Andrea Lončarević, Leonard Bauer, Anamarija Rogina, Hrvoje Ivanković

Preparation of chitosan-copper complex microspheres modified by bioactive glass

Faculty of Chemical Engineering and Technology University of Zagreb, Trg Marka Marulića 19, HR-10000 Zagreb Croatia

Abstract

The aim of this work was to prepare composite microspheres with high sphericity and narrow size distribution based on chitosan-copper complex and bioactive glass (bioglass). The influence of the bioactive glass content on the size and morphology of chitosan-copper complex microspheres was investigated. The electrohydrodynamic atomization process was used to produce highly spherical particles of narrow size distribution and with defined surface morphology. The addition of bioglass particles caused the surface changes, from smooth to wrinkled surface by increasing the bioglass quantity. The size of the obtained microspheres was estimated to be between 40 and 100 μm, depending on the bioglass content.

***Keywords**: chitosan, bioactive glass, electrospraying, microspheres*

1. Introduction

Natural human bone undergoes continuous process of formation, resorption and remodeling. In small defects of damage, bone possesses the self-healing ability [1]. However, larger defects which usually originate from surgical resection require implants, i.e., temporary supports for cell adhesion, proliferation and tissue regeneration. Furthermore, sufficient vascularization is necessary for the repair of the large bone defects, providing transports of oxygen, nutrients and osteoprogenitor cells to facilitate bone regeneration [2]. Bioactive glasses (bioglass, BG) are recognized as promising biomaterials in bone repair application due to their bioactivity, pro-osteogenic and pro-angiogenic abilities [3]. On the other hand, chitosan-based microspheres have been exploited as carriers of bioactive molecules due to the targeted delivery [4,5]. More significantly, polycationic nature of chitosan allows it to interact with metal ions involved in biological processes of bone formation and remodeling, called therapeutic metal ions (TMIs) [6].

The application of therapeutic metal ions emerges as a potential alternative for tissue regeneration without using expensive biomolecules, such as growth factors [7]. Copper, zinc, magnesium and strontium belong to a family of therapeutic metal ions due to their key role in regulating physiological pathways, such as angiogenesis, osteogenesis, proliferation, differentiation, and antibacterial properties. Combining biologically active ions with pH-responsive chitosan microspheres and bioactive glasses could generate multifunctional carriers and delivery systems for bone tissue engineering. The physical nature of complexation interactions of chitosan and doped BGs can optimize the delivery of necessary therapeutic metal ions, which play an important role during angiogenesis and homeostasis.

This work proposes the production of microsized biodegradable matrices functionalized by copper (II) ions through complexation reactions with chitosan and their modification by bioactive glasses. The electrohydrodynamic atomization process was used to produce microspheres with a narrow size distribution. Furthermore, the different content of bioglass affected the morphology and size of the microspheres.

2. Experimental part

2.1. Materials

In this work, Chitosan with DD = 87% (Chitoscience, CHT85/100) was purchased from Heppe Medical Chitosan GmbH (Halle (Saale), Germany). Copper acetate monohydrate (BDH Prolabo, Leuven, Belgium) was used as a precursor of copper (II) ions (Cu^{2+}) for the complexes' preparation. Other chemicals for materials preparation were 99.8% acetic acid (HAc; Lach–Ner, Neratovice, Czech Republic), sodium hydroxide (NaOH; Honeywell, Seelze, Germany), 96% ethanol (EtOH; Kefo, Ljubljana, Slovenia) and acetone (T.T.T. doo, Sveta Nedjelja, Croatia). All chemicals were of analytic grade. Bioactive glass powder (45S5 [8], particle size ~4 μm) was kindly provided by Prof. A.R. Boccaccini for the preparation of composite microspheres.

2.2. Preparation of chitosan-Cu/BG solutions

1.0 wt.% chitosan solution was prepared by dissolving polymer powder in 1% solution of acetic acid for 2 h at ambient conditions.

The chitosan–copper (II) ions complex solutions were prepared by mixing the appropriate weight of copper acetate monohydrate into 1.0 wt% chitosan solution followed by stirring for 2 h. The prepared CHT–copper (II) solution was designated as CHT–Cu3. As described in detail in our previous work [9], the amount of Cu^{2+} ions was added with respect to the molar ratio of Cu^{2+} ions and amino groups in chitosan as $n(Cu^{2+}) : n(-NH_2) = 0.0549:1$.

The chitosan-Cu solutions modified by bioglass particles (CHT-Cu/BG) were prepared by mixing the CHT-Cu3 solution with the 1, 2, 3, 4, 5 and 10 wt.% of bioglass and were designated as CHT-Cu/1BG, CHT-Cu/2BG, CHT-Cu/3BG, CHT-Cu/4BG, CHT-Cu/5BG and CHT-Cu/10BG, respectively. Furthermore, 5 wt.% sodium hydroxide solution (NaOH) was used as a gelation medium during the electrohydrodynamic atomization process. All solutions were freshly prepared for each microsphere production.

2.3. Chitosan-Cu/BG microsphere production

The electrospraying process was performed using the setup shown in Figure 1, applying the constant process parameters given in Table 1.

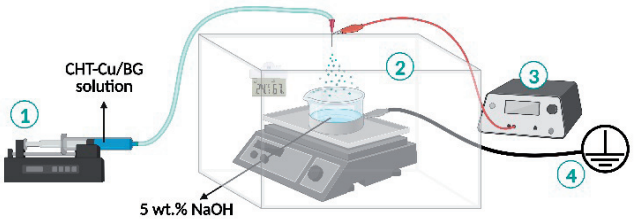

Fig. 1. Schematic illustration of electrospraying setup. 1 - syringe pump; 2 - isolating chamber; 3 - high voltage generator; 4 - grounding. Created with BioRender.com.

Table 1. Parameters of electrospraying process.

Parameter	Unit	Value
Concentration of acetic acid	%	1.0
Initial concentration of chitosan solution	wt.%	1.0
Flow rate of solution	mL h^{-1}	5
Needle gauge	G	23
Applied voltage	kV	20
Distance between the needle tip and collector	cm	10
Concentration of NaOH	wt.%	5
Volume of NaOH (collector)	mL	50
Duration of electrospraying	min	60
Temperature (in the chamber)	°C	25 ± 1
Relative humidity (in the chamber)	%	66 ± 5

The electrospraying process was conducted as follows: the syringe (Becton Dickinson, Le Pont-de-Claix, France) was filled with the prepared chitosan–Cu complex solution (10 mL) and processing parameters presented in Table 1 were set. The needle was positively charged, and the collector was grounded. The container with 50 mL of gelation medium isolated with aluminum foil was used as the collector. The distance between the collector and the blunt tip needle was 10 cm. The required voltage for the electrospraying process was determined according to the formation of stable Taylor cone-jet mode. For CHT85/100-Cu3/BG-based complex solutions, a stable jet was observed at the voltage of 20 kV. The flow rate of solutions was 5 mL h^{-1} and the process was performed for 60 min without interruption, while gentle stirring the gelation medium.

After electrospraying, the gelation medium containing produced microspheres was stored for the next 2 h to complete gelation. Microspheres were then washed with distilled water until neutral pH, dehydrated with 96% EtOH for 2 h and acetone for several times. At the end, microspheres were left at the ambient conditions for solvent evaporation.

2.4. Characterization of chitosan-Cu/BG microspheres

The composite microspheres were analyzed with a BA200 binocular microscope (light microscope; Motic Instruments, Barcelona, Spain). Pictures were taken using the Motic Images Plus 2.0 program and processed by ImageJ 1.53e software used for the estimation of the microspheres' size. The diameter was calculated from the area of at least 100 randomly chosen microspheres, assuming the total sphericity of microspheres.

The morphology of composite microspheres was investigated by scanning electron microscopy (SEM; Tescan Vega III Easyprobe, Brno-Kohoutovice, Czech Republic) with an electron beam energy of 10 keV. Prior to imaging, microspheres were sputtered with gold and palladium for 60 s.

3. Results and discussion

The functionalization of chitosan with divalent metal ions can be accomplished by simple complexation chemistry, where amino, amide and hydroxyl groups of chitosan act as ligands in metal coordination. Such physical interactions can improve mechanical properties of chitosan [10]. Previously [11], we prepared copper (II)-chitosan complex microspheres, where Cu–chitosan complexes with higher Cu^{2+} amount showed more stable microspheres produced by electrospraying. Moreover, FTIR spectroscopy indicated structural changes of chitosan where free hydroxyl and amino groups are involved in metal coordination. Such coordination can form structures that can be described by two models, 'pendant' and 'bridge' model [12]. Several types of $[CuNH^2(OH)^2X]$ complexes (where X can be water molecules or hydroxyl groups of chitosan) with 'pendant' configuration were proposed [13]. Depending on the pH of aqueous solution, copper can be

coordinated by amino group, hydroxide ions and water molecules. On the contrary, the 'bridge' model was described by the tetrahedral coordination with amino groups of the same or a different chitosan chain.

In the present study, the previously developed chitosan-copper complex microspheres were modified by bioactive glass particles to extend their functionality for bone tissue engineering application. The successful production of bioglass-modified microspheres was observed using optical microscope, shown in Figure 2. The produced composite microspheres were spherical regardless of the bioglass content. In our previous work [11], we showed that the concentration of copper (II) ions influences the production of spherical particles. Chitosan-Cu complex systems with lower $c(Cu^{2+})$ yielded deformed particles or precipitates of an undefined shape as a result of less physical crosslinks between amino and hydroxyl group and metal ion. Taking into account good sphericity of composite microparticles, we could assume that the addition of BG particles did not significantly affect the physical crosslink between chitosan and copper (II) ions.

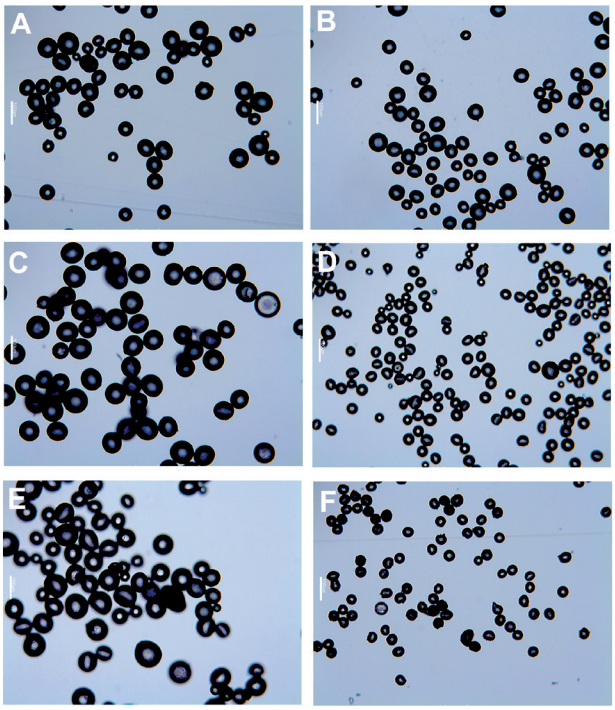

Fig. 2. Chitosan-copper complex microspheres with different amount of BG particles: A) 1%, B) 2%, C) 3%, D) 4%, E) 5%, F) 10%. Scale bar: 100 µm.

The size distribution of composite microspheres was estimated by light microscopy (Figure 3).

The size of composite microspheres ranged from 60 to 100 µm, depending on the BG content. The addition of bioglass particles had an impact on the distribution of particles size, i.e. the composite microspheres with 10% of BG showed the narrowest size distribution. Furthermore, the highest amount of BG particles resulted in the smallest composite microspheres (size of 40 – 60 µm).

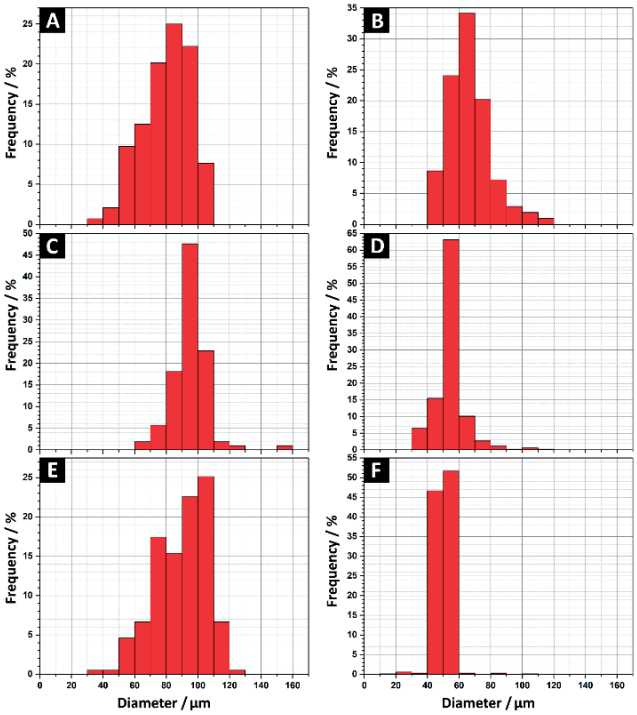

Fig. 3. Size distribution of A) CHT-Cu/1BG, B) CHT-Cu/2BG, C) CHT-Cu/3BG, D) CHT-Cu/4BG, E) CHT-Cu/5BG and F) CHT-Cu/10BG microspheres.

The viscosity and surface tension of the polymer solution are the main parameters that influence the electrospraying process [14]. A previous study indicated that larger particles are obtained when a higher viscosity of solution is applied [15]. For our chitosan-Cu complex solutions [11], the viscosity was decreased by a higher Cu concentration as a result of the complex formation. Here, a possible reason for obtaining smaller particles of the composite with 10% of BG could be changes in viscosity caused by physical interactions between polymer macromolecules and BG particles.

The influence of BG particles on microsphere morphology was observed in SEM micrographs (Figure 4). Gradual surface changes were observed with an increase of the BG content.

Microspheres with 1% of BG particles showed a smoother surface, while 10% of BG resulted in a non-homogeneous surface with noticeable wrinkling. Furthermore, some microspheres were slightly deformed. During the electrohydrodynamic atomization process, the applied electric field needs to overcome the surface tension of the polymeric solution to generate a drop. It has been observed [16] that a stronger field is required for a higher surface tension in order to obtain the regular shape of the microsphere. In the present study, all composite systems were produced at constant voltage, where only composite 10% BG yielded microspheres with different shape. It can be assumed that a higher addition of BG particles significantly changes the surface tension of the chitosan-Cu solution which in the end influences the microsphere shape and morphology. However, this effect needs to be confirmed by further in-

vestigation including electrospraying performed at different processing parameters.

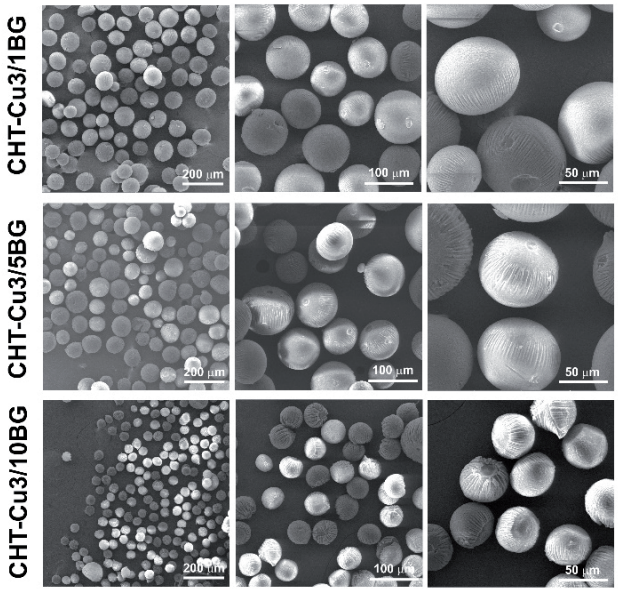

Fig. 4. SEM micrographs of chitosan-copper complex microspheres with 1%, 5% and 10% of BG microparticles at different magnification.

4. Conclusion

The electrohydrodynamic atomization process was used to produce chitosan-copper (II) microspheres modified by bioactive glass microparticles in this research. The increase of bioglass amount resulted in smaller microspheres with a narrow size distribution. Furthermore, microspheres containing 10% bioglass had greater surface roughness and minor shape deformation. The electrohydrodynamic atomization process could be a promising method for producing chitosan-Cu microspheres modified by inorganic phase.

5. References

[1] R. Dimitriou, E. Jones, D. McGonagle, P. V. Giannoudis, Bone regeneration: Current concepts and future directions, BMC Med. 9 (2011) 1–10.

[2] W. Yu, T.-W.W. Sun, Z. Ding, C. Qi, H. Zhao, F. Chen, Z. Shi, Y.-J.J. Zhu, D. Chen, Y. He, Copper-doped mesoporous hydroxyapatite microspheres synthesized by a microwave-hydrothermal method using creatine phosphate as an organic phosphorus source: application in drug delivery and enhanced bone regeneration, J. Mater. Chem. B. 5 (2017) 1039–1052.

[3] H. Sun, K. Zheng, T. Zhou, A.R. Boccaccini, Incorporation of Zinc into Binary SiO2-CaO Mesoporous Bioactive Glass Nanoparticles Enhances Anti-Inflammatory and Osteogenic Activities, Pharm. 13 (2021) 2124.

[4] Y. Zhou, H.-L. Gao, L.-L. Shen, Z. Pan, L.-B. Mao, T. Wu, J.-C. He, D.-H. Zou, Z.-Y. Zhang, S.-H. Yu, Chitosan microspheres with an extracellular matrix-mimicking nanofibrous structure as cell-carrier building blocks for bottom-up cartilage tissue engineering, Nanoscale. 8 (2016) 309–317.

[5] M.A. Islam, J. Firdous, Y.J. Choi, C.H. Yun, C.S. Cho, Design and application of chitosan microspheres as oral and nasal vaccine carriers: An updated review, Int. J. Nanomedicine. 7 (2012) 6077–6093.

[6] M. Rhazi, J. Desbrières, A. Tolaimate, M. Rinaudo, P. Vottero, A. Alagui, M. El Meray, Influence of the nature of the metal ions on the complexation with chitosan.: Application to the treatment of liquid waste, Eur. Polym. J. 38 (2002) 1523–1530.

[7] V. Mouriño, J.P. Cattalini, A.R. Boccaccini, Metallic ions as therapeutic agents in tissue engineering scaffolds: an overview of their biological applications and strategies for new developments, J. R. Soc. Interface. 9 (2012) 401–419.

[8] M.L. Dittler, P.M. Zelís, A.M. Beltrán, R. Destch, C.A. Grillo, M.C. Gonzalez, A.R. Boccaccini, Magnetic 3D scaffolds for tissue engineering applications: bioactive glass (45S5) coated with iron-loaded hydroxyapatite nanoparticles, Biomed. Mater. 16 (2021) 055006.

[9] A. Rogina, A. Lončarević, M. Antunović, I. Marijanović, M. Ivanković, H. Ivanković, Tuning physicochemical and biological properties of chitosan through complexation with transition metal ions, Int. J. Biol. Macromol. 129 (2019) 645–652.

[10] J. Nie, Z. Wang, Q. Hu, Chitosan Hydrogel Structure Modulated by Metal Ions, Sci. Rep. 6 (2016) 1–8.

[11] A. Lončarević, M. Ivanković, A. Rogina, Electrosprayed chitosan–copper complex microspheres with uniform size, Materials (Basel). 14 (2021) 5630.

[12] K. Ogawa, K. Oka, T. Yui, X-ray Study of Chitosan-Transition Metal Complexes, Chem. Mater. 5 (1993) 726–728.

[13] M. Rhazi, A. Tolaimate, M. Rhazi, J. Desbriéres, A. Tolaimate, M. Rinaudo, A. Alagui, P. Vottero, Contribution to the study of the complexation of copper by chitosan and oligomers, Polymer 43 (2001) 1267–1276.

[14] J.A. Tapia-Hernández, P.I. Torres-Chávez, B. Ramírez-Wong, A. Rascón-Chu, M. Plascencia-Jatomea, C.G. Barreras-Urbina, N.A. Rangel-Vázquez, F. Rodríguez-Félix, Micro- and Nanoparticles by Electrospray: Advances and Applications in Foods, J. Agric. Food Chem. 63 (2015) 4699–4707..

[15] N. Ardila, Z. Ajji, M.C. Heuzey, A. Ajji, Chitosan electrospraying: Mapping of process stability and micro and nanoparticle formation, J. Aerosol Sci. 126 (2018) 85–98.

[16] A. Bateni, A. Ababneh, J.A.W. Elliott, A.W. Neumann, A. Amirfazli, Effect of gravity and electric field on shape and surface tension of drops, in: Adv. Sp. Res. (2005) 64–69.