inhibition of bacterial enzymatic activity<sup>36</sup>. The preparation of chitosan-based particles has been successfully reported for antibacterial purposes<sup>37-40</sup>. For instance, chitosan can be easily formed through self-aggregation<sup>41</sup>. Furthermore, chitosan tripolyphosphate nanoparticles have been reported to present antibacterial activity<sup>42</sup>

In this part of the study, the aim was to use calcium carbonate microparticles as a sacrificial template to develop a new antibacterial carrier. For this, charged polysaccharides (chitosan and a dextran derivative) were used for multilayer coatings on the surface of CaCO<sub>3</sub> microparticles (Figures 1 B and 4 5). In comparison to other synthetic routes, this approach is attractive because only water is used as the solvent in all steps, instead of organic solvents which are often toxic. Calcium carbonate microparticles allow for controlling the final size of the materials, and after coating is removed without affecting the multilayer shell, leading to the formation of a capsule. The antibacterial activity of these biocapsules was studied against

pathogenic bacteria (*E. coli* and *S. aureus*). In this study, we are interested in using capsules instead of self aggregation particles made of polysaccharides because i) the used process (i.e. templating method and self assembly process described in Figure 1B) enables to easily control the size of the resulting particles and the surface charge of particle surface and ii) the aim is to show if the capsule can act as an antibacterial-agent by itself without any addition of antibiotic. We want to evidence that as the capsules are supposed to be more flexible than the coated calcium carbonate particle, that they may interact more easily with bacteria walls and then disturb/inhibit their growth.

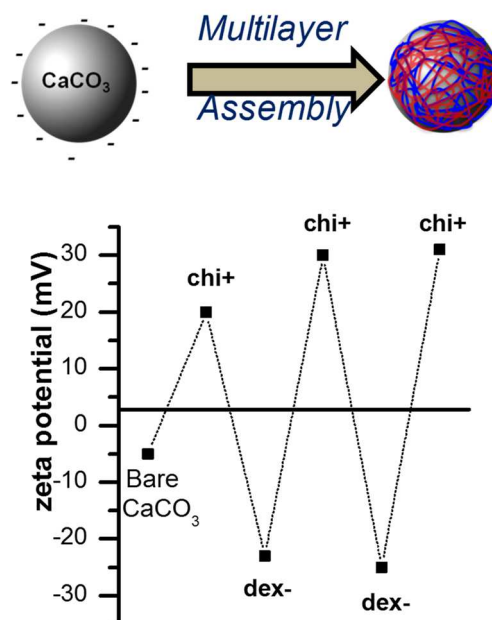


**Figure 4 5.** Polyelectrolytes implied used for the coating of the calcium carbonate microparticles, i.e. the chitosan and the dextran sulfate Chemical structure of the chitosan and the dextran sulfate.

The zeta potential of the microparticles was measured after the deposition of each polyelectrolyte layer on  $\text{CaCO}_3$  microparticle cores (Figure 5 6). As expected, the zeta potential of the bare calcium carbonate microparticles indicated a negatively charged surface ( $\sim -5$  mV). The addition of a chitosan layer led to a positive value (+30 mV), followed by a negatively charged surface after the deposition of the dextran sulfate layer (-25 mV), which could be correlated to complete charge reversal. Alternating zeta-potential values were observed, which is an excellent indicator of multilayer film growth on the surface of  $\text{CaCO}_3$  particles. This result suggests that multilayers were formed on the particles via electrostatic adsorption as the main driving force<sup>55</sup>. Our results are in accordance with those of Tripathy *et al.* who reported the adsorption of sodium carboxymethyl cellulose and poly(allylamine

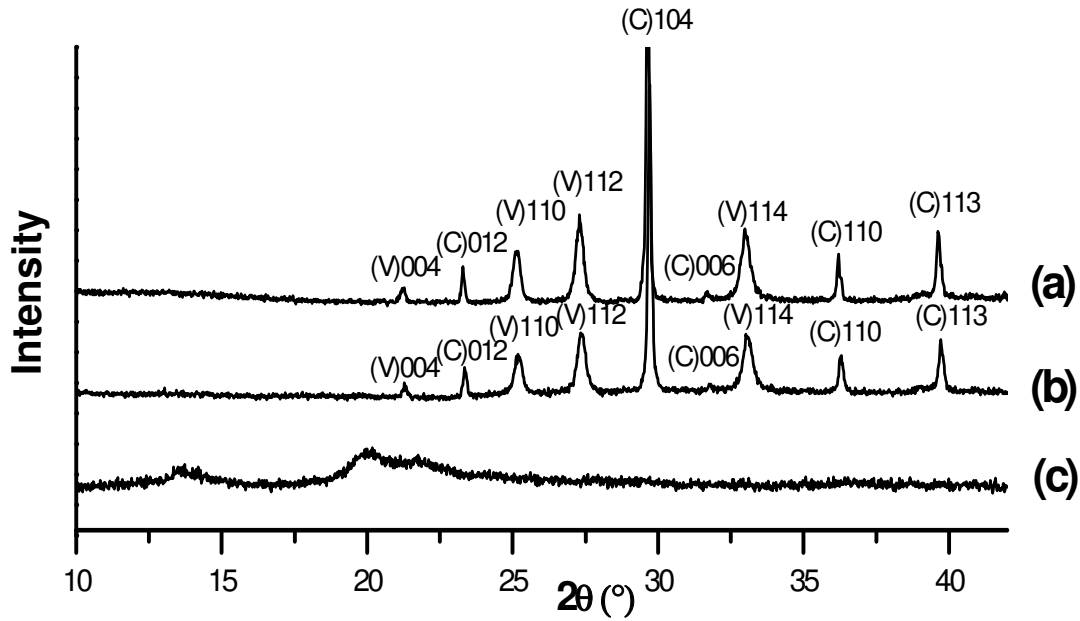


hydrochloride) on calcium carbonate microparticles <sup>56</sup> and those of Belbekhouche *et al.* who showed the adsorption of cationic-polycyclodextrin and alginate on CaCO<sub>3</sub> particles <sup>57</sup>.



**Figure 5.6.** Zeta potential variation during the coating of the calcium carbonate microparticle by chitosan and dextran sulfate polyelectrolyte.

X-ray powder diffraction (XRD) analysis was performed to monitor the calcium carbonate microparticle removal (Figure 6 7a). The diffraction patterns of CaCO<sub>3</sub> microparticles MPs showed characteristic peaks, indicating the crystallinity of the synthesised calcium carbonate microparticles. A dominant phase of vaterite was observed evidenced, although a trace of calcite was also seen. The broad diffraction peaks indicates that the fabricated vaterite microparticles were composed of small nanoparticles. Calcite and vaterite were the principal crystalline polymorphs corresponding respectively to *hkl*: 012, 104, 006, 110, 113 and 004, 110, 112, 114.



**Figure 6 7.** XRD pattern of (a) pristine  $\text{CaCO}_3$  microparticle, (b) modified  $\text{CaCO}_3$  microparticle  $(\text{chi}^+/\text{dex-SO}_3^-)_n$  and (c) capsule  $(\text{chi}^+/\text{dex-SO}_3^-)_n$ . Note that C and V correspond to calcite and vaterite, respectively.

From Rao's equation (equation 3)<sup>58</sup>, which defines the relative fraction of vaterite ( $f_v$ ) and the XRD pattern, we can report that the surface modification of  $\text{CaCO}_3$  did not have an impact on the composition or crystalline structure (Table 1).

$$f_v = \frac{(I_{110V} + I_{112V} + I_{114V})}{(I_{110V} + I_{112V} + I_{114V} + I_{104C})} \quad \text{equation 3}$$

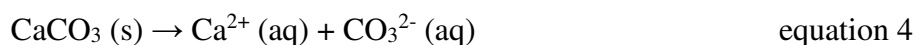
**Table 1:** Relative fraction of vaterite and calcite

	$f_v^*$	$f_c^{**}$
pristine $\text{CaCO}_3$ microparticle	0.33	0.67
modified $\text{CaCO}_3$ microparticle $(\text{chi}^+/\text{dex-SO}_3^-)_n$	0.33	0.67

\*  $f_v$ : fraction of vaterite; \*\*  $f_c$ : fraction of calcite

For the modified  $\text{CaCO}_3$  microparticles, as expected, the peaks correlated to the  $\text{CaCO}_3$  structure were still present (Figure 6a compared to Figure 6b)<sup>35</sup>.

To fabricate hollow microparticles, the CaCO<sub>3</sub> template was removed after a selected number of layers using EDTA<sup>59</sup>. The dissolution process of the particle could be described as follows (equations 4 and 5):



X-ray powder diffraction studies were performed on the capsules after drying to investigate the extent of core dissolution. Upon comparison of the X-ray powder diffraction results of CaCO<sub>3</sub> microparticles and the hollow microparticles (Figure 6b compared to Figure 6c), the crystalline polymorphs had completely disappeared, leaving only the polysaccharides. It can conclude that there was no peak due to the CaCO<sub>3</sub> core, indicating complete removal. Moreover, no presence of calcium was detected by ICP analysis in such suspension; which strongly comforts the calcium carbonate core removal. We confirm the capsule formation by SEM measurement (Figure S1A) and that capsules in suspension kept a spherical shape by confocal microscopy by using rhodamine-labelled chitosan during the multilayer build-up (Figure S1B).

An antibacterial assay was performed on the obtained coated particles and capsules. The kinetics of bacterial growth was monitored in LB (Luria-Bertani lysogeny broth) medium via the variation in optical density (OD) versus time. The influence of the presence or absence of the core was studied. Each assay was replicated eight times. It is worth noting that no inhibition was seen with bare calcium microparticles or with physiological solution under these experimental conditions.

For *E. coli* (Table 2), the following OD values were measured at the end of the study at the end of the bacterial assay: 0.55, 0.45, 0.40 and 0.30 for the reference and calcium carbonate microparticles coated with (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> (n = 1, 2 and 3), respectively. For microcapsules, the OD values were as follows: 0.33, 0.28 and 0.06, still according to the number of layers. Interestingly, the same behaviour was noted for *S. aureus* (Table 2); the measured OD values were 0.43, 0.36, 0.32 and 0.22 for the reference and calcium carbonate microparticles coated with (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> (n = 1, 2 and 3), respectively. For microcapsules based on (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> (n = 1, 2 and 3), the OD values were 0.25, 0.18 and 0.03. The percentage of inhibition was calculated for each bacterium. Thus, for *Escherichia coli*, this parameter varied from 18.2% to 45.5% for the calcium carbonate nanoparticles coated with (chi<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> and

from 40% to 89.1% for  $(\text{chi}^+/\text{dex}^-)_n\text{-chi}^+$  microcapsules ( $n$  increasing from 1 to 3). For the coated particles (supposed to be more rigid particles than the capsule), we observed same tendency as Prodana *et al.*, who studied the functionalisation of carbon nanotubes by introducing COOH groups. They observed an increase in the percentage inhibition of *E. coli* growth from 19% to 34%<sup>60</sup>. Adams *et al.* showed 38% growth inhibition in these bacteria in presence of oxide nanoparticles<sup>61</sup>. A study with *S. aureus* led to the same observation, i.e. this parameter was enhanced from 16.3% to 48.8% for calcium carbonate microparticles coated with  $(\text{chi}^+/\text{dex}^-)_n\text{-chi}^+$  and from 41.9% to 93.0% for  $(\text{chi}^+/\text{dex}^-)_n\text{-chi}^+$  nanocapsules ( $n$  increasing from 1 to 3). Namasivayam *et al.* obtained inhibition close to that obtained in the present study using coated silver nanoparticles (50-60 nm)<sup>61</sup>. Interestingly, the number of bacteria in suspension was confirmed by counting bacteria with a Mallassez Cell. Compared to some systems reported in the literature, the systems studied here were found to be more efficient.

Comparing the two strains, quite similar effects were shown, i.e. a significant inhibitory effect on the growth of both bacteria with an increase in the number of layers and the removal of calcium carbonate. For the same conditions of layer-by-layer assembly (number of layers and concentration), capsules presented remarkable inhibitory effects compared to the calcium carbonate coated particles. This could only be related to the rigidity of the particles. Stiff particles (capsules) seem to impact bacterial growth more than rigid ones. The last layer of the particle was chitosan. The antibacterial activity of chitosan can be correlated directly with the characteristics of bacterial cell walls<sup>37</sup>.

For the two bacteria, a significant inhibitory effect on their growth was observed with an increase in the number of the polyelectrolyte layers and the removal of calcium carbonate (formation of capsule). For the same conditions of layer-by-layer assembly (number of layers and concentration), capsules show a remarkable inhibitory effects compared to the calcium carbonate coated particles. We assume that this is due to the fact that the capsules are more flexible than the coated particles which may interact more efficiently/easily with the bacterial cell walls<sup>37</sup> and then inhibit their growth. According to previous work<sup>38-39</sup>, the antibacterial activity of chitosan could be related to its polycationic form. Positively charged chitosan can interact with the bacterial cell wall, which is negatively charged. This involves the disruption of the normal functions of the bacterial wall, for instance by inhibiting the transport of nutrients into cells or by promoting the leakage of intracellular components<sup>40, 62-63</sup>.

**Table 2.** Inhibitions of *E. coli* (EC) and *S. aureus* (SA) by coated CaCO<sub>3</sub> microparticles and microcapsules

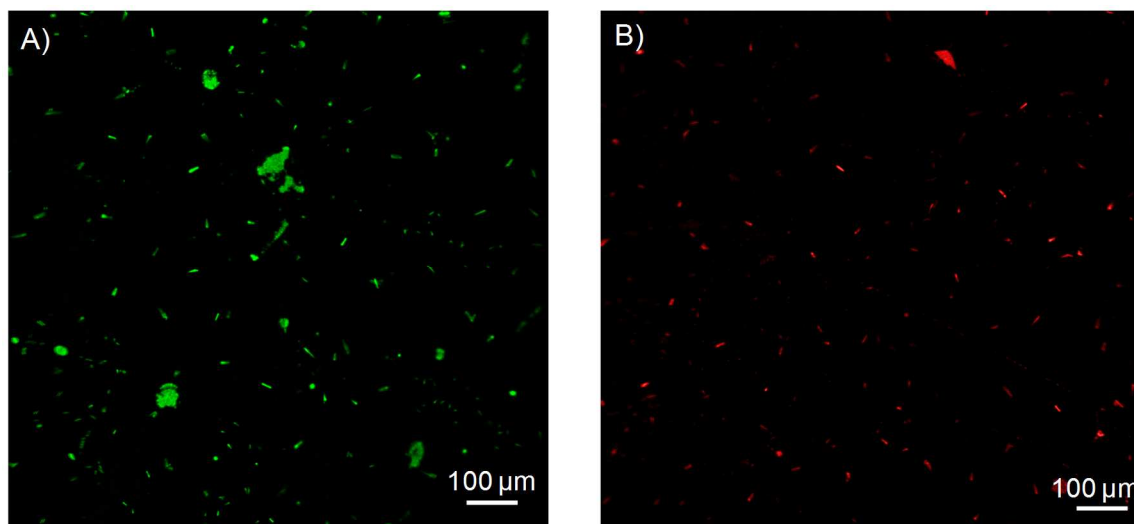
EC n value in the multilayer (chi <sup>+</sup> /dex <sup>-</sup> ) <sub>n</sub> -chi <sup>+</sup>	CaCO <sub>3</sub> coated particle			Microcapsules		
	OD	Bacteria per mL	I (%)	OD	Bacteria per ml	I (%)
0	0.55	4.40.10 <sup>8</sup>	0	0.55	4.40 10 <sup>8</sup>	0
1	0.45	3.6 10 <sup>8</sup>	18.2	0.33	2.6 10 <sup>8</sup>	40.0
2	0.4	3.2 10 <sup>8</sup>	27.3	0.28	2.2 10 <sup>8</sup>	49.1
3	0.3	2.4 10 <sup>8</sup>	45.5	0.06	4.8 10 <sup>7</sup>	89.1

SA n value in the multilayer (chi <sup>+</sup> /dex <sup>-</sup> ) <sub>n</sub> -chi <sup>+</sup>	CaCO <sub>3</sub> coated particle			Microcapsules		
	OD	Bacteria per mL	I (%)	OD	Bacteria per ml	I (%)
0	0.43	3.40 10 <sup>8</sup>	0	0.43	3.4 10 <sup>8</sup>	0
1	0.36	2.8 10 <sup>8</sup>	16.3	0.25	2.0 10 <sup>8</sup>	41.9
2	0.32	2.5 10 <sup>8</sup>	25.6	0.18	1.4 10 <sup>8</sup>	58.1
3	0.22	1.7 10 <sup>8</sup>	48.8	0.03	2.4 10 <sup>7</sup>	93.0

Note that for enterobacteria like *E. Coli* and *S. Aureus*, 1 OD at 620 nm is equivalent to 8 x 10<sup>8</sup> CFU/mL <sup>64</sup>

After having showing After observing the impact on bacterial growth, bacteria viability was assessed. For this purpose, acridine orange (AO) was used. This is a cell-permeant nucleic acid binding dye that can differentiate between double-stranded and single-stranded nucleic acids. Bound to denatured single stranded DNA, AO emits red fluorescence, highlighting dead of bacteria. In contrast, when bound to double stranded DNA, AO emits green fluorescence, showing that the bacteria are alive. Herein, at the end of the experiment, bacteria were stained with AO to assess bacterial death <sup>65</sup>. As seen in Figure 7, red fluorescence was observed after staining bacteria, showing the death of bacterial cells as a result of exposure to the capsules. Note that green fluorescence was seen in *E. coli* without capsules (negative control, Figure 7 8), pointing out that the bacteria were alive in the absence of capsules or coated calcium carbonate microparticles.



**Figure 7 8.** *E. coli* stained with acridine orange after the antibacterial assay, A) without capsules (negative control) and B) in contact with the capsules (magnification: x200)

In the literature, interactions between materials and bacteria have been found to depend upon multiple factors such as electrostatic attraction, as well as hydrophobic and Van der Waals forces. Positively charged particles are known to favourably interact with the negatively-charged bacterial surface, leading in most cases to a bactericidal effect. Below its pKa (below 7), chitosan is polycationic and acts as an antimicrobial agent. The antibacterial mechanism is recognised to be due to its adsorption onto the surface of bacteria, involving aggregation and the leakage of bacterial intracellular contents. Another mechanism could also involve alterations in cell permeability. This leads to chitosan binding with bacterial DNA, which is a

polyanion, as well as the chelation of trace metals, which interferes with both the production of virulence factors and bacterial growth.

#### 4. Conclusion

Herein, we showed that calcium microcarbonate microparticles are good candidates for developing tailor-made antibacterial systems with controlled antibiotic release loaded with antibiotic (penicillin, ampicillin or ciprofloxacin). Furthermore, our approach is simple and able to tackle the problems of poor solubility in aqueous media and the resistance of bacteria against antibiotics. The percentage loading content was found to be above 5 % and the encapsulation efficiency was up to 95%. These particles could be stably loaded with a sufficient amount of antibiotics to prevent the growth of *E. coli* and *S. aureus*.

We also propose a new approach concerning the development of particles presenting inherent antibacterial properties. The particles were synthesized via an easy and robust templating method based on the capacity of cationic-chitosan to be assembled layer-by-layer with anionic-dextran sulfate onto calcium carbonated used as sacrificial particles. The resulting charge-charge interaction was followed at each step in aqueous solution by zeta potential measurements. Capsules were obtained through the in-situ etching of the calcium carbonate core into aqueous EDTA solution evidenced by X-ray diffraction measurement. According to the rigidity of these particles, they have been evidenced to prevent the growth of pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*). Moving forwards, we plan to start from the elaborated materials (rigid and stiff particles) and study how the mechanism of bacteria is impacted (mainly by proteomic assay). Membrane dysfunction and denaturation of cellular proteins are the two mechanisms of bacterial inhibition which may be implied. This behaviour may be facilitated with the use of “flexible” capsules rather than “rigid” one, explaining the greater inhibition seen for capsules than coated particles. We prospect to do both biochemical analysis and electron microscopy to determine the dominant inhibition mechanism of the bacteria.

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## Graphical Abstract

