Co\textsuperscript{0} superparamagnetic nanoparticles stabilized by an organic layer coating with antimicrobial activity

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Introduction

Magnetic nanoparticles (MNPs) have attracted attention due to their potential applications in a wide range of technologies, including environmental remediation, catalysis, magnetic fluids, and magnetic resonance imaging.\textsuperscript{1-5} In the field of nanotechnology and nanomedicine, magnetic nanoparticles are of interest to researchers in studies of hyperthermia therapy, temperature-triggered drug release,\textsuperscript{4,5} and cytotoxicity on cancer cells,\textsuperscript{6} because of their unique physical and chemical properties.\textsuperscript{7,8} Small sizes and large surface area to volume ratios make nanomaterials very reactive and enable them to interact with the cell wall and genome of bacteria, leading to their antimicrobial activity.\textsuperscript{9} Among the MNPs, cobalt (Co) is one of the most promising materials and has attracted a great deal of interest for many years from researchers from a wide range of fields for different applications.\textsuperscript{10} Co nanoparticles exhibit high resistance to oxidation, corrosion, and wear.\textsuperscript{11} Most importantly, Co-NPs also have an intrinsic advantage in biomedical related fields.\textsuperscript{12} Due to their superior magnetic properties, Co nanoparticles could be a promising candidate for biomedical applications substituting the almost uniquely used biocompatible iron oxide nanoparticles.\textsuperscript{1} The activity in biological systems of Co-NPs and nanocomposites has been little studied. Because Co has high cytotoxicity, its passivation with an inert shell is a necessary prerequisite.\textsuperscript{6} However, some publications show that cobalt nanoparticles and cobalt oxide nanoparticles have been used as antitumor, anticancer, and antimicrobial agents against the proliferation of \textit{Acanthamoeba castellani},\textsuperscript{13} an amoeba that can be found in most ecosystems and is responsible for causing many diseases in humans.\textsuperscript{14} Other studies have published positive results of the antimicrobial action of cobalt cellulose nanocomposite against Gram-positive and Gram-negative bacteria.\textsuperscript{15} \textit{Staphylococcus aureus} is a pathogen responsible for serious infections, which can often be treated with broad-spectrum antibiotics. However, due to the growing concern of antimicrobial resistance, alternative antimicrobials are needed.\textsuperscript{15} Besides, little information is available in the literature on the antimicrobial activity of metallic cobalt nanoparticles (Co\textsuperscript{0}-NPs). However, studies show the use of complex nanostructured systems based on cobalt, such as, for example; rare earth element (REE) cerium (Ce\textsuperscript{3+}) doped CoFe\textsubscript{2}O\textsubscript{4} nanoparticles,\textsuperscript{16} nitrogen-rich carbon-coated bismuth/cobalt...
nanoparticles,7 cobalt ferrite-chitosan,18 and Co-doped TiO2 nanostructures,19 which have presented good antimicrobial behavior in Gram-positive and Gram-negative bacteria. Many synthetic methods have been developed to prepare MNPs, including the thermal decomposition method,20 hydrothermal microemulsion process,21 high temperature solution phase method,22 and solvothermal process.23 Solvothermal synthesis has allowed obtaining stable cobalt nanoparticles (Co0-NPs) with sizes smaller than 20 nm and spherical shape.24 Thus, the aim of this study was to develop stable and superparamagnetic Co0@C-NPs through solvothermal technique and analyzed its antimicrobial activity against Staphylococcus aureus.

Experimental

Materials

All the starting materials were commercially available reagents of analytical grade and were used without further purification.

Synthesis of Co0@C-NPs

The Co0@C-NPs were obtained using solvothermal synthesis in the same synthetic conditions, as reported by Paredes-Garcia et al.23 Due to the high stability obtained in nickel nanoparticles synthesis. The NPs were obtained from L-2-amino-3-hydroxypropanoic acid (l-serine) and CoCl2·6H2O in a molar ratio of 1 : 1, using DMF in a 23 mL Teflon-lined stainless steel autoclave.

Characterization

The Co0@C-NPs were characterized by powder X-ray diffraction (PXRD) using a Bruker diffractometer, model D8 Advance with Cu Kα1 radiation and Bragg–Brentano geometry in the 5° ≤ 2q ≤ 80° range. The morphological features of nanoparticles were observed by Transmission Electron Microscope (TEM) model JEM-1001L and Scanning Electron Microscope (SEM) Zeiss EVO MA10 equipped with an Oxford X-Act Energy Dispersive Spectrometer (EDS). A Vibrating Sample Magnetometer (VSM) PPMS Dyna Cool 9T was used to characterize the magnetic properties of as-prepared nanoparticles. The nanoparticles stability was study through Thermogravimetric Analysis (TGA) on PerkinElmer Thermogravimetric Analyzer, model TGA4000. The FTIR spectroscopy technique was used for identify and characterize the organic coating on Fourier Transform Infrared Spectrometer Jasco FT/IR-4600.

Antibacterial activity

Staphylococcus aureus (ATCC 25923) was provided by the Chilean Institute of Public Health (ISP). The strain was cultured overnight in trypticase soy broth (TSB) at 37 °C. Afterwards, the culture was diluted 100-fold with TSB and incubated at 200 rpm until obtaining an optical density (OD) 600 nm of mid-log phase. Cells were then washed and recovered by centrifugation at 1000 g for 2 min. The resultant pellet was resuspended in 1% TSB in 10 mM phosphate buffer saline (PBS) and adjusted to 1 × 107 CFU mL−1 (between 0.1 to 0.7 corresponding to 1 × 107 CFU mL−1). Then was exposed to 0.5, 1, and 2 mM Co0@C-NPs concentrations for 1 h at optimum growth temperature. Following exposure, the bacteria culture was diluted ten-fold with the same buffer and incubated for 18 h in fresh TSB media. Independent experiments were repeated a minimum of three times.25

Scanning Electron Microscopy (SEM) of Gram positive bacteria

Aliquots 100 µL of mid-log phase S. aureus was harvested by centrifugation at 3000 × g for 5 min and resuspended in HEPES buffer 10 mM with 1% TSB. The cell suspension was incubated at 37 °C for 30 minutes with the Co0@C-NPs at 0.5 and 2 mM. After incubation, the cells were centrifuged and washed 3 times at 4000 × g for 5 min with PBS and resuspended in the same buffer. The bacterial pellet was deposited on a glass coverslip and Gram stained. Afterwards it was sputter coated with approximately 30 nm thickness of gold. Finally, the microscopic examination was performed in a Carl Zeiss EVO MA10 scanning electron microscope at 20 kV.

Statistical analysis

All values obtained through antimicrobial assay were expressed as mean ± S.E. Statistical differences between means were calculated with GraphPad Prism 6 software by using the Student's t test correcting for differences in sample variance.

Results and discussion

Characterization of Co0@C-NPs

X-ray powder diffraction. The chemical nature and crystallinity of the synthesized product were identified by X-ray powder diffraction. Fig. 1 show the diffraction patterns obtained for the Co0@C-NPs. The nanoparticles display three peaks of different intensity at 2q values of 44.2; 51.5 and 75.8° which correspond to the Miller Indices (111), (200), (220) respectively, and are characteristic of face centered cubic Co0 (JCPDS file no. 15-0806, cubic system, spatial group: Fm3m, a = 3.5447 Å). Only one metallic phase is observed.
TEM and SEM microscopy analysis of Co\textsuperscript{0}@C-NPs

Morphological and structural details of Co\textsuperscript{0}@C-NPs were investigated by scanning and transmission electron microscopy. The observation of TEM images of these nanoparticles revealed that all the particles have spherical shape with an average diameter less than 100 nm. Besides, the TEM micrographic (Fig. 2A and B) permits the observation of a thin organic coating layer. This layer was supposedly associated with the amino acid used as a starting material in a previous study, which was also responsible for the stability of the nickel nanoparticles and prevented their oxidation.\textsuperscript{23,24} Fig. 2C and D show the SEM images for Co\textsuperscript{0}@C-NPs at two different magnifications. It is observed that they are forming homogeneous metallic agglomerates with spherical shapes and sizes around 2 \( \mu \text{m} \) in diameter.

In order to investigate the surface composition of the organic layer attached to the synthesized Co\textsuperscript{0}@C-NPs organic layer SEM/EDS and FTIR analysis were carried out. Through spectra obtained by SEM/EDS we observed peaks corresponding to carbon, oxygen and cobalt elements (Fig. 3).

The presence of organic material on the Co\textsuperscript{0}@C-NPs surface was confirmed by FTIR spectrum (Fig. 4), where we can see characteristic bands for the functional groups of amino acid. The peak noticed at 3428 cm\textsuperscript{-1} could be attributed to (\(-\text{NH stretching}\))\textsuperscript{16} 1632 cm\textsuperscript{-1} and 1456 cm\textsuperscript{-1} vibration band could be ascribed to COO- asymmetric (\(\nu_{as}\)) and COO– symmetric (\(\nu_{s}\)) stretching respectively.\textsuperscript{27–29}

Thermogravimetric analysis

In order to investigate the thermal stability, Co\textsuperscript{0}@C-NPs were subjected to thermogravimetric analysis (TGA) from 30 to 800 °C under air and nitrogen condition at the heating rate of 10 °C min\textsuperscript{-1}. TGA curves show that Co\textsuperscript{0}@C-NPs are stable until 400 °C (Fig. 5). Over this temperature was observed a significant mass increase of 30.32% and 26.00% in air and nitrogen atmosphere, respectively. This mass increase could be attributed to the generation of cobalt oxides due to the decomposition of organic material present in the Co\textsuperscript{0}@C-NPs surface, which is corroborated with the results obtained through TEM, SEM/EDS images and FTIR spectrum.
Magnetic characterization

The magnetic behaviour for Co@C-NPs was investigated by vibrating sample magnetometer at 300 K under an external magnetic field sweeping from +20 to −20 kOe. The magnetic hysteresis loop for Co nanoparticles is shown in Fig. 6.

The magnetic parameters values obtained from Co@C-NPs such as saturation magnetization ($M_s$), coercivity ($H_c$), remnant magnetization ($M_r$) and ratio of the remnant to saturation magnetization ($M_r/M_s$) are 148.6 (emu g$^{-1}$), 56 (Oe), 3.95 (emu g$^{-1}$) and 0.027 respectively. The results demonstrate that the magnetization curve shows typical superparamagnetic behavior with very low values in both remnant magnetization and coercivity. The value of $M_s$ for Co@C-NPs is lower than multidomain bulk particles of Co (168 emu g$^{-1}$). This is due to the size in which the nanoparticles are obtained. The coercive force ($H_c = 56$ Oe) and residual magnetization ($M_r = 3.95$ emu g$^{-1}$) are small, which indicates the Co@C-NPs are close to superparamagnetic. Furthermore, the ratio of $M_r/M_s = 0.027$, is very small, which is typical for superparamagnetic behaviour.

Superparamagnetic nanoparticles exhibit a monodomain structure with a high magnetic susceptibility exclusively under an external magnetic field. This unique property combining with a biocompatible capability is very promising because allow applying this kind of nanostructure in several biomedical application like theragnostic, hyperthermia tumor therapy, drug delivery, and magnetic resonance imaging.

Antimicrobial effect of Co@C-NPs in bacteria

Since it has been shown that bacteria are becoming resistant to antimicrobial agents, it is necessary to find novel materials with antimicrobial activity. Nanoparticles are being used with this purpose. The Co based nanoparticles has been studies against Staphylococcus aureus, these include cobalt ferrite nanoparticles and cobalt oxide nanoparticles, but there are a few investigation that used only metallic cobalt nanoparticles without conjugated at other nanoparticles or nanomaterials. For this reason, in this study, we evaluated the antimicrobial properties of Co@C-NPs synthesized by a solvothermal technique against Gram-positive bacteria (S. aureus) (Fig. 7). At 0.5 and 1 mM of Co@C-NPs, no significant inhibition was observed in the growth of S. aureus, but at 2 mM, it was observed that Co@C-NPs have major antimicrobial activity against this strain (Fig. 7), inhibiting the amount of bacteria from 0 up to $5.5 \times 10^8$ CFU mL$^{-1}$ after 1 h of exposition. In two investigations have been demonstrated that Co@C-NPs have antimicrobial activity. One of them have described that cobalt nanoparticles, in the range of 20-49 nm synthesized by adopting an eco method, showed activity against S. aureus above 25 mg mL$^{-1}$, which is 200 to 400 times higher than what we used in this research. The second investigation, cobalt nanoparticles of 10 nm synthesized by chemical methods are capable of killing $1 \times 10^2$ CFU mL$^{-1}$ of Escherichia coli after 3 h of exposition at $1.86$ mM (0.11 mg mL$^{-1}$), which is a lower inhibition than was obtained in this investigation.

Fig. 6 Magnetization curve of Co@C-NPs measured at 300 K.

Fig. 7 Antimicrobial activity of Co@C-NPs. Growth inhibition of S. aureus in the absence of Co@C-NPs and in the presence of Co@C-NPs concentrations of 0.5, 1 and 2 mM after 1 hour of exposure. The standard error was determined from the results of two experiments carried out in triplicate.

Fig. 8 Scanning electron microscopy of S. aureus. SEM micrographs of Staphylococcus aureus (A) without Co@C-NPs, (B) in the presence of Co@C-NPs at 0.5 mM, and (C) in the presence of Co@C-NPs at 2 mM.
A SEM study was performed for the direct observation of cell morphological changes after Co\(^0\@C\)-NPs treatment at 0.5 and 2 mM. The untreated \(S.\)\(\text{aureus}\) cells were used as a control, exhibiting a smooth and intact surface (Fig. 8), but when we treated \(S.\)\(\text{aureus}\) with Co\(^0\@C\)-NPs at 0.5 mM, it was observed that NPs form an aggregate-like structure on the bacteria surface. It could be presumed that this is an interaction observed between clustering of NPs and the cell surface that occurs during co-incubation and is not reflective of damage induced to the bacteria membrane as previously described by Sousa et al.\(^\text{44}\) At 2 mM a similar aggregate structure was observed but with fewer NPs. Probably, since there is a higher concentration of Co\(^0\@C\)-NPs, it is easier for them to aggregate and precipitate at the time of centrifugation, leaving individual NPs or apparently loose NPs aggregates\(^\text{44}\) on the bacteria surface. Further research is necessary to demonstrate if Co\(^0\@C\)-NPs have effects on bacterial surfaces or on intracellular components of bacteria at different times.

Given that Co\(^0\@C\)-NPs have a thin organic coating layer associated to L-serine amino acid and DMF which is responsible for their stability and prevention of oxidation, the effect of metallic cobalt in bacterial growth-inhibition may be explained for the generation of oxidation products derived from L-serine amino acid in agreement to general reaction of oxidation for \(\alpha\)-amino acids: \(R-\text{CH(NH}_2\text{)}\cdot\text{CO}_2\text{H} + O \overset{\text{R-CHO} + \text{NH}_3 + \text{CO}_2}{\longrightarrow}\) described by Hough et al.\(^\text{45,46}\) and oxidation of DMF to a carboxylic acid as well as the reduction of Ni\(^{2+}\) (or Co\(^{2+}\)) to Ni (or Co), which can be briefly formulated as follows: HCONMe\(_2\) + Ni\(^{2+}\) (or Co\(^{2+}\)) + \(\text{H}_2\text{O} \overset{\text{Ni(}\text{or Co)} + \text{Me}_2\text{NCOOH} + 2\text{H}^+}{\longrightarrow}\) described by Zhang et al.,\(^\text{44}\) where functional groups of Me\(_2\)NCOOH could be able to form hydrogen bonds with the bacterial cell wall.

On the other hand, it has been described that different types of nanoparticles generate reactive oxygen species (ROS), that could damage bacterial DNA, cell membranes (lipid peroxidation) and cause protein dysfunction.\(^\text{15,35,47,48}\) In this study, cobalt ions could be released from metallic cobalt nanoclusters which could interact with thiol groups on bacterial enzymes leading to cell death, as previously described by Alahmadi et al.\(^\text{47}\) Further experiments are essential before concluding about the antibacterial behavior of such oxidation products generated in the synthesis of Co\(^0\@C\)-NPs, which may lead to the release of cobalt ions or possibly both. Experiments will be important for determining the potential use of these nanoparticles as a new antimicrobial agent to combat bacterial infections.

**Conclusions**

In conclusion, we report the structural, morphological and magnetic properties of cobalt nanoparticles stabilized with an organic layer (Co\(^0\@C\)-NPs) and its potential antimicrobial activity. The solvothermal technique permits us to obtain stable and spherical fcc-Co\(^0\) with an organic layer that avoids the oxidation process until 400 °C. The elemental analysis confirm the presence of carbon and oxygen elements associated to organic layer and through spectroscopy was identified functional groups of amino acid like –NH and COO–.

Furthermore, this study reveals promissory results given that Co\(^0\@C\)-NPs shows antimicrobial activity against \(S.\)\(\text{aureus}\) at 2 mM, being 55 times more effective in less time than the previous studies. SEM analysis shows that Co\(^0\@C\)-NPs form an aggregate-like structure probably due interaction observed between clustering of NPs and the cell surface. Further research is necessary to establish the mechanism of action of the Co\(^0\@C\)-NPs.

The organic coating Co\(^0\@C\)-NPs and superparamagnetic property could be biocompatible with biological systems and in the future could be use in several biomedical application without additional processes required such as encapsulation, but previously must be evaluated its biosafety through in vitro biological assay.

**Conflicts of interest**

The authors declare that they have no competing interest.

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