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Domination of volumetric toughening by silver nanoparticles over interfacial strengthening of carbon nanotubes in bactericidal hydroxyapatite biocomposite

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ABSTRACT

In order to address the problem of bacterial infections in bone-substitution surgery, it is essential that bone replacement biomaterials are equipped with bactericidal components. This research aims to optimize the content of silver (Ag), a well-known antibacterial metal, in a multiwalled carbon nanotube (CNT) reinforced hydroxyapatite (HA) composite, to yield a bioceramic which can be used as an antibacterial and tough surface of bone replacement prosthesis. The bactericidal properties evaluated using *Escherichia coli* and *Staphylococcus epidermidis* indicate that CNT reinforcement supports growth of Gram negative *E. coli* bacteria (~8.5% more adhesion than pure HA); but showed a strong decrease of Gram positive *S. epidermidis* bacteria (~diminished to 66%) compared to that of pure HA. Small amounts of silver (2–5 wt.%) already show a severe bactericidal effect when compared to that of HA–CNT (by 30% and ~60% respectively). MTT assay confirmed enhanced biocompatibility of L929 cells on HA–4 wt.% CNT (~121%), HA–4 wt.% CNT–1 wt.% Ag (~124%) sample and HA–4 wt.% CNT–2 wt.% Ag (~100%) when compared to that of pure HA. The samples with higher silver content showed decreased biocompatibility (77% for HA–4 wt.% CNT–5 wt.% Ag sample and 73% for HA–4 wt.% CNT–10 wt.% Ag). Though reinforcement of 4 wt.% CNT has shown an increase of fracture toughness by ~62%, silver reinforcement has shown enhancement of up to 244% (i.e. 3.43 times). Accordingly, isolation of toughening contribution indicates that volumetric toughening by silver dominates over interfacial strengthening contributed by CNTs towards enhanced fracture toughness of potential HA–Ag–CNT biocomposites.

1. Introduction

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is widely used in orthopedic surgery due to its similarity to apatite in human bone skeleton (Ca/P ratio 1.67), which displays an exceptional biocompatibility [1–3]. The poor hardness and fracture toughness and the promotion of bacterial growth, which leads to bacterial infection, are the two big challenges that are addressed in order to obviate implant rejection [4–7]. Carbon nanotube (CNT) reinforcement of HA has been widely studied to enhance the mechanical properties because of its high stiffness (Young's modulus of up to 1 TPa) [8–11]. Also CNTs are an attractive addition due to its physiochemical and biological properties [12–16]. Balani et al. and Agarwal et al. report superior optimized tribological and mechanical performance (fracture toughness increase of 56%) for HA–CNT composites containing 3–5 wt.% multiwalled CNTs, while showing good cytocompatibility [8,12]. The interaction of CNT with different bacteria as well as mammalian cells is still being discussed controversially. The literature implies the dependence on various different factors like tube length and diameter, structure of the CNTs (multi- or singlewalled), functionalization of the CNTs, type of the cell and structure of the cell membrane (Gram positive/negative for bacteria). For mammalian cells, referring to bone forming, cell's multiwalled CNTs are reported to have good interaction with osteoblasts and fibroblasts [8–11,13,17–21], while depending on the size, singlewalled CNTs show cytotoxicity by blocking potassium channel activities and therefore might decrease the biocompatibility of implants [18,22].

Elimelech et al. has documented bactericidal properties for single- and multiwalled CNTs on *Escherichia coli* (Gram negative), eliciting severe bactericidal property of singlewalled CNTs due to the smaller diameter of the tubes [23]. Similar results exist for Gram positive and negative bacteria [24,25]. Then again, enhanced *E. coli* growth and proliferation on multiwalled CNTs are also reported in the literature [5]. To further handle the missing bactericidal properties of HA, several studies have been done on adding a second phase with antimicrobial agents [4,26–28]. Silver is a bactericidal metal with rising significance in biomedical applications [5,29]. It is known that Ag^+ ions are able to diffuse into the cytoplasm through bacterial membranes, where they

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interact with the DNA, RNA and bacterial enzymes, particularly to their SH-groups, leading to dysfunction of the cell metabolism and protein biosynthesis leading to suppression of cell division and ensued cell death [5,29–33]. Special attention has to be drawn on the influence of silver ions on human cells. Micrometer sized silver particles as well as silver nitrate or lactate are reported to show a strong cytotoxic effect which questions the application of silver in biocomposites [34]. On the other hand, recent studies demonstrate remaining biocompatibility upon addition of low amounts of silver nanoparticles [33,27]. Valiyaveettil et al. elaborated the mechanisms of AgNPs acting toxic on human fibroblast glioblastoma cells and stated a dependency of the cytotoxicity on the applied dose of AgNPs, making appearance by disruption of the mitochondrial respiratory chain of the cell, a reduced ATP content, increased production of reactive oxygen species and damaged DNA [35].

But, only a limited literature exists on simultaneously evaluating the effect of AgNPs on both, bacteria and mammalian cells that show antibacterial effects while retaining biocompatibility [27,33]. This indicates a different sensitivity towards a certain silver amount of eukaryotic and prokaryotic cells. Possible reasons are, for instance, differences in size and metabolic functions. Since eukaryotic cells are usually larger than prokaryotic cells, a far bigger concentration of silver ions is necessary to achieve a similar toxic effect. Also, high structural organization of eukaryotic cells through compartmentalization, which enables decentralized energy conservation in mitochondria or the separated DNA localization in the core, weakens the impact of silver ions compared to prokaryotes [33]. Referring to silver reinforced implants this observation suggests that an AgNP dose range can be found, in which prokaryotic cell metabolism is successfully hampered, while no constraint eukaryotic viability can yet be observed.

To achieve densification of novel materials, spark plasma sintering (SPS) has been established over the last decades, evolving from the idea of activating the sintering process by the use of electrical current that was introduced in 1933 [36]. Evident advantages compared to conventional methods like pressureless sintering or hot pressing reside in decreased sintering temperature (by 150–200 °C) and shortened dwell times (sintering time of 5–15 min). These innovations allow sintering of nanometer-sized powders to near theoretical values with sparse grain growth [36–38]. The high densification of the powder sample obtained by sintering via the SPS route is favorable for the enhanced mechanical properties of the composite. Still, CNTs tend to cluster and entangle during sintering, which can lead to marginally higher porosity of the sample [5]. At moderate sintering temperatures of 900–1200 °C, decomposition of HA with formation of a TCP phase (at 1400 °C) [7,39] and the carbon nanotubes in spheroid particles at temperatures higher than 1700 °C can be avoided [5,40–43].

For samples containing silver which has a melting point of ~960 °C, sintering can bear the benefit of densification through a liquid silver phase, but de-mixing of the phases needs to be avoided [5]. Functionally graded materials are a new approach to meet the requirements of bioceramics to interact best possible with the surrounding tissue and still bear the high hardness and fracture toughness in the core to handle the loads of a long lasting implant. In a non-monolithic bone replacement material, highly bioactive components like reinforced HA on the outside can be combined with inner layers of strong materials like yttria stabilized zirconia or alumina oxide [44]. Challenges are to durably bond the different layers to a single composite which means that a high gradient of mechanical properties is not favorable. It has been shown, that the implication of intermediate layers with adjusted qualities can lead to a gradual change among properties from surface to core, and therefore elicit reliable mechanical performance [44–47].

The main focus of the present study is the enhancement of antibacterial properties with the highest possible content of silver without influencing the good attachment of body cells and without decreasing the mechanical properties. In regard to the application of HA–CNT–Ag, as a part of functionally graded material, HA is reinforced with 4 wt.% of multiwalled carbon nanotubes to improve the mechanical strength and to make it compatible with potential other layers. The CNT reinforced HA matrix is complemented by a varying silver-nanoparticle content of 0; 1; 2; 5 and 10 wt.%. The influence of the mechanical properties is characterized by Vickers indentation and indentation fracture toughness. Pure HA is processed under the same conditions and is used as a reference. Bactericidal properties are evaluated using Gram positive (*Staphylococcus epidermidis*) as well as Gram negative (*E. coli*) bacterial colonies. In order to establish the optimal content of silver, cytocompatibility of sintered HA–CNT–Ag biocomposites is studied though *in vitro* cell culture with L929 mouse fibroblast cell line, which is quantified via MTT assay.

2. Materials and methods

2.1. Material processing

Starting powders were hydroxyapatite (HA), multiwalled carbon nanotubes (CNTs) and silver nanoparticles (AgNPs). HA powder (~15–55 nm) was synthesized using a suspension–precipitation method [5,10] and it was ball-milled for 16 h with a ball-mass ratio of 10:1 in ethanol. Multiwalled CNTs (95% purity, inner dia 20 nm, and outer dia 40 nm, 1–2 μm long) were purchased from Nanostructured & Amorphous Materials Inc., United States. The dried HA powder and the CNTs were sieved separately for homogenization with a sieve of mesh size 120 (125 μm). The AgNPs were procured from Sigma Aldrich, United States, with high purity (99.5%) and with particle size of less than 100 nm. Powder mixtures of six different compositions were prepared which were pure HA, HA with 4 wt.% CNT, HA with 4 wt.% CNT and x wt.% AgNPs where x = 1, 2, 5 and 10. The nomenclature of the prepared powders is provided in Table 1. The SEM image of powder mixture of HA with 4 wt.% CNT and 10 wt.% Ag has been shown in Fig. 1(a). The powders were mixed in ethanol and ultrasonicated for 5 min followed by magnetic stirring while heating at 80 °C in order to evaporate the solvent. This obtained paste was dried for 12 h at 200 °C and subsequently sieved with mesh size 170 (90 μm). Quantitative elemental analysis, Fig. 1(b), shows the presence of silver in HA–CNT matrix.

The powders were subsequently processed by rapid spark plasma sintering (SPS, Dr. Sinter 1050 SPS apparatus, Sumitomo, Japan/Dr. Sinter 511 S SPS) in a cylindrical graphite die with an inner diameter of 15 mm. Sintering was performed at temperature of 950 °C (with ramp rate of 100 °C/min) and a dwell time of 5 min under uniaxial pressure of 30 MPa in vacuum (~6 Pa) in order to obtain dense ~3 mm thick pellets.

2.2. Phase and microstructural characterization

The density of the pellets was measured via Archimedes' water immersion principal using distilled water on a commercial weighing machine (Citizen CX 220) with decimal accuracy of 0.1 mg. The theoretical densities of the composites while taking the density of pure component were taken as 3.16 g cm^{-3}, 2.10 g cm^{-3}, 10.49 g cm^{-3} and 0.988 g cm^{-3} for HA, CNT, Ag and H_2O respectively. Porosity may affect cell adhesion due to affected roughness and enhanced surface area [48].

The pellets were polished with emery paper followed by cloth polishing with an alumina particle suspension, particle size 5 μm and 1 μm. Phase analysis was conducted with X-ray diffraction technique (X-ray diffraction; ISO Debyeflex-2002, Rich Seifert & Co., Germany) with $Cu_{K\alpha}$ radiation, $\lambda = 0.15418$ nm at a step size of 0.02°, and scan rate of 1°/min. The critical analysis of the X-ray diffraction-data for confirming phase stability and absence of undesirable formed products during sintering was performed with the help of Pearson's Crystal Data Base (PCD). Raman spectroscopy (WITec GmbH, Germany, alpha 300 series microscope equipped with a furnace cooled charge coupled device (CCD)) was performed on the samples to detect the presence of CNT using argon laser (of wavelength of 514 nm).

The fractured surfaces and distribution of the CNTs and Ag were examined via scanning electron microscopy (SEM; SUPRA 40VP, Carl Zeiss NTS GmbH, Germany), and elemental analysis was carried out using