Antibacterial ability of supported silver nanoparticles by functionalized hydroxyapatite with 5-aminosalicylic acid

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ARTICLE INFO

ABSTRACT

Antimicrobial performance of silver nanoparticles supported by functionalized hydroxyapatite with 5-aminosalicyle acid was tested against Gram-negative bacteria *E. coli*, Gram-positive bacteria *S. aureus* and yeast *C. albicans*. Thorough characterization of materials (electron microscopy, nitrogen adsorption−desorption isotherms, diffuse reflectance spectroscopy) followed each step during the course of nanocomposite preparation. Synthesized powder consists of rod-like hydroxyapatite particles (40−60 × 10−20 nm, length × diameter) decorated with nano-sized spherical silver particles whose content in nanocomposite was found to be 1.9 wt.-%. Concentration- and time-dependent bacterial reduction data indicated that use of silver nanoparticles even at concentration as low as 1 μg mL^{-1} lead to complete reduction of both bacteria (*E. coli* and *S. aureus*). On the other hand, non-toxic behavior of nanocomposite in broad concentration range (0.05−2.0 mg mL^{-1}) was found towards *C. albicans*. Successful inactivation of *E. coli* and *S. aureus* in five repeated cycles proved that synthesized nanocomposite can perform under long-run working conditions.

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1. Introduction

In the last few decades, hydroxyapatite ((Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, HAP) has been thoroughly investigated due to chemical and thermal stability, as well as sorption properties and biocompatibility [1–6]. Because of that, this bio-material has widespread applications, for example, in catalysis, liquid chromatography, industry of fertilizers and pharmaceutical products, for isolation and purification of proteins, nucleic acids and viruses, production of biocompatible ceramics and coatings, drug delivery, waste water treatment, soil remediation, etc. [7–9]. In the nature, HAP occurs in the form of geological deposits and as the inorganic component of bones and teeth in vertebrates [10]. However, prior to any use, purification/isolation of HAP originating from natural sources must be performed. This is one of the main reasons why different approaches for the synthesis of HAP have been proposed in the literature. Generally, three methods can be recognized for the synthesis of

HAP: wet, dry and hydrothermal [1].

Silver and silver compounds are historically recognized as powerful biocides. Despite the excellent antimicrobial activity, soluble silver compounds, such as AgNO$_3$, are unsuitable for the long term use. However, a desired level of antimicrobial activity can be achieved with silver nanoparticles (Ag NPs). The antimicrobial activity of Ag NPs is highly influenced by their morphology − the smaller the particles, the higher the antimicrobial efficiency [11–15]. In addition, for many applications, it is necessary to immobilize Ag NPs on support in order to control the contact time between microbial species and antimicrobial agent, as well as release rate of Ag$^+$ ions [16–21].

Biocompatible HAP with incorporated silver, either in ionic or metallic form, is frequently utilized to modify a variety of metallic implant surfaces. Generally, silver-doped HAP was prepared taking advantage of pronounced ion-exchange ability of HAP [22–27]. On the other hand, metallic silver − HAP composites were prepared either by linking separately prepared Ag NPs to HAP or by *in situ* reduction of co-precipitated Ag$^+$ ions with HAP powders using variety of methods [28–33]. Although content of silver was low in

both types of HAP based materials, silver-doped HAP and metallic silver − HAP composites, excellent efficiency against common bacteria was found [22–26,28–34]. However, antifungal activity was less studied, and, so far, data were reported for yeasts *C. albicans* [22] and *I. orientalis* [29].

In this study, we present a new method to chemically link Ag NPs to rod-like HAP particles over 5-aminosalicylic acid (5-ASA), widely used an anti-inflammatory drug. It is known that coordination of salicylate-type of ligands to the surface of wide band gap oxides (TiO_2 and Mg_2TiO_4) over adjacent hydroxyl and carboxyl groups leads to the formation of charge transfer (CT) complexes with enhanced optical properties [35–39]. In this particular case, advantage was taken of introduced free amino groups for *in situ* reduction of Ag^+ ions to metallic silver upon surface modification of HAP with 5-ASA. To the best of our knowledge, this approach has never been used for preparation of metallic silver − HAP composites. The thorough microstructural and optical characterization of synthesized composite was performed. Special attention was paid to the antibacterial and antifungal ability of Ag NPs supported by functionalized HAP with 5-ASA. Detailed concentration- and time-dependent measurements of inactivation of Gram-negative bacteria *E. coli*, Gram-positive bacteria *S. aureus*, and fungus *C. albicans* by metallic silver − HAP composite were conducted, as well as reduction of bacterial cells in successive cycles.

2. Material and methods

2.1. Synthesis and characterization of supported Ag nanoparticles by HAP

The chemicals used were all of the highest purity available and were used without further purification (Alfa Aesar, J.T. Baker). Milli-Q deionized water was used as solvent (resistivity 18.2 MΩ cm^{-1}).

The nanocomposite consisting of HAP, functionalized with 5-ASA, and decorated with Ag nanoparticles was prepared in three steps. In the first step, HAP powder was synthesized by neutralization of a $Ca(OH)_2$ suspension with H_3PO_4 following the procedure described in details in our previous report [7]. In the second step, HAP was surface-modified with 5-ASA. Briefly, 100 mg of HAP was dispersed in 30 mL of water containing 46 mg of 5-ASA. Mixture was stirred overnight and coloration of powder indicated its successful surface modification. Surface-modified powder was separated by centrifugation, washed several times with deionized water in order to remove excess ligand, and, finally, dried at 40 °C in the vacuum oven. In the third step, nanocomposite consisting of supported Ag nanoparticles by functionalized HAP with 5-ASA was prepared by the reduction of Ag^+ ions with amino groups from 5-ASA molecules. Typically, mixture of 50 mg of surface-modified HAP with 5-ASA and 7.9 mg of $AgNO_3$ in 25 mL of de-aerated water with Ar was stirred for 90 min at 60 °C. The synthesized nanocomposite was separated by centrifugation, washed several times with water, and dried at 40 °C in vacuum oven. For the sake of clarity, as-prepared HAP, surface-modified HAP with 5-ASA, and nanocomposite consisting of Ag nanoparticles supported by surface-modified HAP with 5-ASA will be further in the text named SHAP, SHAP/5-ASA, and SHAP/5-ASA/Ag, respectively.

Content of Ag in SHAP/5-ASA/Ag nanocomposite was determined using the Thermo iCAP ICP-MS instrument. Prior to measurements, SHAP/5-ASA/Ag samples were digested in Milestone Start D microwave using concentrated HNO_3 and H_2O_2. Morphology of synthesized powders was studied using TEM (JEOL JEM-2100 LaB_6 instrument operated at 200 kV). TEM images were acquired with a Gatan Orius CCD camera at 2 × binning. Nitrogen adsorption-desorption isotherms were determined on Sorptomatic 1990 Thermo Finnigan automatic system. Optical properties of synthesized powders were investigated by diffuse reflectance spectroscopy (Shimadzu UV-Visible UV-2600 spectrophotometer equipped with an integrated sphere ISR-2600 Plus).

2.2. Antimicrobial activity of SHAP/5-ASA/Ag nanocomposite

The antibacterial activity of SHAP/5-ASA/Ag nanocomposite was evaluated against pathogenic bacterial species, Gram-negative bacteria *E. coli* (ATCC 25922) and Gram-positive bacteria *S. aureus* (ATCC 25923), while antifungal activity was evaluated against yeast *C. albicans* (ATCC 10259). Details of concentration- and time-dependent measurements of microbial reduction are presented in Supporting Information 1.

In addition, the time-dependent antibacterial activities of SHAP/5-ASA/Ag nanocomposite against *E. coli* and *S. aureus* were tested under long-run conditions in five repeated cycles using concentration of 1.0 mg mL^{-1} of nanocomposite. Successive introduction of live bacterial cells (approximately 10^5 CFU mL^{-1}) was performed with the frequency of 24 h. Determination of viable bacterial cells is described in Supporting Information 1.

3. Results and discussion

3.1. Synthesis and characterization of SHAP and SHAP/5-ASA/Ag powders

The white SHAP powder was prepared by neutralization of CaO with H_3PO_4. The microstructural characterization of SHAP including TEM, selected area electron diffraction (SAED), as well as nitrogen adsorption−desorption isotherms and pore size distribution is presented in Fig. 1. The TEM imaging (Fig. 1A) indicated the presence of agglomerated rod-like SHAP particles (40−60 × 10−20 nm, length × diameter). Analysis of the SAED pattern (Fig. 1B) revealed the presence of the diffraction rings consistent with the (002), (102), (211), (130), (213), and (004) planes of the hexagonal phase of $Ca_5(PO_4)_3(OH)$ (Card No. 9013627). Nitrogen adsorption−desorption isotherms of SHAP are shown in Fig. 1C. The specific surface area was calculated using BET method [40], and it was found to be 62 m^2 g^{-1}. The average pore diameter was estimated from the desorption branch of isotherm using BJH method [41], and it was found to be 55 nm (see Fig. 1D). According to type of isotherm (Type II) and determined average pore diameter SHAP samples can be classified as macroporous. Also, it is well-known that SHAP is amphoteric and the zero point charge is at $pH_{zpc} = 6.5$ [7].

The functionalization of SHAP samples with 5-ASA induced immediate coloration of powders. The diffuse reflection spectra of as-prepared SHAP and surface-modified SHAP with 5-ASA (SHAP/5-ASA) powders are shown in Fig. 2 (curves a and b, respectively). Also, photographs of SHAP and SHAP/5-ASA are presented as inset to Fig. 2. As expected, SHAP does not absorb in UV-Vis spectral range due to large band gap (>6 eV) [42]. On the other hand, the appearance of absorption in visible spectral range upon surface modification (absorption threshold at 750 nm) can be explained in terms of the charge transfer (CT) complex formation between surface of wide band gap material and small colorless organic molecule with specific molecular structure. So far, the CT complex formation between surface atoms and benzene derivatives with adjacent hydroxyl and carboxyl groups (salicylate-type of ligands) has been reported for TiO_2 particles [35–38] and Mg_2TiO_4 [39]. To the best of our knowledge, this approach has never been used to extend optical absorption of HAP in the visible spectral range.

However, the investigation of optical properties of surface-modified SHAP with 5-ASA is not the main focus of this study. Our intent was to achieve linkage between organic molecules (5-