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**Multilayer Dextran Derivative Based Capsules Fighting Bacteria Resistant to Antibiotic:  
Case of Kanamycin-Resistant *Escherichia coli***

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**Abstract.**

Bacteria resistance to antibiotics has emerged as a major health problem. Developing new antibacterial systems is then of major interest. In this sense, we present biocapsules presenting inherent antibacterial capacity. The self-assembly of charged biopolymer, namely diethylaminoethyl-dextran hydrochloride ( $\text{dex}^+$ ) and dextran sulfate ( $\text{dex}^-$ ), were done on calcium carbonate microparticles, used as a template. Zeta potential measurements have shown the successful alternate adsorption of these biopolymers and related charge reversal upon the multilayer film construction onto the particles surface. The shape of the capsules was characterized by scanning electron microscopy (SEM). These particles were tested against bacteria resistant to antibiotics, namely kanamycin-resistant *Escherichia coli*. An inhibitory effect of the particles was observed during bacterial growth in liquid medium, i.e. in the range of 10 % for  $(\text{dex}^+/\text{dex}^-)_n$  coated  $\text{CaCO}_3$  materials and of 50% for  $(\text{dex}^+/\text{dex}^-)_n$  capsules. These findings evidence the high potential of capsules to act as antimicrobial agents in future and in treatments against infections.

**Keywords:** calcium carbonate-based particles; capsule; bacteria resistant to antibiotic, antibacterial agent; polysaccharide

## 1. Introduction

Microorganisms have a high impact on human beings.<sup>1</sup> For instance, microorganism contamination involves severe problems in various industries (pharmaceutical, food...) where they contaminate products, raw materials, and equipment.<sup>2</sup> Antimicrobial agents can be considered as materials that kill or simply prevent the growth of microorganisms. Various mechanisms of microorganism' inhibition have been reported in the literature. They are mainly related to the chemical composition and structure (organic, inorganic, charged...) of the antimicrobials, each presenting benefits and limitations.<sup>3</sup>

Antibacterial resistance is a growing issue affecting the entire world<sup>4</sup>. Intracellular bacterial infections and resistance in pathogenic microbes are a challenging problem in human medicine<sup>5</sup>. To fight these infections, antibiotics are required, but due to their poor solubility and/or stability in biological environment, their vectorization is often required<sup>6-7</sup> to deliver the therapeutic agents at adequate concentration within the intracellular medium and to overcome antibacterial resistance<sup>8-9</sup>. Antibacterial carriers have been evidenced to avoid/prevent antibiotic efflux and increase the intracellular retention of the antibiotic. Several studies have reported on the increased antibacterial activity of antibiotic-conjugated particles<sup>10-12</sup>.

As an alternative to antibiotics, some charged polymers being inherently bactericidal exist, like phosphonium or quaternary ammonium groups. These polycations are able to interact with the negatively-charged bacterial cell wall, which compromises the integrity of the bacterial cell wall.<sup>13</sup> For developing materials with antibacterial activity, these polymers have then advantageously been used in self-assembly processes (e.g. the layer-by-layer,<sup>14-17</sup> (LbL) approach) through the alternating deposition of the oppositely charged polymer on substrate as charged particles. Particles present the interest to offer an appealing alternative to antibiotic as an entirely different mechanism of antibacterial activity is implied compared to antibiotics. In the present work, we focused on derived polyelectrolytes from dextran. Polycations present generally beneficial physiological effects: antibacterial, antifungal, and antitumor.<sup>18</sup> Diethylaminoethyl dextran ( $\text{dex}^+$ ) has been used as the polycation in the polyelectrolyte film, as it presents an ammonium group (amine groups have a positive charge) in physiological pH. This polysaccharide is recognized for being harmless to the plasma membranes of cells at low concentrations.<sup>18</sup> Dextran sulfate ( $\text{dex}^-$ ) has been selected as the polyanion. This polyanion presents antithrombotic<sup>19</sup> and anticoagulant properties<sup>20</sup>. We have proposed to self-assemble these biopolymers, the diethylaminoethyl-dextran hydrochloride ( $\text{dex}^+$ ) as polycation and

dextran sulfate (dex<sup>-</sup>) as polyanion, onto the surface of charged particles (calcium carbonate particles) as a sacrificial template.<sup>21-24</sup> Its selective removal enables us to obtain hollow particles, also called capsules. One of the issues of the current antimicrobial material is its highly toxic and irritant action in humans. Developing new approaches for the fabrication of cost-effective and safe biocide systems is thus of major interest. This is the reason why we propose to exploit polysaccharides, mainly because of their effective antibacterial properties and their weak toxicity toward mammalian cells<sup>25</sup>.

Highly-resistant Gram-negative bacteria have become very difficult to treat pathogens worldwide.<sup>26</sup> Among them, we can cite *Pseudomonas aeruginosa*, *Acinetobacter*, *Klebsiella*, and *Escherichia* species.<sup>27</sup> Gram-positive bacteria can also be included, e.g. methicillin-resistant *Staphylococcus aureus*.<sup>28</sup> In this context, there has been a boost in the work aimed at developing new antibiotic analogs and identifying new antibacterial therapeutics and strategies.<sup>29</sup> Different approaches have then been reported for the use of antibiotic sensitizers<sup>30</sup> or antibiotic-metallic micronutrient combinatorial treatments<sup>31</sup>.

In this paper, we aim at investigating the impact of coated particles and capsules on the growth of bacteria resistant to antibiotics, namely kanamycin –resistant *Escherichia coli*. Kanamycin A, often simply referred to kanamycin, is an antibiotic used for treating severe bacterial infections and tuberculosis. This antibiotic belongs to the aminoglycoside family. Its antibacterial mechanism is due to its binding to the bacterial ribosome and its blocking effect on the synthesis of bacterial proteins required for bacterial survival.<sup>32</sup> Kanamycin-resistant bacteria have merged. This is due to phosphorylation of kanamycin, which has been identified as the main mechanism involved<sup>33</sup> or due to several adenylating and acetylating enzymes conferring a resistance to kanamycin<sup>34</sup>.

Here, the proposed approach is new because the aim is not to study an effect of particle charge or concentration but to compare the impact of coated particles to particles with aqueous liquid cores and multilayer shell materials on the kanamycin-resistant *Escherichia coli*.

## **2. Experimental section**

### **2.1. Materials**

These following chemicals were used as received: dextran sulfate (dex<sup>-</sup>, 40 000 g/mol, Sigma-aldrich), diethylaminoethyl-dextran hydrochloride (dex+, 40 000 g/mol, Sigma-

aldrich), and calcium chloride ( $\text{CaCl}_2$ , rechapur, 99%), sodium carbonate ( $\text{Na}_2\text{CO}_3$ , rechapur, 99%) and ethylenediaminetetraacetic acid (EDTA).

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

The calcium carbonate microparticles were fabricated by mixing equal volume of calcium chloride ( $\text{CaCl}_2$ ) and sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solutions under a vigorous stirring for 30 min, resulting in a rapid precipitation (formation of  $\text{CaCO}_3$  microparticles). All the residual ions were removed by centrifugation process (10 min, 7000 rpm). The particles were washed three times with water and then were dried at 50 °C.

## **2.3. Fabrication of capsules using calcium carbonate microparticles as template**

Surface modification of the calcium carbonate microparticles was done by the LbL process as these particles present negatively charged surface. 10 mg of calcium carbonate microparticles were suspended in 1 mL of water then was added under stirring of a solution of the polycation namely the diethylaminoethyl-dextran ( $1 \text{ g.L}^{-1}$  prepared in NaCl 0.15 M). After 15 min, the particles were centrifuged (5 min, 7 000 rpm) for removing the unadsorbed polycation and washed twice with NaCl 0.15 M. To these positively-charged coated calcium microparticles, 1000  $\mu\text{L}$  of a solution of the polyanion namely the dextran sulfate was added similarly ( $1 \text{ g.L}^{-1}$  NaCl 0.15 M and mixed 15 min) and free polyanion was removed by centrifugation technique (5 min, 7 000 rpm). The above process described the assembly of a single bilayer written:  $(\text{dex}^+/\text{dex}^-)_n$  (with n representing the number of bilayers). Zeta potential measurement was done via a Zetasizer Nano-ZS (Malvern Instrument) to follow the adsorption of each polyelectrolyte onto the  $\text{CaCO}_3$  microparticle (Zetasizer 4700 Malvern Instruments, Brookheaven Instruments Corporation, USA).

A solution of EDTA (0.2 M) was used to dissolve the calcium microparticles template leading to the formation of capsules. A centrifugation process was used for removing the complex.

Scanning Electron Microscopy (SEM) was employed to investigate the shape of the particles materials (Leo Elektronenmikroskopie, Oberkoche in Germany). Prior to analysis, the particles were covered with palladium/carbon. 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.

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

*Microbial cultures.* Kanamycin-resistant *Escherichia coli* were grown aerobically at 37°C during 24h in liquid medium. For the assay, 500 µL of bacterial suspension was added to 5 mL of the culture medium then 100 µL of the suspension of particle (1 mg/mL) were added (incubated under agitation at 37°C). As reference, a suspension of bacteria without particles was used. Bacterial growth was followed every hour by reading the optical density of bacterial suspensions at 630 nm using a UV-visible spectrophotometer.

The percentage of inhibition was estimated using equation 1:

$$I (\%) : \text{percentage of inhibition } (I = 100 - ((OD_{\text{sample}} / OD_{\text{ref}}) \times 100)) \quad \text{equation 1}$$

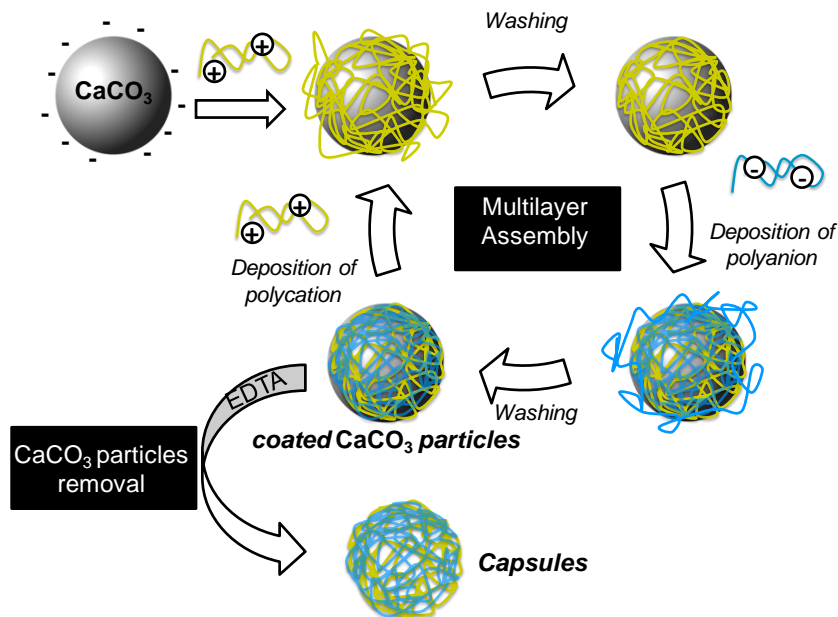
OD<sub>ref</sub> and OD<sub>sample</sub> correspond to the optical density (OD) of the reference (suspension of bacteria) and sample (suspension of bacteria containing a suspension of sample), respectively.

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

The effect of the particles on bacterial viability was assessed using trypan blue followed by optical microscopy observation. Trypan blue was added to bacterial suspension taken at the end of the antibacterial assay (described in section 2.4). A drop was then introduced on a cover slip to observe the bacteria. Images were obtained with an optical microscope.

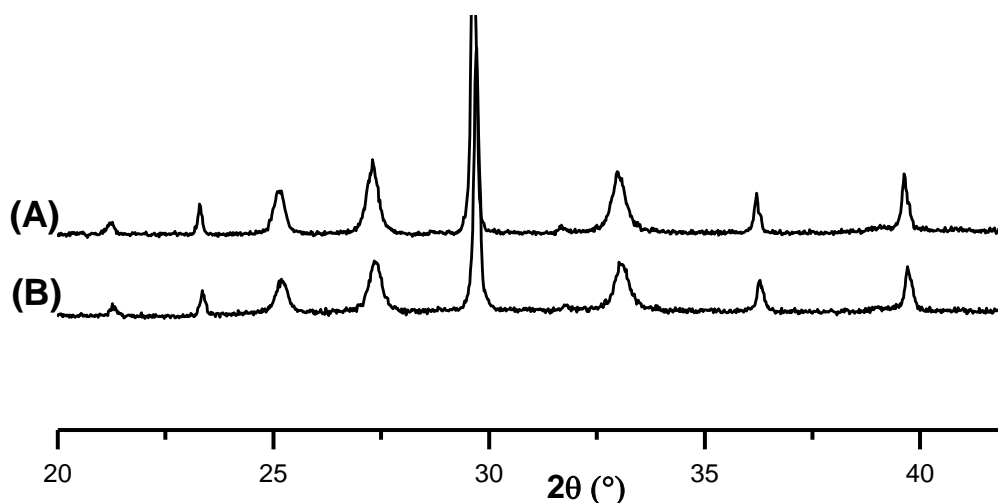
### **3. Results and discussion**

As shown in Figure 1, the overall strategy relied on the use of calcium carbonate microparticles as a template for the fabrication of coated particles and capsules. The antibacterial activities of these materials were investigated on kanamycin –resistant *Escherichia coli*. *Escherichia 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. It is worth noting that both non- and pathogenic strains of *E. coli* exist. Indeed, non-pathogenic *E. coli* strains provide the host with benefits, such as vitamin K; however, some *E. coli* strains can induce disease. For instance, enterohemorrhagic *E. coli* is a pathotype of diarrheagenic *E. coli*, which can lead to mild to severe bloody diarrhoea in humans which can progress to haemolytic uremic syndrome.



**Figure 1.** Schematic illustration of the fabrication of the capsules coated with charged biopolymer (the diethylaminoethyl-dextran hydrochloride ( $\text{dex}^+$ ) as polycation and dextran sulfate ( $\text{dex}^-$ )) using calcium carbonate microparticles as template.

Calcium carbonate microparticles were chosen due to their low price, non-toxic nature, and easy preparation. We prepared them by mixing soluble salts containing both  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$ . X-ray powder diffraction (XRD) analysis was done to show the formation of calcium carbonate particles (Figure 2). The diffraction patterns of the particles presented characteristic peaks of the crystallinity of the synthesised calcium carbonate microparticles. A trace of calcite was observed compared to a dominant observed phase of vaterite. Vaterite and calcite were the main crystalline polymorphs corresponding to  $hkl$ : 012, 104, 006, 110, 113 and 004, 110, 112, 114.

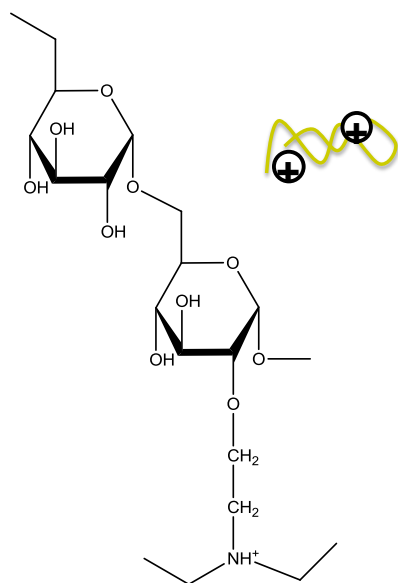


**Figure 2.** XRD pattern of (a) bare  $\text{CaCO}_3$  microparticle and (b) modified  $\text{CaCO}_3$  particle with the  $(\text{dex}^+/\text{dex}^+)_n$

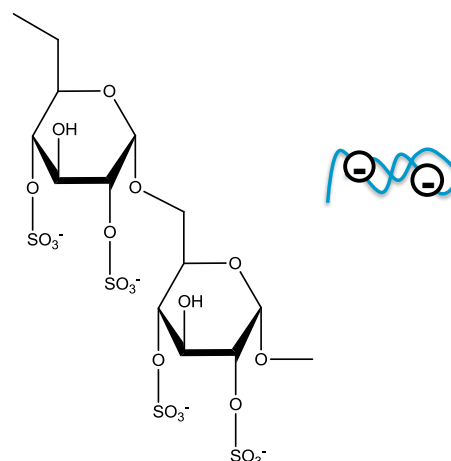
Charged polysaccharides, namely the diethylaminoethyl-dextran hydrochloride ( $\text{dex}^+$ ) and dextran sulfate ( $\text{dex}^-$ ), were then used for the multilayer assembly on the surface of  $\text{CaCO}_3$  microparticles (Figure 3). In comparison with other synthetic approaches, this route is highly promising as only water is employed as a solvent in all steps, instead of using organic solvents which are usually toxic. Calcium carbonate particles are considered for controlling the final size of the materials. After the multilayer coating on the substrate,  $\text{CaCO}_3$  microparticles are removed without affecting the multilayer shell for fabrication capsule. Herein, we are interested in employing LbL multilayer capsules, instead of self-aggregation particles, made of charged polysaccharides as: i) the process that we use here (described in Figure 1B) enables us to easily control the size and the surface charge of the final particles and ii) the objective is to study if the capsule can behave as an antibacterial-agent by itself without any supplementary addition of antibiotic. We want to demonstrate that as the capsules are supposed to be more flexible than the coated solid particle, they may interact more easily with bacteria walls and inhibit their growth.



**Dextran ammonium, dex<sup>+</sup>**

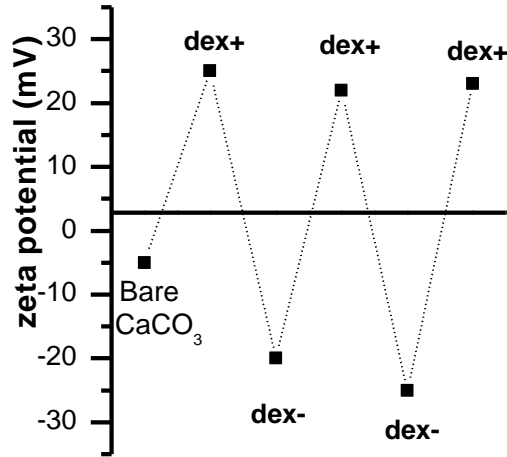


**dextran sulfate, dex<sup>-</sup>**



**Figure 3.** Chemical structure of the diethylaminoethyl-dextran hydrochloride (dex<sup>+</sup>) and dextran sulfate (dex<sup>-</sup>)

After the deposition of each polyelectrolyte layer on CaCO<sub>3</sub> particles, the zeta potential of the particles was measured (Figure 4). The bare calcium carbonate microparticles present a negatively-charged surface (~ -5 mV). The addition of a diethylaminoethyl-dextran hydrochloride layer led to a positive value (up to +20 mV) then the deposition of the dextran sulfate layer resulted in a negatively-charged surface after (below to -20 mV), in accordance with a complete charge reversal. Successive alternating of zeta potential values is shown, indicating the multilayer film growth via electrostatic adsorption as the driving force<sup>35</sup> on the CaCO<sub>3</sub> particles surface. The results are in agreement with the those of Tripathy *et al.* who reported the adsorption of poly(allylamine hydrochloride) and sodium carboxymethyl cellulose on calcium carbonate microparticles<sup>36</sup> and those of Belbekhouche *et al.*<sup>17</sup>.



**Figure 4.** Zeta potential variation during the coating of the calcium carbonate microparticle by diethylaminoethyl-dextran hydrochloride ( $\text{dex}^+$ ) and dextran sulfate ( $\text{dex}^-$ ) polyelectrolyte.

As expected, the peaks correlated to the  $\text{CaCO}_3$  structure were still present for the modified  $\text{CaCO}_3$  particles (Figure 2).<sup>37</sup> The coated film is too thin to be able to see multilayer films using XRD. However, the zeta potential measurements allow us to highlight the coating on the  $\text{CaCO}_3$  particles. From Rao's equation (Equation 2)<sup>38</sup>, we show that the surface modification of  $\text{CaCO}_3$  did not impact the composition or crystalline structure (Table 1)

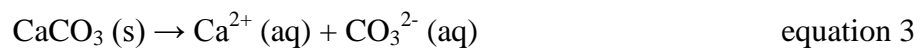
$$f_V = \frac{(I_{110V} + I_{112V} + I_{114V})}{(I_{110V} + I_{112V} + I_{114V} + I_{104C})} \quad \text{equation 2}$$

**Table 1:** Relative fraction of vaterite and calcite in the fabricated particle

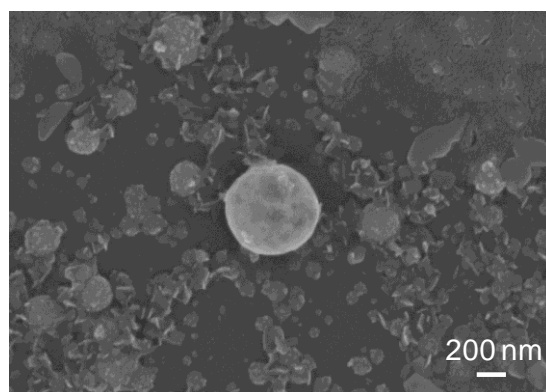
	$f_V^*$	$f_C^{**}$
$\text{CaCO}_3$ particle	0.33	0.67
modified $\text{CaCO}_3$ particle ( $\text{dex}^+/\text{dex}^-$ ) <sub>n</sub>	0.33	0.67

\*  $f_V$ : fraction of vaterite; \*\*  $f_C$ : fraction of calcite

To fabricate hollow microparticles, the  $\text{CaCO}_3$  template was removed after a selected number of layers using EDTA<sup>39</sup>. The dissolution process of the particle could be described as follows (equations 3 and 4):



SEM measurements (Figure 5) demonstrated that the  $(\text{dex}^+/\text{dex}^-)_n$  capsules formed from coated  $\text{CaCO}_3$  particles with  $(\text{dex}^+/\text{dex}^-)_n$  multilayer as well as their spherical shape. It deserves noting that further verification by high-resolution transmission electron microscopy (HRTEM) is needed to better evidence the hollow nature of the particle.

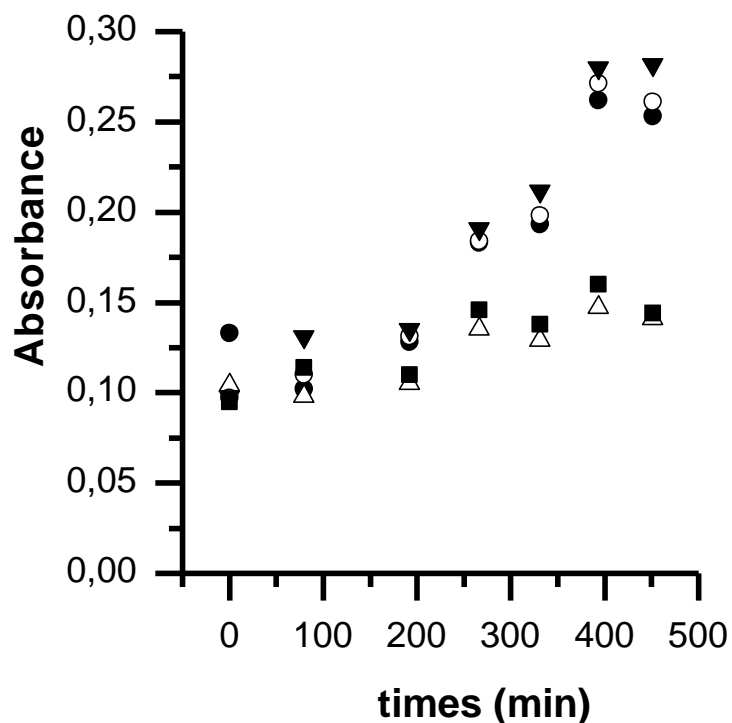


**Figure 5.** SEM image of  $(\text{dex}^+/\text{dex}^-)_n$  capsules

Finally; an antibacterial assay was done on the coated  $\text{CaCO}_3$  particles and capsules. The bacterial growth kinetics were followed in liquid medium by monitoring the variation in optical density (OD) versus time. It is worth noting that no impact on the bacteria growth was observed with bare calcium microparticles or with the physiological solution under the studied experimental conditions.

For *E. coli* (Table 2) at the end of the bacterial assay, the following OD values were measured: 0.282, 0.253, and 0.262 for the reference and calcium carbonate microparticles coated with  $(\text{dex}^+/\text{dex}^-)_n$  ( $n = 2$  and  $4$ ), respectively. For capsules, the OD values were as follows: 0.141 and 0.144, still according to the number of layers. The percentage of inhibition was then calculated using equation 1. This parameter varied from 10.3 % to 7.4% for the calcium carbonate particles coated with  $(\text{dex}^+/\text{dex}^-)_n$  and from 50.0% to 48.9% for  $(\text{dex}^+/\text{dex}^-)_n$  capsules ( $n$  increasing from 2 to 4). A significant inhibitory effect on the bacteria growth was noticed by increasing the number of the biopolyelectrolyte layers and the removal of calcium carbonate particle template (in other words with the multilayer capsule). For the same experimental conditions of multilayer layer-by-layer assembly (number of layers and particles concentration), capsules present a remarkable inhibitory effect in comparison with the calcium carbonate-coated particles. We hypothesize that the capsules, which are more flexible

then the coated particles, may interact more efficiently/easily with the membrane of the bacterial cell<sup>40</sup> then induce an inhibition of their growth.



**Figure 6.** Growth study of kanamycin-resistant *Escherichia coli* in liquid medium inoculated with bacteria alone (▼) and in the presence of: (dex<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub> coated CaCO<sub>3</sub> particles ( n = 2 (○) and n = 4 (●)) and (dex<sup>+</sup>/dex<sup>-</sup>)<sub>n</sub> capsules ( n = 2 (■) and n = 4 (△))

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

Sample	CaCO <sub>3</sub> coated particle		Microcapsules	
	OD	I (%)	OD	I (%)
No particles	0.282	0	0.282	0
A	0.253	10.28	0.141	50
B	0.261	7.44	0.144	48.9

After studying the impact on bacterial growth (kanamycin-resistant *Escherichia coli*), the bacteria viability was assessed. For this, trypan blue was used because the trypan blue dye exclusion test is known to enable the estimation of viable cells present in a cell suspension. The principle is based on the fact that live cells possess intact cell membranes that exclude this dye, whereas dead ones do not. No coloration was observed after staining bacteria, showing that upon exposure to the capsules, the bacterial cells growth is inhibited but they remain alive.

#### 4. Conclusion

In the present work, we propose a new route concerning the development of particles presenting antibacterial properties against bacteria resistant to antibiotics (kanamycin-resistant *Escherichia coli*). The particles were fabricated based on the capacity of biopolymer, presenting oppositely charged, to be layer-by-layer assembled onto charged particles used as sacrificial particles. The resulting charge-charge interaction was successfully evidenced by zeta potential measurements. Capsules were obtained by treating the calcium carbonate core with aqueous EDTA solution (through the in-situ etching). We show that the rigidity of these particles has an impact on the growth of bacteria, i.e. only flexible particles inhibit the bacteria growth. In the future, we plan to test the impact of flexible particles (capsules) on other antibiotic-resistant *Escherichia coli* and other types of bacteria to evidence the possibility to generalize the impact for all types of bacteria.

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