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Preparation and *in vitro* characterization of silver-doped bioactive glass nanoparticles fabricated using a sol-gel process and modified Stöber method

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ABSTRACT

The study aimed at preparing Ag-doped bioactive glass (BG) nanoparticles containing 5 wt% Ag_2O using a new modified Stöber method (Ag-BGm) and the conventional sol-gel method (Ag-BGsg). The aim of employing the new Ag-doping mechanism is to implement a relatively short process to incorporate higher concentration of silver (Ag^+) ions in an attempt to combine the antibacterial activity of silver with the bioactivity of BG. SEM and TEM micrographs showed that Ag-BGm nanoparticles were mondispersed, and spherical, while Ag-BGsg nanoparticles were formed of highly porous irregular agglomerated particles. A significantly higher silicon, calcium and silver ions release from the Ag-BGsg was evident, as compared to Ag-BGm, in deionized water (DIW). *In vitro* bioactivity test showed rapid bioactive properties of Ag-BGsg in simulated body fluid (SBF), as compared to Ag-BGm. Both BGs showed antibacterial effect against *E. coli* O157: H7 wild type strain 93111 and *S. aureus* ATCC 25923 as evident by the disc diffusion assay, but Ag-BGsg showed significantly higher mean inhibition zone compared to Ag-BGm. It may be concluded that both Ag-BGsg and Ag-BGm has the potential to be used as bone substitute materials with the Ag-BGsg being more promising due to its high bioactivity and antibacterial effect.

1. Introduction

A characteristic feature of bioactive materials, including bioactive glasses (BGs) and bioactive ceramics, are the time-dependent, dynamic interactions that occur at the surface upon contact with biological fluids. The formation of highly reactive carbonated hydroxyapatite layer (HCA) provides the bonding interface with bone, as well as soft tissues for special compositions [1].

Silica-based BGs are one of the most promising bioceramics proposed as bone substitutes and tissue regeneration matrices. This was attributed to their bioactivity, tissue compatibility (with both hard and soft tissues), osteoconductivity, possibly even osteoinductivity and the ability to induce bone-like mineral phase on its surface in direct contact with the host tissues [2–4].

Such bioactive role is achieved through releasing critical concentrations of biologically active soluble silicon (Si), calcium (Ca), phosphorus (P) and sodium (Na) ions, during its controlled dissolution, at the rate needed for cell proliferation and differentiation [5].

The most common techniques for the production of BGs are the traditional melt quenching and the novel sol-gel methods. In the melt quenching technique, glass is prepared through melting a mixture of the required stoichiometric amounts of high purity different constituent oxides or carbonates at high temperatures. In 1991, Li et al. [6] introduced a novel low temperature sol-gel chemical process for BGs fabrication. Sol-gel process is a room temperature process, involving the formation of an inorganic network through mixing of metal alkoxides in solution, followed by hydrolysis, gelation, and low-temperature firing (600–700 °C) to form the glass [6]. It was proposed by Orcel et al. [7] that sols are made of primary particles (~2 nm in diameter), which form secondary particles (~6 nm in diameter) upon aggregation. A gel is then formed through agglomeration of the secondary colloidal nanoparticles into a highly connected 3D network, when the conditions are acidic [8,9], which accounts for the nanoporosity of the sol-gel-derived BGs, as compared to the dense melt-derived glasses. This nanoporosity results in higher specific surface area with higher bioactivity and more rapid degradation rates. Also the nanotopography, mimicking the *in vivo* environment, could result in improved cellular response [10].

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However, if the preparation process is done in basic environment, a different scenario occurs where the colloidal nanoparticles form spherical monodispersed particles. Preparing the silica nanoparticles under such basic conditions is known as the Stöber process [11]. Using ammonium hydroxide as a catalyst, instead of nitic acid, raises the pH value quickly to ~11 which is above the isoelectric point of the silica particles, causing particles to be electrostatically repulsed from each other, terminating polycondensation and no agglomeration occurs thus, the secondary particles remain as discrete particles [9,12]. To produce BG nanoparticles, modification of the classical Stöber process is performed through addition of $Ca(No_3)_2$ to impart bioactive properties to the prepared particles [9].

Development of bacterial infections in the implant site often complicates wound healing [13–15]. The local release of antimicrobial agents at the implant site is considered a potent solution for such an issue [13–15], this could be achieved by loading antibiotic molecules onto the scaffold surface, through dipping it into antibiotic solutions [14–17]. Yet, this approach carries the risk of biofilm formation and the development of resistant, mutant bacterial strains by the prolonged release of prophylactic inhibitory amounts of antibiotics [14,18].

Bioactive glasses were reported to show antibacterial effect against different types of bacteria [19,20]. The antibacterial activity of BG was reported to be attributed to the high pH levels [20] and osmotic effects caused by the non-physiological concentration of ions dissolved from BG. The release of high concentrations of Ca and alkalis could cause disturbances in the membrane potential of bacteria [21]. Hence, lowering the particle size have been suggested to enhance the antibacterial action by increasing the surface area available for the release of alkaline species [19]. Although other investigations reported lack of antibacterial activity for the undoped BGs [22,23].

The antimicrobial properties of silver ion (Ag^+) is well documented, characterized by its broad-spectrum antimicrobial action [13,24–29]. Several theories have been proposed to explain its antibacterial effect; including loss of bacterial DNA replicative ability, altering their membrane permeability and inactivation of some cellular proteins and enzymes, essential to ATP production. Still, the exact mechanism of antibacterial effect of silver nanoparticles is not yet fully understood [24,25,30].

Thus, silver addition to bioactive glasses would benefit from the potential bacteriostatic and bactericidal activity of the leaching Ag^+ ions in minimizing the risk of microbial contamination, without damage to human cells, while preserving the bioactivity. The leached Ag^+ ions were proven to provide bacteriostatic and bactericidal effects on gram-positive and gram-negative bacteria [13,14,23,25–27,31–33].

Although Ag-doping of the sol-gel-derived BG nanoparticles has been widely studied, only one research, to our knowledge, has been conducted to investigate the antibacterial effect of monodispersed Ag-doped BG prepared by the modified Stöber method [34]. This latter study incorporated a relatively low concentration of Ag_2O and implemented a long post-preparation surface modification process, that needed 6 h. In addition, this doping concentration and mechanism imparted an antibacterial effect only against Gram-negative *E. coli*.

Therefore, the aim of the present study was to employ a new doping mechanism that incorporates a higher concentration of Ag^+ ions into the BG during its fabrication and that requires a much shorter preparation time.

Another objective was to determine which of the two BGs, this new monodispersed Ag-doped glass or the conventional Ag-doped sol-gel-derived glass, would be the better choice for bone tissue engineering applications.

2. Materials and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS) and calcium nitrate tetrahydrate $Ca(NO_3)_2{\cdot}4H_2O$ purity were ≥ 99.0% and were purchased from Sigma-Aldrich (Germany). Triethyl phosphate (TEP) purity was ≥ 99.0% and purchased from Merck (Germany). Silver nitrate $AgNO_3$, Ammonia solution, 28%, nitric acid, 65%, and ethanol, 96%, were purchased from VWR Chemicals (Germany). Both nitric acid and ammonia solutions were diluted to 2 M using distilled water. All the reagents used were of analytical grade and were used as received without further purification.

Table 1
The nominal compositions (wt%) for the prepared bioactive glass nanoparticles; undoped (BGsg and BGm) and Ag-doped (Ag-BGsg and Ag-BGm).

Fabrication method	BG groups[a]	Composition (wt%)			
		SiO_2	Cao	P_2O_5	Ag_2O
Conventional sol-gel method	**BGsg**	58	33	9	0
	Ag-BGsg	58	28	9	5
Modified Stöber method	**BGm**	50	50	0	0
	Ag-BGm	50	45	0	5

[a] **BGsg**: undoped sol-gel-derived glass, **Ag-BGsg**: silver-doped sol-gel-derived glass, **BGm**: undoped monodispersed glass, **Ag-BGm**: silver-doped monodispersed glass.

2.2. Methods

2.2.1. Sol-gel-derived bioactive glass nanoparticles

Both bioactive glass nanoparticles; undoped (BGsg) and 5 wt% Ag-doped (Ag-BGsg), were prepared through the sol-gel process, according to method described by Xia et al. [35] and El-Kady et al. [13]. The nominal composition of the two powders is shown in Table 1.

TEOS (21.6 ml) as a precursor to silicon dioxide (SiO_2), distilled water (13.9 ml) and 2 M nitric acid (2.8 ml) (as a hydrolysis catalyst), were mixed in 50 ml ethanol. The mixture was allowed to react for 60 min at room temperature under continuous magnetic stirring to allow for the acid hydrolysis of TEOS. Next, 2.2 ml of TEP as a precursor to phosphorus pentoxide (P_2O_5) was added and stirred for 30 min. Ca $(NO_3)_2{\cdot}4H_2O$ (14.04 g) as a precursor of calcium oxide (CaO) was added and stirred for 60 min to allow for the completion of hydrolysis. At the end of the reaction, 10 ml of 2 M ammonia solution (a gelation catalyst) was added dropwise to the mixture while vigorously stirring. Gelation of the mixture took place in 2–3 min. At this stage, mechanical rather than magnetic stirring was performed to avoid formation of a monolithic gel.

For preparing 5 wt% Ag-BGsg, the same procedure was performed, except that 11.81 g $Ca(NO_3)_2{\cdot}4H_2O$, instead of 14.04 g, was added and stirred for 30 min. Then, silver nitrate ($AgNO_3$) (0.733 g) was added and stirred for 60 min.

Finally, the resulting gels were dried (Nabertherm More Than Heat 30–3000 °C, Nabertherm GmbH, Germany) at 60 °C for one day to remove the residual water and ethanol. The dried gel powders were calcined at 700 °C for 2 h, with a heating rate of 2 °C/min.

2.2.2. Monodispersed bioactive glass nanoparticles

Both undoped (BGm) and 5 wt% Ag-doped (Ag-BGm) monodispersed spherical bioactive glass nanoparticles were prepared by a modified Stöber method, according to method described by Kozon et al. [34] with modification. The nominal composition of the two powders is shown in Table 1.

First, a solution containing 155 ml ethanol and 18.6 ml TEOS, was added to another solution containing 77 ml distilled water, 50.4 ml ethanol and 28 ml ammonia solution (28%), under magnetic stirring. After 30 min of reaction, 21.25 g $Ca(NO_3)_2{\cdot}4H_2O$ was added to the suspension and stirred for another 1.5 h.

For preparation of Ag-doped monodispersed particles (Ag-BGm), after mixing the two solutions for 30 min of reaction, $Ca(NO_3)_2{\cdot}4H_2O$ (18.9) g was added to the suspension and stirred for another 30 min, followed by the addition of 0.75 g $AgNO_3$ to the suspension and stirring