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## Chitosan Based Self-Assembled Nanocapsules as Antibacterial Agent

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### Abstract.

Creating an Achieving appropriate antibacterial disinfection system without forming any harmful compounds is still a major challenge challenging and calls for new technologies for efficient disinfection and microbial control. Towards this aim, we report on the elaboration of biodegradable and biocompatible polymeric nanocapsules, also called hollow nanoparticles, for potential applications in antibiotic therapy. These nanomaterials are based on the self-assembly of charged polysaccharides, namely chitosan and alginate, onto gold nanoparticles as a sacrificial matrix (60 nm). Electrostatic interactions between the protonated amine groups of chitosan (+ 35 mV) and the carboxylate groups of alginate (- 20 mV) are the driving attraction force enabling the elaboration of well-ordered multilayer films onto the spherical substrate. The removal of the colloidal gold, via cyanide-assisted hydrolysis, is evidenced by time-dependent variation of the gold spectroscopic signature (30 min is required). TEM shows the obtention of nanocapsules. An inhibitory effect of these particles has been demonstrated during the growth of two representative bacteria in a liquid medium: *Staphylococcus aureus* (Gram-positive) (from 4.6% to 16.3 % for gold nanomaterials + and from 18.6 % to 34.9 % for (chi<sup>+</sup>/alg<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> nanocapsules) and *Escherichia coli* (Gram-negative) (from 5.4% to 20 % for gold nanomaterials + and from 23.7 % to 40 % for (chi<sup>+</sup>/alg<sup>-</sup>)<sub>n</sub>-chi<sup>+</sup> nanocapsules) in a liquid medium. Acridine orange staining demonstrated the bactericidal effect of chitosan-based capsules. These findings demonstrate that (chitosan/alginate)<sub>n</sub> capsules can be exploited as a new antibacterial material. Thus, we We then presented a complementary approach to classical nanoparticles prepared by complexation between alginate and chitosan or other materials.

**Keywords:** particle-templated layer-by-layer assembly, chitosan, nanocapsule, pathogenic bacteria, antibacterial agent, polysaccharide, gold nanoparticle

## 1. Introduction

Microorganisms are recognised ~~recognized~~ to have a strongly impact ~~the~~ on Human beings.[1] Indeed, ~~the~~ contamination by microorganisms ~~such~~ as bacteria, fungus and viruses ~~involves-can lead to~~ serious damages in ~~both the~~ food and pharmaceutical ~~industry-industries~~. [2] Furthermore, due to the abuse of antibiotics, an increasing threat of bacterial antibiotic resistance is observed. One of the ~~encountered~~ problems ~~frequently encountered~~ is the ~~phenotypic trend presented~~ by several organisms ~~present-phenotypic-trend~~ that renders them capable of surviving adverse environmental conditions, for example ~~One may cite~~, *Streptococcus mutans* which ~~have-has~~ developed an acid tolerance response ~~allowing it~~ to adapt and survive against the acidic conditions caused by an excess sugar intake. [3] Therefore, novel approaches for developing antimicrobial agents ~~is are~~ required for blocking ~~their~~ key mechanisms of phenotypic variation or simply limiting/preventing their growth. In this regard, ~~One one~~ may cite ~~the~~ chitosan,[4] metal oxide or metallic nanoparticles [5, 6] and some carbonaceous nanomaterials [7, 8].

The antimicrobial activity of some polyelectrolytes could be ~~correlated~~ ~~related~~ to the interaction of these ionic polymers with ~~the~~ negatively charged bacteria cell walls compromising the integrity of ~~the~~ bacterial cell envelope. In this context, self-assembly of polymer ~~as implying~~ peptides [9] or polyelectrolytes [10] to name but a few, ~~was were~~ reported to ~~behave-as~~ have antimicrobial ~~agents~~ ~~properties~~ [9, 11]. Furthermore, ~~the development of developing~~ nanoscale systems as antibacterial carriers ~~is increasingly gaining gained-increased-of~~ interest. For instance, nanocarriers could effortlessly diffuse across all biofilm structures. [12]

The LbL (Layer-by-Layer) approach is one of the most useful methods ~~for synthesising to~~ ~~synthesize~~ multifunctional nanocapsules. For instance, if ~~implying~~ ~~applying~~ polyelectrolytes of opposite charges, the main driving force for the assembly build-up remains ~~the~~ electrostatic attraction between each ~~of the~~ polyelectrolyte layers of opposite charge, ~~which-This~~ makes the deposition procedure very simple and ~~it is~~ straightforward to modify spherical surfaces.[13] ~~The An~~ alternative deposition of polymer layers is achieved on the surface of a ~~given~~ sacrificial template. [14-17] ~~Its-selective~~ ~~Selective removal of the sacrificial template~~ allows the formation of nanocapsules (hollow nanoparticles). Nanocapsules obtained through this strategy exhibit highly controlled dimensional features.

Gold nanoparticles are one ~~of-them~~ ~~such~~ ~~sacrificial template~~ and combine very interesting properties. Indeed, they are biocompatible and commercially available in a wide range of

sizes including diameters as low as 5 nm. For instance, the preparation of nanocapsules based on poly(diallyldimethylammonium chloride) (PDADMAC) and on sodium poly(styrene sulfonate) (PSS) by using gold nanoparticles with different diameters ( $11 \pm 2$  nm to  $54 \pm 13$  nm) was reported by Gittins and Caruso [18, 19]. Schneider and Decher [20] have evidenced how gold colloids can efficiently be coated with up to 20 layers of a polycation (poly(allylamine hydrochloride), PAH) and ~~the a~~ polyanion (poly(styrene sulfonate), PSS), approach finally followed by the gold core degradation. Belbekhouche ~~et al. and co-workers~~ reported on the elaboration of ultra-low sized (20 and 60 nm) biodegradable polymeric capsules based on ~~the~~ chitosan/poly(acrylic acid) ~~based on~~ initial assembly on a gold core. [21] The authors took advantage of the electrostatic interactions for both building up the multilayer films and loading a small molecule containing an amine group, namely ~~the~~ amoxicillin, an antibiotic.

The main ~~issue of the concern around~~ current antimicrobial agents is ~~correlated~~ related to their highly toxic and irritant ~~behaviour~~ to humans. ~~Developing-Therefore,~~ developing an efficient approach for the elaboration of cost-effective and safe biocidesystems is ~~then~~ crucial. Using biopolymers like polysaccharides is an interesting solution, mainly due to their low toxicity toward mammalian cells and ~~their~~ effective antimicrobial properties. Nanocapsules can be elaborated ~~from-with~~ charged polysaccharides and ~~then-be-useful~~ subsequently used in the biomedical domain due to their intrinsic advantages ~~such~~ as biodegradability or biocompatibility.[22]

Among ~~them~~ polysaccharides, chitosan is a natural biopolymer presenting interesting biological features, ~~including as~~ biocompatibility and ~~or~~ antibacterial activity ~~to name but a few~~. The inhibitory or antibacterial effects of chitosan are explained in the literature by (1) the electrostatic attraction between the negatively charged membrane of the bacterial cell and the polycationic chitosan, resulting in ~~disruption of~~ intracellular components ~~disruption~~ [23] or (2) the inhibition of bacterial enzymatic activity [24].

Several works ~~have~~ reported ~~on~~ the successful preparation of chitosan-based nanoparticles for antibacterial purposes. [25-28] For instance, ~~it~~ chitosan can be easily formed through self-aggregation. [29] ~~Furthermore,~~ Chitosan tripolyphosphate nanoparticles have been reported to present antibacterial activity. [30]

In this study the dual objective is (1) to explore an easy and efficient approach for the preparation of capsules based on chitosan and investigate their antibacterial activity and (2) to demonstrate qualitatively the impact of rigid vs flexible particles on bacterial growth. Both

aspects have not yet been investigated in the literature. Indeed, in our study we have investigated how two natural polymers, alginate and chitosan, may be combined to elaborate nanocapsules with tunable antibacterial properties. The antibacterial activities of these nanoparticles were evaluated on a representative gram-positive bacterium, *Staphylococcus aureus* and gram-negative bacterium, *Escherichia coli*. We then proposed a complementary approach to the one achieved by chitosan nanoparticles formed by crosslinking with TPP (tripolyphosphate), or chitosan-alginate nanocapsules prepared by complexation. In addition to the capsule side, the interest of the present study lies in the fact that in most of the reported study studies reported to date, the antibacterial activity is tuned through the an increase of in the concentration of the nanoparticles. Herein, we present the possibility to impact modify this property by keeping a constant concentration of the nanomaterials but, instead, by increasing the number of the-chitosan layers.

## 2. Experimental section

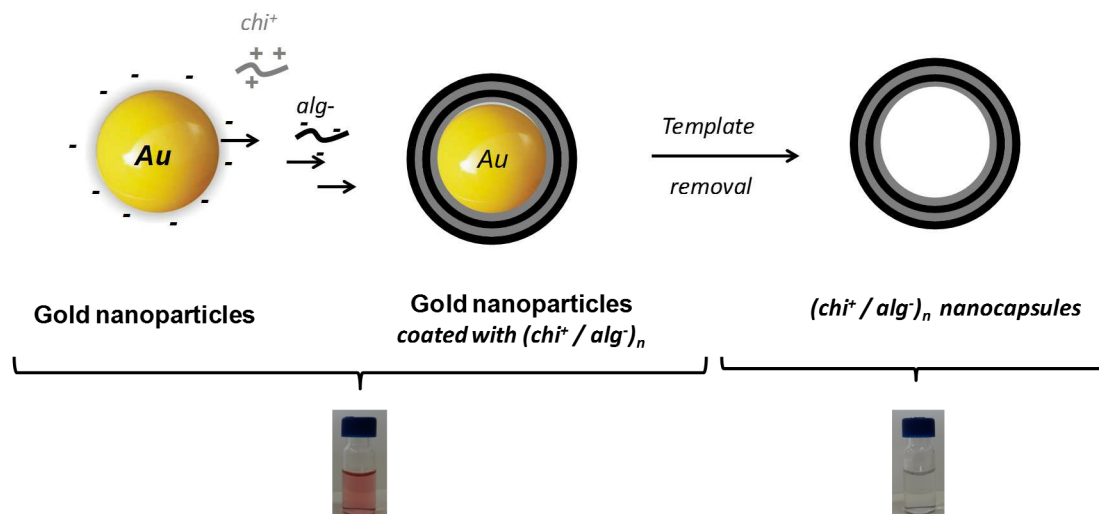
### 2.1. Materials

These following chemicals were purchased and used as received: chitosan (chi<sup>+</sup>, Sigma-aldrich), alginate (alg<sup>-</sup>, Sigma-aldrich), acetic acid (Alfa aesar) and gold nanoparticles (60 nm). 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 chitosan] were eluted with 0.1 mol.L<sup>-1</sup> LiNO<sub>3</sub> or acetate buffer 0.1M, respectively at 0.5 mL min<sup>-1</sup>. The sample (1 g.L<sup>-1</sup> for alginate (dn/dc = 0.140 mL.g<sup>-1</sup> [31] and (2 g.L<sup>-1</sup> for chitosan (dn/dc = 0.187 mL.g<sup>-1</sup> [32] filtered on 0.45  $\mu$ m unit filter (Millipore) was injected (100  $\mu$ L) with an automatic injector (Shimadzu SIL-20A). The flow rate was fixed at 1 mL.min<sup>-1</sup>. For the alginate, the  $\overline{Mn}$  value is  $2.7 \cdot 10^5$  g.mol<sup>-1</sup>, the  $\overline{Mw}$  value is  $3.8 \cdot 10^5$  g.mol<sup>-1</sup>. For the chitosan, the  $\overline{Mn}$  value is  $8.5 \cdot 10^4$  g.mol<sup>-1</sup>, the  $\overline{Mw}$  value is  $2.0 \cdot 10^5$  g.mol<sup>-1</sup> and the degree of acetylation of chitosan is  $0.14 \pm 0.02$  (estimated from <sup>1</sup>H NMR [22]).

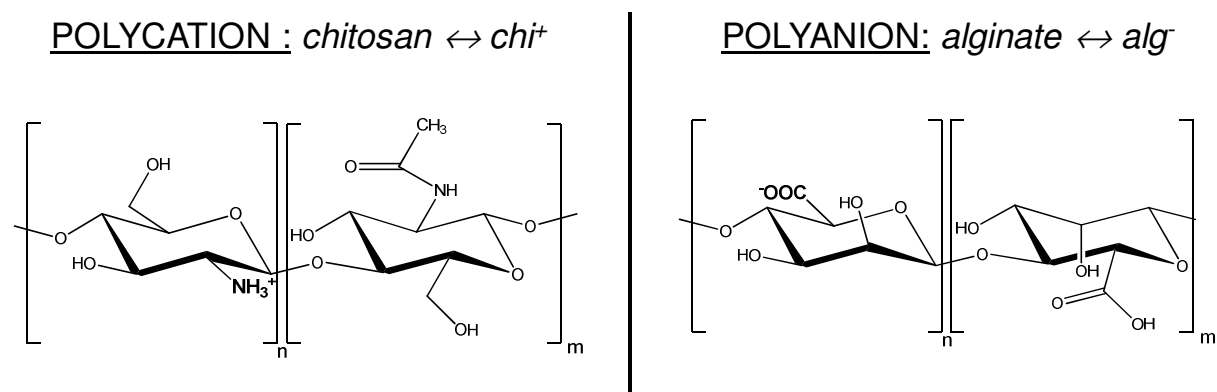
## 2.2. Preparation of chitosan-based nanomaterials.

~~The surface~~ Surface modification of the negatively charged gold nanoparticles was performed by a direct LbL saturation method (Figure 1). This means neither washing nor purification steps. ~~In the literature, this method is widely described~~ [33-35]. This elegant strategy proposes the possibility ~~to~~ of directly ~~incorporate~~ incorporating the required quantity of polyelectrolytes to efficiently coat all nanoparticles on the surface. ~~This presents the interest of avoiding/limiting~~ Interestingly, this helps to avoid/limit the presence of free polyelectrolytes in the aqueous phase. The saturation concentration of each polyelectrolyte ~~has to~~ must be empirically determined ~~over~~ by zeta potential measurements. First, 900  $\mu\text{L}$  of the negatively charged gold nanoparticles template (60 nm) ~~were~~ was progressively covered with the polycation, namely ~~the~~ chitosan, starting from a solution of  $1 \text{ g.L}^{-1}$  prepared in acetate buffer pH 4, 10 mM. Then, the polyanion (alginate) was added similarly (~~starting from a solution of  $1 \text{ g.L}^{-1}$  prepared in water~~). Each polyelectrolyte addition was made every 15 min, at room temperature. A zeta potential measurement was performed between each addition of polyelectrolyte and this allowed us to determine the required volume of each polyelectrolyte to saturate the gold nanoparticle surface. The assembly of a single bilayer is then denoted as follows: ~~described~~: (chitosan/alginate)<sub>n</sub> (with n representing the number of bilayers). ~~During the process, a concentrated solution of polyelectrolyte was implied, i.e.  $1 \text{ g.L}^{-1}$  (in 10 mM buffer acetate, pH 4 solution for chitosan and water for the alginate).~~ Zeta potential and absorbance measurements enable one to (i) determine the saturation concentration of each polyelectrolyte and (ii) monitor their adsorption onto the gold nanoparticles (Zetasizer Nano-ZS, Malvern Instruments, Brookhaven Instruments Corporation USA and Varian, Cary 100). Dissolution of the gold template enabled the elaboration of capsules. This was performed ~~via treatment~~ addition of ~~with~~ 100  $\mu\text{L}$  of potassium cyanide solution (10 mg in 1 mL of water) to the coated gold nanoparticles, whilst stirring at room temperature. A dialysis method was applied to remove the gold complex from the capsules for two days using a cellulose dialysis bag (3.5 kDa cut off) against distilled water. ~~Water~~ The water was refreshed every hour. ~~Kinetic~~ The kinetics of the core removal was investigated by monitoring the spectroscopic signatures of the gold cores (15 min, 25 min and 30 min) ~~The detail condition of the preparation of the nanomaterials will be determined and then discussed in the text.~~

A)



B)



**Figure 1.** A) Schematic illustration of the elaboration of the chitosan-based nanomaterials (gold coated nanoparticles and nanocapsules based on chitosan/alginate ( $\text{chi}^+/\text{alg}^-$ ) multilayers). B) Chemical structure of the polyelectrolytes used, i.e. chitosan as polycation and alginate as polyanion.

### 2.3. Assay for antibacterial activity: chitosan-based nanomaterials impact on bacterial growth

*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°C overnight in Luria-Bertani medium (LB, Sigma Aldrich). To estimate the inhibitory impact of the chitosan-based nanomaterials on heterotrophic bacterial growth, *Escherichia coli* and *Staphylococcus aureus* were grown under shaking in Luria broth medium (LB) at



37°C. For the assay, eight parallel wells of a 96-well microplate received 180 µL of bacterial suspension (eight replicates). 20 µL ( $1.7 \times 10^{10}$  particles/mL) of the suspension of nanomaterials ~~was~~ were added to each well and then incubated at 37°C. The final volume ~~was~~ is 200 µL. The initial optical density of the bacteria medium was 0.05 at 620 nm. ~~Physiological-A physiological~~ solution ~~as reference~~ ( $9 \text{ g.L}^{-1}$  NaCl in ~~distillated~~ 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 spectrophotometer from multiscan FC. The percentage of inhibition was estimated using equation 1:

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

$OD_{\text{ref}}$  and  $OD_{\text{sample}}$  correspond to the optical density (OD) of the reference (suspension of bacteria containing water) and sample (suspension of bacteria containing a suspension of chitosan-based nanomaterial), respectively.

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

## 2.4. Bacterial viability assay: microscopic observation

The effect of capsules 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 2.3). A 10 µL drop was then introduced on a cover slip to observe the bacteria.[36]

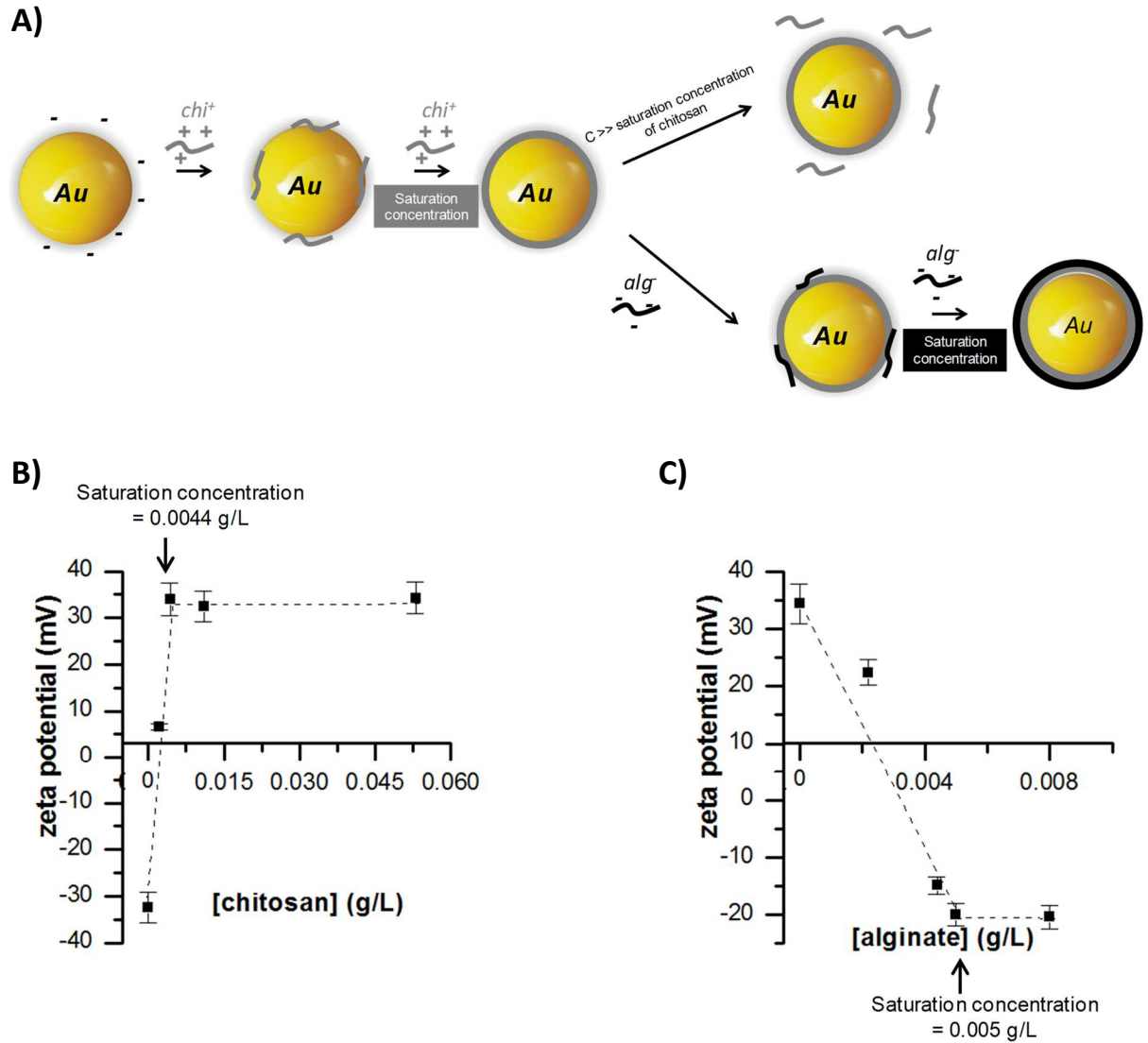
## 3. Results and discussion

Herein, the proposed approach consists of ~~in~~ multilayer coatings of gold nanoparticles with polymers bearing opposite charges (Figure 1 A). The present strategy is advantageous in comparison with other synthetic routes because, at all steps, only water is used as a solvent, rather than organic solutions ~~and not an organic one~~. Towards this aim, biopolymers were ~~applied-implied~~, namely chitosan and alginate, as polyelectrolyte pairs (Figure 1 B 2) to form ~~the a~~ multilayer film on the surface of gold nanoparticles. Interestingly, the dispersion of bare or modified gold colloid is ~~characterized~~ characterised by a red colour. The removal of this

core leads to a colourless solution (Figure 1 A 4). Lastly, the antibacterial activity of these chitosan-based nanomaterials will be investigated according to the number of polyelectrolyte layers and the presence of the gold template.

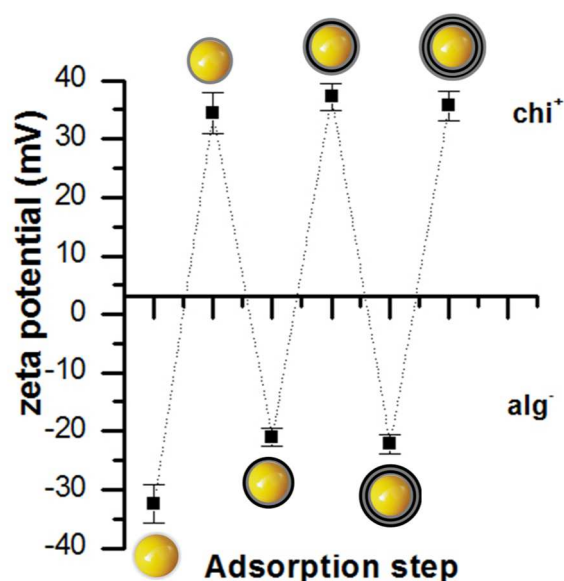
Applying the ~~The~~ LbL technique ~~applies~~ to modify macroscopic flat substrates ~~is easier then rather than~~ onto the surface of colloidal particles ~~is easier~~, because ~~in this last case, it lies in the the latter case relies on~~ separation of the coated colloids from free and excess polyelectrolyte before the addition of the next deposition cycle. ~~The wrapping~~ A phenomena of polyelectrolytes ~~wrapping~~ around particles may then occur. [37, 38] The centrifugation method is the most used techniques to separate adsorbed polyelectrolytes from free polyelectrolytes but this technique presents some drawbacks as it is time-consuming or aggregation problems may occur. ~~We~~ Consequently, we ~~then looked~~ investigated into an alternative ~~method one~~. The saturation method is an attractive ~~one~~ option, as this methodology offers ~~the~~ great benefit to avoid post-processing procedures such as ~~centrifuge centrifugation~~ and washing steps.

The required polycation and polyanion concentration (called “saturation concentration”) to form each layer of the nanocapsules shell ~~were was~~ experimentally determined by zeta-potential ~~potential~~ measurements. [35, 39] The addition of polycation/polyanion was stopped as soon as the zeta potential of the measured surface nanoparticles reached a value close to ~~those that~~ obtained for the free polyelectrolyte in solution. It can then be estimated that the amount of free polyelectrolyte in the suspension was very low/insignificant, as illustrated in Figure 2A 3A. Since particles are only subjected to a slow and adjustable stirring, they are not stressed. The optimal concentration to form a stable first layer was 0.0044 g/L for the polycation (chitosan) as shown in Figure 2B 3-B. Then, the deposition of the negatively charged layer was ~~done-completed~~. The polyanion (alginate) was added to the positively charged nanoparticles to form a stable second layer. As seen in Figure 2C 3C, the zeta potential progressively decreases to reach a negative zeta potential value. The optimal concentration to form the second layer was 0.005 g/L.



**Figure 2. 3.** A) Illustration of the "saturation method". Determination of the saturation concentration of B) chitosan and C) alginate over zeta potential measurements.

Figure 3 4 presents a typical variation of the zeta potential value of coated nanoparticles after the successive layer adsorption of the polycation/polyanion shell on the colloidal gold core. The negatively ~~charged~~ charged gold particles were first coated with the first layer of the polycation (chitosan) alternated with a layer of negatively charged alginate and again a final layer of chitosan until the selected number of layers. We demonstrate regular layer to layer variations of zeta potential from +35 mV to -20 (accuracy 5%) for alginate and chitosan polyelectrolytes pairs. This charge reversal after each polyelectrolyte deposition ~~that~~ proved the ~~coating of gold nanoparticles was~~ successful ~~gold nanoparticles coating~~.

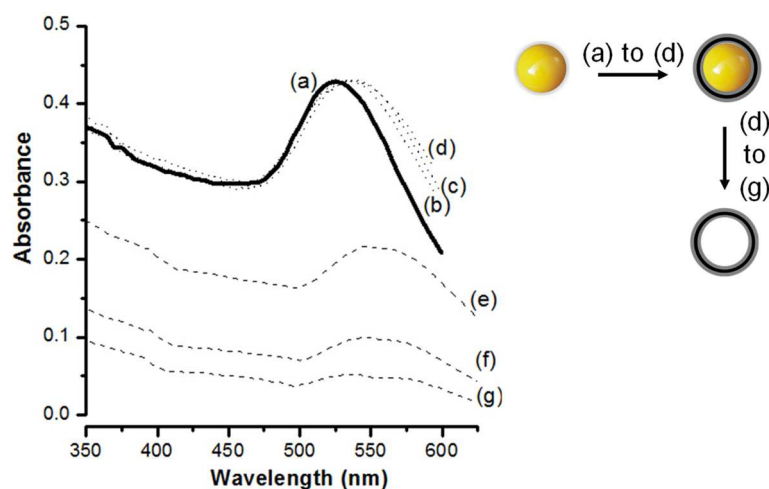


**Figure 3. 4.** Zeta potential variation during the elaboration of (chitosan/alginate)<sub>2</sub>-chitosan multilayer on gold nanoparticles.

Exploiting the sensitivity of the gold nanoparticle's surface ~~Plasmon~~ plasmon band due to the adsorbed species also enables ~~one~~ to follow the alternative deposition of the polyelectrolyte multilayers onto gold nanoparticles. [40-42] ~~Studying~~ UV-vis spectroscopy ~~study then permits to monitor then enables the monitoring of~~ the successive deposition of polyelectrolyte layers onto gold nanoparticles. For instance, Schneider and Decher [20] reported the elaboration of multilayer films (PAH/PSS) using gold nanoparticles as matrix. The authors monitored changes in the ~~Plasmon~~ plasmon band of the gold colloids ~~in with the aim of proving the aim to prove~~ the adsorption of the polyelectrolyte layer on the colloidal surface (red shift of the absorption band from 0.5 nm to about 10 nm at the saturation limit) and ~~also~~ to discriminate between interparticle bridging and aggregation (red shift of typically more than 80 nm to about 150 nm).

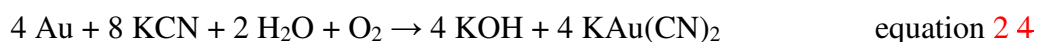
UV-vis spectra of gold nanoparticles, bare and modified with successive coatings with chitosan and alginate, are shown in Figure 4 5 (A to D) ~~(a to d)~~. The absorption peak above 525 nm is correlated ~~to with~~ the transverse surface ~~Plasmon~~ plasmon band of the bare ~~gold~~ nanoparticles ~~of gold~~. No variation of the absorbance value but a red-shift of ~ 10 nm in the ~~Plasmon~~ plasmon band maxima are observed upon the multilayer coating of the colloidal gold. This result may be correlated ~~to with~~ the changes in the local refractive index from that of water to that of the polyelectrolyte layer upon its adsorption. The work of Caruso and co-

workers [43-45], Gole and Murphy [46] and Belbekhouche et coll.[21] ~~comfort-support~~ this hypothesis. This result also ~~evidences~~ demonstrates that there is no variation ~~of~~ in the gold concentration upon ~~coating with~~ the multilayers ~~coating~~. Combined with the zeta potential result, the successful coating of the gold nanoparticles is evidenced.



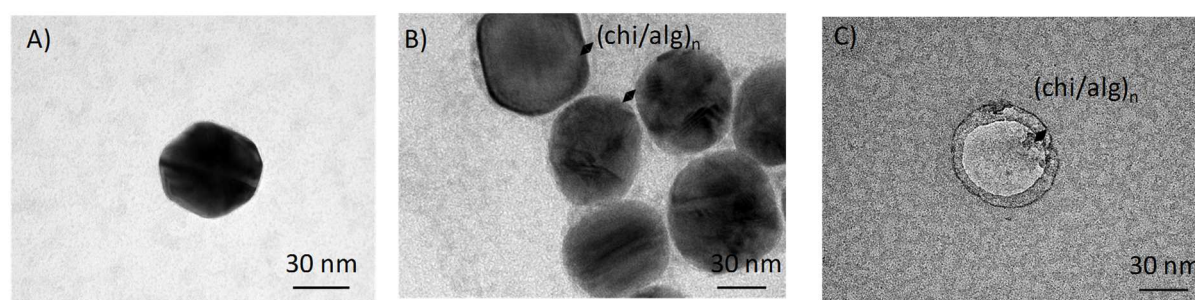
**Figure 4. 5.** ~~UV-vis spectra of polyelectrolyte-coated gold nanoparticle as a function of the number of adsorbed polymer layers: curve a: uncoated gold nanoparticles; curve b: one (chi<sup>+</sup>/alg<sup>-</sup>) bilayer; curve c: two (chi<sup>+</sup>/alg<sup>-</sup>) bilayers; curve d: four (chi<sup>+</sup>/alg<sup>-</sup>) bilayers. Impact of the cyanide treatment on (chi<sup>+</sup>/alg<sup>-</sup>) coated gold nanoparticles: curve (e):15 min, curve (f):25 min and curve (g): 30 min.~~ Monitoring of the LbL build-up of the (chi<sup>+</sup>/alg<sup>-</sup>)<sub>n</sub> film onto gold nanoparticles by UV-vis experiment: curve a: uncoated gold nanoparticles; curve b: one (chi<sup>+</sup>/alg<sup>-</sup>) bilayer; curve c: two (chi<sup>+</sup>/alg<sup>-</sup>) bilayers; curve d: four (chi<sup>+</sup>/alg<sup>-</sup>) bilayers. Monitoring of the nanocapsules formation upon cyanide treatment: curve (e):15 min, curve (f):25 min and curve (g): 30 min.

To obtain nanocapsules, dissolving the sacrificial ~~Au<sup>0</sup>~~ gold core is achieved through the formation of a water-soluble gold complex. Potassium cyanide is typically used to enable gold hydrolysis (equation 2 4).



~~Such~~ The process of core dissolution ~~can be easily could easily be~~ investigated by following the disappearance of the ~~Plasmon~~ plasmon absorbance band [19, 47]. From Figure 4 5 (E to G) ~~(e to g), it can be observed~~ a decrease ~~of~~ in the absorbance at the same wavelength, i.e. a decay of the ~~Plasmon~~ plasmon band absorbance peak intensity over time, ~~can be observed~~.

This result could ~~directly~~ be directly correlated ~~to~~ with a decrease in a gold concentration ~~decreases~~ due to the cyanide treatment and then to the elaboration of capsules (hollow nanoparticles). This interpretation is in accordance with the work of Gittins and Caruso [18] or Belbekhouche *et al.* ~~coll.~~ [21] The TEM observations ~~comforted~~ confirmed the success of the strategy depicted in Scheme 1 for the formation of nanocapsules (Figure 5 6). Furthermore, the presence of atomic gold was not detected by energy dispersive X-ray (EDX, results are shown in Figure S1 in the supporting information) analysis which is in accordance with the elaboration of polymer capsules and ~~corroborated~~ corroborates the cyanide removal (or the water-soluble salt of the potassium gold cyanide complex). There is no variation of the size of the particles after the removal of the gold core as evidenced by TEM pictures (figure 5) and DLS measurements (see Figure S2 in the supporting information).



**Figure 5 6.** TEM micrographs of: A) bare gold nanoparticle with diameter of 60 nm, B) (chitosan/alg)<sub>n</sub>-gold hybrid nanoparticles and C) (chitosan/alg)<sub>n</sub> nanocapsules.

After having successfully evidenced the elaboration of the new tailor-made chitosan-based nanomaterial, an antibacterial assay ~~has-been~~ was performed to ~~evidence~~ describe their possible impact. In the LB (Luria-Bertani) medium, the kinetics of bacterial growth was monitored through the variation of the optical density versus time. The influence of the number of layers as well as the presence or ~~not~~ absence of the core was investigated. Each test was ~~done-in replicated~~ eight times ~~replicates~~. Note that no inhibition was observed with bare gold nanoparticles or the polyelectrolytes under the studied experimental condition.

As observed ~~from~~ in Figure 6 7, upon the addition of particles or even capsules in the microbial suspension, a decrease ~~of~~ in the optical density is observed, as compared to the reference (see ~~t~~Table 1). This result could directly be correlated ~~to~~ with a decrease in the rate of growth. The bacterial density reached at the end of the experiment ~~is-affected~~ was altered and ~~is~~ was much lower in the presence of capsules as compared to coated gold nanoparticles.

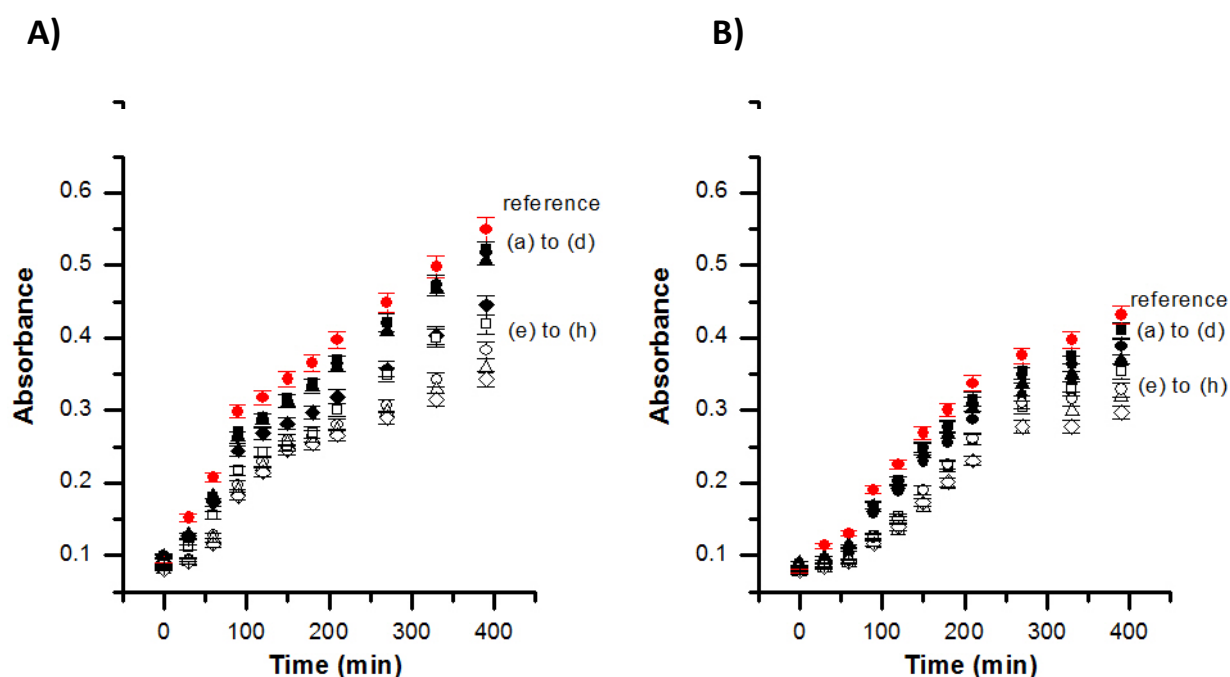


For *Escherichia coli*, the following OD (Optical Density) values ~~has-been~~ were measured at the end of the experiment 0.55, 0.52, 0.51, 0.50 and 0.44 for the reference and gold nanoparticles coated with  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  ( $n = 1, 2, 3$  and  $4$ ), respectively. For nanocapsules, the OD values ~~were are~~ 0.42, 0.35, 0.34 and 0.33, according to the same parameters. The same trend ~~is-was~~ observed for *Staphylococcus aureus*; the OD values at the end of the bacterial incubation ~~are were~~ 0.43, 0.41, 0.38, 0.37 and 0.36 for the reference and gold nanoparticles coated with  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  ( $n = 1, 2, 3$  and  $4$ ), respectively. For nanocapsules based on  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  ( $n = 1, 2, 3$  and  $4$ ), the OD values are 0.35, 0.32, 0.29 and 0.28. The percentage of inhibition for *Escherichia coli* varies from 5.4% to 20 % for the gold nanoparticles coated with  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  and from 23.7 % to 40 % for  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  nanocapsules ( $n$  increasing from 1 to 4). Our results are similar to those obtained by Prodana et al., who studied the functionalisation of carbon nanotubes by introducing -COOH groups. They observed an increase in the percentage inhibition of growth of *Escherichia coli* bacteria from 19 % to 34 %. [48] Adams et al. evidenced 38% inhibition of growth of this bacteria due to oxide nanoparticles. [49] The same trend is observed for *Staphylococcus aureus*; i.e. this parameter ~~increased-increases~~ from 4.6 % to 16.3 % for ~~the~~ gold nanoparticles coated with  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  and from 18.6 % to 34.9 % for  $(\text{chi}^+/\text{alg}^-)_n\text{-chi}^+$  nanocapsules ( $n$  increasing from 1 to 4). Namasivayam et al. obtained inhibition near to that obtained in our present study by using silver nanoparticles (50-60 nm). [49] Note that the number of bacteria in a suspension was confirmed by counting them with a Mallassez Cell.s

~~The~~ A comparison of the results for the two strains shows ~~a~~ quite ~~a~~ similar effect; i.e. a significant inhibitory impact on the growth of bacteria ~~growth~~ due to ~~the~~ an increase in the number of layers ~~of-the-layers-numbers~~ and ~~to-the~~ removal of gold ~~removal~~. For ~~a~~ the same conditions of assembly (number of layers and concentration), ~~the~~ results show that capsules present remarkable inhibitory effects, greater than for gold coated particles (~~two-times~~ twice more according to the percentage of inhibition). This result may only be related to ~~correlated~~ ~~to~~ the rigidity of the particles ~~rigidity~~. Flexible particles seem to have a ~~more~~ greater impact on bacteria growth than rigid ~~particles one~~. The antibacterial activity of chitosan can ~~directly~~ be directly correlated with the surface characteristics of bacteria ~~surface-characteristics~~. [25] The surface of the bacteria contains a variety of surface appendages, such as fimbriae, pili or flagella. Furthermore, the cell surfaces are chemically heterogeneous and structurally complex. Different polymers are presented onto the surface, such as lipoteichoic acids mycolic acids, polysaccharides or proteins. In combination with the topography of the cell

surface, these biopolymers have a crucial role in interactions with surfaces.[50] According to previous work, [26, 27] the antibacterial activity of chitosan may be correlated ~~to~~ with its polycationic form (protonation of an amino group on the C-2 position of the D-glucosamine moiety). Positively charged chitosan can interact with the surface of the bacterial cell surface, i.e. bind to its surface. This induces ~~the~~ a disruption of the normal functions of the membrane; ~~For~~ for instance, by inhibiting the transport of nutrients inside the cells or by ~~favouring~~ the leakage of intracellular components [28, 51, 52].

Based on this, the ~~antimicrobial activity of the~~ cationic elaborated chitosan-based nanomaterial may be due to adsorption onto - or interaction with - the cell surface of the bacteria. A phenomenon of diffusion may then be induced through the cell wall due to the nanoscale dimension of the particle. ~~Followed~~ This is followed by an adsorption onto the cytoplasmic membrane which leads to the disruption of the cytoplasmic membrane (leakage of the cytoplasmic compound). Consequently, this induces the death of the cell. ~~They~~ This may involve membrane damage, enzyme inhibition and ~~denaturation of~~ other proteins ~~denaturation~~. [53] ~~In the future, biochemical analysis and electron microscopy would allow us to know which the dominant inhibition mechanism is. The phenomenon may be facilitated/favorized for the “flexible” capsules than the “rigid” coated gold nanoparticles, explaining the more inhibiting effect for capsules than the coated nanoparticles. In the future, electron microscopy as well as biochemical analysis would enable us to evidence which of them is the dominant inhibition one.~~





**Figure 6 7.** Growth study of A) *Escherichia coli* and B) *Staphylococcus aureus* in LB medium inoculated with bacteria in the presence of nanoparticles and capsules. curve a: ● (chi<sup>+</sup>/alg<sup>-</sup>)-chitosan nanocapsules; curve b: ■ (chi<sup>+</sup>/alg<sup>-</sup>)<sub>2</sub>-chitosan nanocapsules; curve c: ● (chi<sup>+</sup>/alg<sup>-</sup>)<sub>3</sub>-chitosan nanocapsules; curve d:▲ (chi<sup>+</sup>/alg<sup>-</sup>)<sub>4</sub>-chitosan nanocapsules, curve e: □ gold nanoparticles coated with (chi<sup>+</sup>/alg<sup>-</sup>)-chitosan; curve f: ○ gold nanoparticles coated with (chi<sup>+</sup>/alg<sup>-</sup>)<sub>2</sub>-chitosan; curve g:Δ gold nanoparticles coated with (chi<sup>+</sup>/alg<sup>-</sup>)<sub>3</sub>-chitosan; curve h: ◇ gold nanoparticles coated with (chi<sup>+</sup>/alg<sup>-</sup>)<sub>4</sub>-chitosan.

**Table 1.** Inhibitions of *E. coli* (EC) and *S. aureus* (SA) by gold nanoparticles and nanocapsules

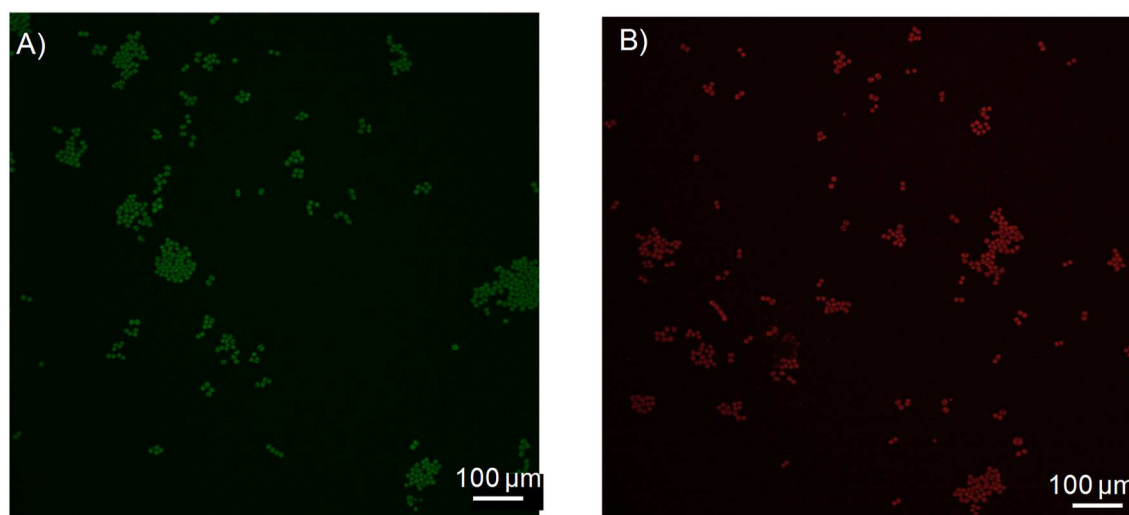
EC	Gold coated particle			Nanocapsules		
number of layer	OD	Bacteria per mL	I (%)	OD	Bacteria per ml	I (%)
1	0.52	$4.2 \cdot 10^8$	5.4	0.42	$3.4 \cdot 10^8$	23.7
2	0.51	$4.1 \cdot 10^8$	7.3	0.35	$2.8 \cdot 10^8$	36.4
3	0.50	$4.0 \cdot 10^8$	9.1	0.34	$2.7 \cdot 10^8$	38.2
4	0.44	$3.5 \cdot 10^8$	20.0	0.33	$2.6 \cdot 10^8$	40.0

SA	Gold coated particle			Nanocapsules		
number of layer	OD	Bacteria per mL	I (%)	OD	Bacteria per ml	I (%)
1	0.41	$3.3 \cdot 10^8$	4.6	0.35	$2.8 \cdot 10^8$	18.6
2	0.38	$3.0 \cdot 10^8$	11.6	0.32	$2.6 \cdot 10^8$	25.6
3	0.37	$3.0 \cdot 10^8$	13.9	0.29	$2.3 \cdot 10^8$	32.6
4	0.36	$2.9 \cdot 10^8$	16.3	0.28	$2.2 \cdot 10^8$	34.9

R : reference (sterile physiological distilled water) for EC OD 0.55 ( $4.4 \cdot 10^8$  Bacteria per mL) and SA 0.43 ( $3.4 \cdot 10^8$  Bacteria per mL)

Note that for enterobacteria like *E. coli* and *S. aureus*, 1 OD at 620 nm is equivalent to  $8 \times 10^8$  CFU/mL [54]

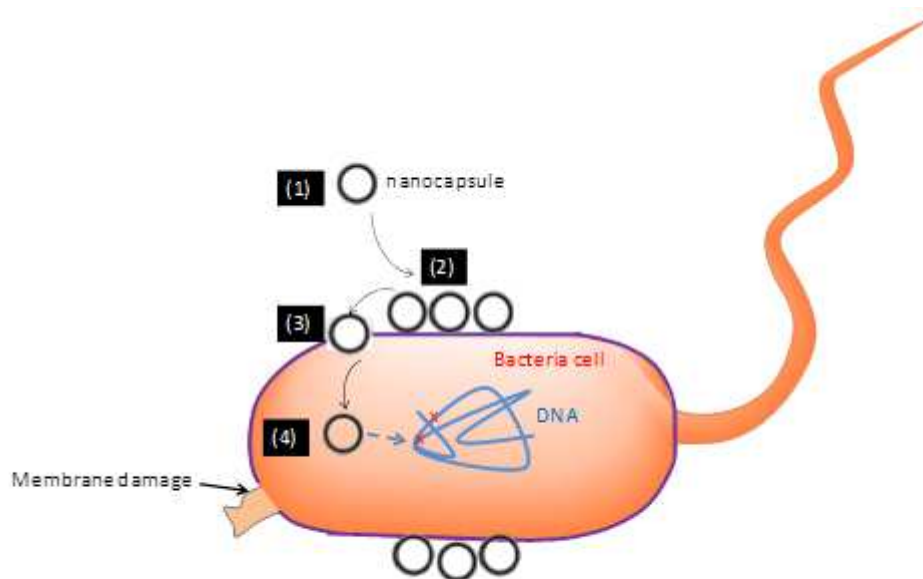
After observing the inhibition of bacterial growth, we investigated if the bacteria were viable. Towards this aim, bacteria were stained with acridine orange (AO) to enable us to discriminate between living and dead bacteria. [55] Note that acridine orange is a cell-permeant nucleic acid binding dye and allows one to differentiate between double-stranded and single-stranded nucleic acids. AO emits red fluorescence when bound to denatured single stranded DNA (ssDNA) found in dead bacteria and emits green fluorescence when bound to double stranded DNA (ds DNA), as found in living bacteria. A red fluorescence was observed after staining bacteria (Figure 7), demonstrating the death of bacterial cells due to exposure to the capsules. It should be noted that green fluorescence was observed for *S. aureus* without capsules (negative control, figure 7A), demonstrating that the bacteria are alive when no capsules are present.



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

Based on the obtained results and mechanism described in the literature, [56] we suggest a hypothetical mechanism and a highly simplified scheme behind the activity of nanocapsules on bacteria (figure 8): (1) interaction with the protein membrane of the bacteria affecting their correct function, (2) accumulation in the bacteria membrane affecting membrane permeability, (3) uptake of nanomaterial and (4) entry of the particles into the bacteria cell

where it can affect DNA, cause damage to cell membranes... and therefore, the death of the bacteria.



**Figure 8:** Schematic illustration of a hypothetical mechanism summarizing nanomaterials interaction with bacterial cells.: (1) interaction with the protein membrane of the bacteria, (2) accumulation in the bacteria membrane, (3) uptake of nanomaterial and (4) entry of the particles into the bacteria cell leading to the death of the bacteria. For clarity purpose the scheme is not to scale.

#### 4. Conclusion.

~~For antibacterial purpose, we first~~ In our study, we demonstrated the possibility ~~to use~~ of using a new cationic chitosan-based nanomaterial (gold coated nanoparticles and capsules) for antibacterial purposes. ~~They~~ The nanomaterials were prepared ~~through~~ via an easy and robust ~~way implying~~ templating method, ~~i.e. relied;~~ relying on the capacity of cationic-chitosan to be assembled layer-by-layer ~~assembled~~ with anionic-alginate onto colloidal gold used as sacrificial particles. The ~~resulting~~ charge-charge interaction ~~built~~ was monitored in aqueous solution by zeta potential measurements. UV-vis measurements confirmed this result.

Indeed, a red-shift ~~of in~~ the surface ~~Plasmon-is plasmon band was~~ observed with the increase ~~of in~~ the number of self-assembled bilayer onto the gold nanoparticles. Capsules ~~are were~~ further obtained through the in-situ etching of the Au<sup>0</sup> gold core into aqueous potassium gold cyanide ~~yielded~~. These particles ~~allow-preventing prevented~~ the growth of pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*). ~~Moving forwards, we plan to start from the elaborated nanomaterials and investigate how several factors, such as the concentration of nanoparticles, the number of layers and the starting matrix, influence bacterial properties.~~

Capsules ~~are rather flexible and (rather-flexible)~~ have been proven to ~~strongly~~ inhibit microbial growth ~~more strongly~~ than rigid particles. Two mechanisms of ~~bacterial~~ inhibition may be implied, ~~i.e membrane dysfunction of-membrane~~ and denaturation of cellular proteins. Further work is required to evidence which ~~of these is predominant one-is-preponderant~~. In the future, biochemical analysis and electron microscopy would allow one to determine the dominant inhibition mechanism. The phenomenon may be facilitated/favoured with the use of “flexible” capsules rather than the “rigid” coated gold nanoparticles, explaining the greater inhibition observed for capsules than coated nanoparticles. In the future, electron microscopy in addition to biochemical analysis would enable us to evidence which of these is the dominant mode of inhibition.

Usually, ~~increasing~~ the concentration of ~~the~~ nanoparticles ~~presenting~~ with antibacterial properties more efficiently ~~property-is-increased-to kills/inhibits bacteria more-efficiently~~. ~~Herein,~~ In this study, we ~~evidence~~ present a new route to obtain antimicrobial nanomaterials ~~with which have an efficient tunable efficient~~ effect. Antibacterial activity was significantly enhanced by ~~increasing~~ the number of ~~the~~ chitosan layers and with ~~the use of~~ flexible particles. This work, following earlier research, offers the possibility ~~to of formulating formulate~~ new types of bactericides. ~~Other-Another~~ interesting aspects to be considered in future work ~~consists-in-taking would be to take advantage of the-multilayered polyelectrolyte assemblies to load an ionic drug, for example an as-some~~ antibiotic.z

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## Supporting information

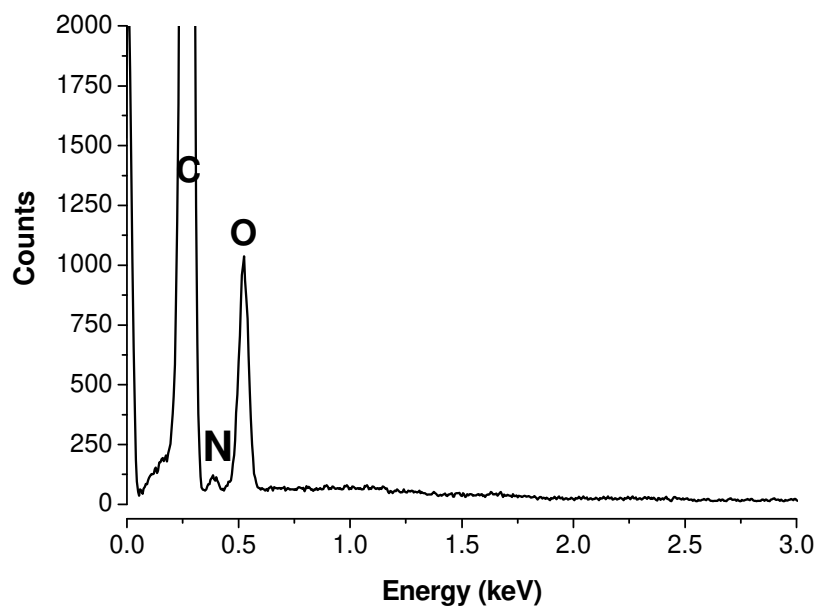


Figure S1: EDX analysis of the capsules

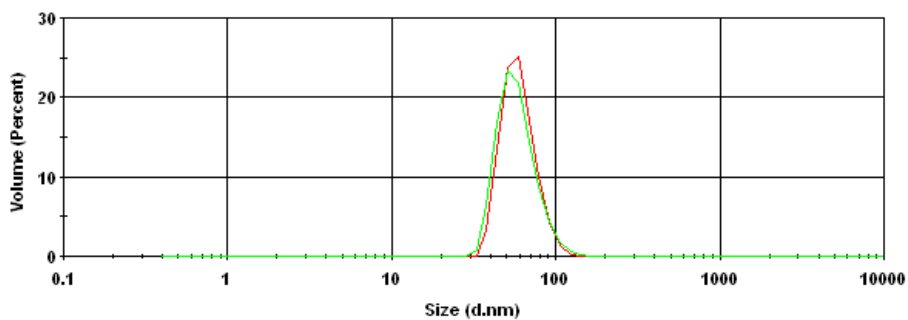


Figure S2 : Light scattering volume distribution of — gold coated nanoparticles and — nanocapsules

