Supporting Information

Development of non-cytotoxic chitosan-gold nanocomposites as efficient antibacterial materials

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Antibacterial test procedure and bacterial cell wall damage SEM visualization

In order to evaluate the antibacterial activity of CS_AuNP nanocomposites in a direct contact form the ASTM E2180-07 standard method was applied (method for determining the antimicrobial effectiveness of agents incorporated into polymeric surfaces). Polymeric samples (CS – as a control, CS_AuNPs) were sterilized overnight in ethanol vapors, before antimicrobial testing. All of the equipment (as Petri plates, beakers, tweezers) and media (agar, broth, PBS, distilled water) were previously sterilized in the autoclave (121 °C/20 min.)

For each test, three pieces of each sample were prepared (squares 3 × 3 cm). Each test was performed in triplicate. Initial culture was cultivated in TSB, as mentioned before, at 37 °C for 18 – 24 h to obtain stationary growth phase bacteria (10^9 CFU/mL). Then, 1 mL of the overnight bacterial culture was transferred to 100 mL of enriched agar medium that had been previously autoclaved and left to cool to approximately 40 °C (~10^5 – 10^6 CFU/mL). Series of dilutions of the initial test inoculum were performed (in a sterile Phosphate Buffered Saline – PBS ; 1 mL of inoculum: 9 mL of PBS, etc.) and subsequently plated on agar plates. Seven dilutions were performed in glass tubes. Spreading of the dilutions on agar plates was performed three times (3 × 25 μL) in order to obtain representative CFU values.

In the next step, 1 mL of the initial inoculum was pipetted on each polymeric sample, placed in a sterile plastic Petri plate inside a sterile glass Petri plate. After the inoculum in agar hardened, glass Petri plates were filled with 10 mL of sterile distilled water. Samples with bacteria were co-incubated at 37 °C for 18 – 24 h.

After incubation, polymeric samples were transferred with sterile tweezers to sterile 50 mL beakers filled with 10 mL of broth (with agar on the bottom). Then, beakers were treated under sonication for 2 minutes, in order to detach potential survived fractions of bacteria. Series of
dilutions were conducted and samples were spread on agar plates, as mentioned above. After 24 h of incubation, bacteria colonies were counted and Colony Forming Units were calculated (CFU/mL). The damage and potential rupture of the bacterial cell walls during the exposure to the chitosan-gold nanocomposites were visualized by SEM. Pieces of chitosan and gold–chitosan films were treated in microtiter plate wells with 1 mL of fixing buffer (sodium cacodylate with 2.5 wt % glutaraldehyde and 0.1 M sucrose) and incubated at 37 °C for 1.5 h. Then, the fixed cells were dehydrated in a graded methanol series. Samples were then air dried, mounted on SEM stubs, and sputtered with a 20 nm gold layer to allow SEM observation.

XPS results

Gold nanoparticles distribution in M2 film was measured (Figure S1). Gold concentration in M2 sample seems to be almost constant a few nanometers (around 10 nm) below the surface.

![Figure S1. Gold nanoparticles distribution in M2 membrane.](image)

The peak at 399.6 eV is related to –NH₂ groups in chitosan (Figure S2.A).¹ In the presence of Au nanoparticles the N 1s spectrum also shows peaks at 400.7 and 405.5 eV (Figure S2.B). The peak around 400 eV could be assigned to amino groups associated with Au surfaces.²
Figure S2. N 1s level spectra for a pure chitosan film (A) and a chitosan-gold nanoparticles film

Chitosan layer on AuNPs surface

Using high resolution TEM and contrasting the polymeric matrix using Phosphotungstic acid on the M10 colloid, a chitosan halo around the particles can be observed (Figure S3) which confirms the strong interactions between the polymer and the noble metal surface.

Figure S3. TEM photograph revealing a chitosan layer on AuNP surfaces.

EDS analysis

Energy dispersive spectroscopy elemental analysis (EDS) was performed to provide evidence of the presence of gold nanoparticles in the nanocomposites (FigureS4).
Figure S4. Confirmation of the presence of gold nanoparticles by using Energy dispersive elemental analysis (EDS).

Thermogravimetric analysis (TGA)

Figure S5 shows the TGA results for CS_L (A), CS_M (B) and CS_H (C) based nanocomposites. The thermograms of pure chitosan films reveal two stages on the polymer thermal degradation. Before the first stage, evaporation of the solvent leftovers occur, up to 180 °C. The first decomposition stage begins above 180-190 °C and ends at about 300-310 °C. The maximum decomposition occurs between 250-280 °C (T_{max}) (weight loss~30%). The maximum decomposition temperatures were listed in Table S1 The first weight loss is a consequence of dehydration of saccharide rings, depolymerisation and decomposition of both, acetylated and deacetylation of carbohydrate monomers.³
Figure S5. Chitosan-gold nanocomposite thermograms.

The final thermo-oxidative decomposition stage is observed above 450 °C. In the TGA curves obtained with the nanocomposites also two stages of decomposition were noticeable; however, due to the presence of NPs as fillers differences in the shape of the thermograms and decomposition progress occur. According to the results of thermogravimetric analysis around a 67% of pure chitosan (L, M, H) disintegrates. The amount of remained undecomposed matter (~30-40 wt %) corresponds to gold particles, other inorganic species and polymer traces (carbon based residues because the TGA was run under Ar atmosphere). The result show the slight change in the nanocomposite thermal stability in comparison to pure chitosan films due to the presence of gold nanoparticles.
Table S1. TGA analysis of the different CS-based films tested including % of decomposition and maximum decomposition temperatures reached

| Gold precursor initial concentration (mM) | CS_L | CS_M | CS_H |
|------------------------------------------|------|------|------|
|                                          | Decomposition (%) | T<sub>max</sub. (°C) | Decomposition (%) | T<sub>max</sub. (°C) | Decomposition (%) | T<sub>max</sub. (°C) |
| 0                                        | 67.56 | 271   | 67.48 | 281   | 67.36 | 287   |
| 1                                        | 63.63 | 254   | 58.38 | 271   | 62.42 | 278   |
| 2                                        | 63.54 | 253   | 59.74 | 264   | 61.43 | 279   |
| 5                                        | 59.27 | 253   | 57.93 | 263   | 61.33 | 251   |

UV–vis spectroscopy (Gold nanoparticles release study)

It is expected that the cytotoxic effect of chitosan-based nanocomposites containing nanoparticulated gold would be enhanced with an uncontrolled AuNP release from the prepared materials. The most important requirement to minimize this effect would be to obtain stable materials which do not release toxic species to the surrounding environment. Gold nanoparticle release rate was determined with UV–Vis spectroscopy and representative spectra are shown in Figure S6 (the intensity of the SPR band (~520 nm) was used as an indicator of the amount of released AuNPs). For all of the materials tested, no gold nanoparticle release to the medium was
observed at the detectable level of the spectrophotometer at least for sizes above 5 nm which are the ones which provide with a measurable UV-vis absorption peak.\textsuperscript{6}

Figure S6. UV–vis gold releasing study from the resulting nanocomposites with different AuNP loadings.

Zeta-potential ($\zeta$) of chitosan-gold nanoparticles dispersions

The surface charge of chitosan-gold nanoparticles after the synthesis was determined by measurement of zeta potential at pH=4.5 (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). The value of zeta potential enables determination of colloid stability and particles aggregation. Analysis was performed in triplicate for each sample and presented as mean ± standard deviation (SD) in Table S2.
Table S2. Zeta potential values for chitosan – gold colloids.

| Sample | $\zeta \pm SD / \text{mV}$ |
|--------|--------------------------|
| L1     | 19 ± 5                   |
| L2     | 26 ± 4                   |
| L5     | 29 ± 5                   |
| M1     | 27 ± 7                   |
| M2     | 30 ± 6                   |
| M5     | 46 ± 7                   |
| M10    | 47 ± 7                   |
| H1     | 29 ± 6                   |
| H2     | 33 ± 7                   |
| H5     | 34 ± 8                   |

Zeta-potential values defining the stable colloids usually occur for $\zeta > (\pm)30 \text{ mV}$. The higher $\zeta$ value, the degree of electrostatic repulsion between nanoparticles increases and thus the aggregation is less probable. For all of the chitosan based gold nanoparticles samples $\zeta$-potential value is positive, indicating positively-charged polymeric layer on AuNPs surface. Zeta potential
of sodium borohydride reduced gold nanoparticles is reversely negative, which supports positively charged chitosan presence. Since chitosan solution provides sufficient charge, obtained gold nanoparticles are electrostatically stable. The effect of molecular weight (Mw) of polysaccharide and gold precursor concentration on charge distribution around the nanoparticles can be observed. The highest ζ-potential values were obtained for medium and high Mw and high gold concentration, where the highest positive charge accumulation of the polymeric chains occurs. Those results together with UV-VIS measurements after months from the synthesis (no shift in SPR band over time) support a long-term stability of chitosan-based nanoparticles.

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