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Water-dispersed bone morphogenetic protein nanospheres prepared by co-precipitation method*

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Abstract: A modified complex coacervation-co-precipitation method was used to prepare bone morphogenetic protein (BMP)-loaded nanospheres. Three natural polymers were used as packing materials to obtain nanoscale delivery device for BMP, in the presence of phosphatidylcholine functioning as stabilizer. Positively charged polysaccharide, N,N-diethylaminoethyl dextran (DEAE-dextran) tended to form stable, uniform and smaller size particles carrying BMP. Negatively charged bovine serum albumin (BSA) induced precipitation of the produced BMP particles due to its weak interaction with BMP molecules, although it produced nanosized BMP spheres. While collagen, a weakly positively charged protein shaped larger particles due to the strong interaction among themselves. A mechanism of co-precipitation process was also deduced to depict the formation of stable nanospheres.

Key words: Co-precipitation, Nanoparticles, Bone morphogenetic proteins, Biopolymers
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INTRODUCTION

Bone morphogenetic proteins (BMPs) are a group of proteins that can induce new bone formation at orthotopical and heterotopical sites in experimental animals (Urist *et al*., 1983; Groeneveld and Burger, 2000). Their biological activity has stimulated interest in its clinical use for bone repair. In clinical use, BMPs are undergoing evaluation including system administration, gene transfer and local matrix delivery vehicle implantation (Kirker-Head, 2000; King *et al*., 1998). In the past decades, local delivery was at a relatively advanced stage of development. Because of the rapid clearance of BMP in serum, appropriate carriers retaining BMP are used for bone induction to increase its serum life. Many delivery devices including reservoir system, polymer matrix and gel have been studied (Winn *et al*., 1998; Bessho *et al*., 2002; Woo *et al*., 2001). However, there were few studies on the use of microspheres containing BMP for intravascular delivery, especially in nanometer size.

Complex coacervation method includes a process of spontaneous phase separation that occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution (Fessi *et al.*, 1989). It has been used to create microspheres and encapsulation for controlled release applications. The colloidal carriers produced are in the micrometer or nanometer size range depending on the substrates or the process used. The encapsulation process can be performed entirely in aqueous solution and at low temperature, and thus has larger chance of preserving the bioactivity of the biomacromole-

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cules. Hence, this method allows the preparation of nanospheres containing BMP packed with some natural polyelectrolytes such as proteins or polysaccharides.

In this paper, modified complex coacervation, i.e. co-precipitation method, was introduced to prepare microspheres of protein and polysaccharide containing BMP. To obtain nano-sized and stabilized BMP particles, biopolymers with different charge property were evaluated as packing materials in the presence of phosphatidylcholine, a stabilizer.

MATERIALS AND METHODS

Materials

Bovine Bone morphogenetic protein (BMP) donated by Antai Science and Technology Stock Ltd. was extracted from bovine femur. Bovine serum albumin (BSA) and N,N-diethylaminoethyl dextran (DEAE-dextran) were commercial products of Sigma. Lecithin was purchased from the Shanghai East China Reagent Corporation. Collagen was extracted from calf tendon by acetic acid and trypsin digestion method (Gao *et al.*, 2003). All other chemicals were of analytical grade and used as received.

Preparation and characterization of BMP-loaded microspheres

In an urea solution at acidic condition (pH 3.0), BMP (1 mg), phosphatidylcholine (5 mg) and BSA, collagen or DEAE-dextran (4 mg) were homogeneously dissolved. After the above composite solution was added dropwise into neutral pH PBS solution with violent magnetical stirring or ultrasonic agitation; the obtained dispersions were then sufficiently washed with the same buffer solution to remove urea and the free phosphatidylcholine, using a stirred ultrafiltration cell (model 8010, Micon), filter size 50 nm.

The produced microspheres were measured by atom force microscopy (AFM, SPI3800N, Seiko), particle analyzer (Mastersizer 2000, U.K.) and scanning electron microscope (SEM, Hitachi S-570, Japan).

RESULTS AND DISCUSSION

Liposome has long been used as drug carrier delivery system because of its convenient preparation, biocompatibility, low toxicity, biodegradability as well as its strong emulsion ability. Hence, phosphatidylcholine was firstly chosen as packing material of BMP to test its emulsion ability. Most particles produced directly packed with phosphatidylcholine were in the range of 200–600 nm in dry state observed by AFM. Some clusters were even several micrometers. Accumulation and precipitation of the dispersed BMP occurred after storage at 4 °C for 3 to 4 days. The results suggested that the phosphatidylcholine molecules can adsorb onto the BMP particles and stabilize them, otherwise big clusters or bulk precipitate of BMP will be formed. However, they may be easy to desorb from the particles because of the low molecular weight and rapid diffusion capability. Therefore, a highly stable macromolecular biomaterial would favor formation of a relatively stable BMP dispersion system, and may further decrease the particle size as well.

The isoelectric point (Ip) of bovine serum albumin (BSA) is about 4.2, while Ip of BMP is 4.9–5.10. In a neutral aqueous solution, both BSA and BMP are negatively charged. Therefore, in the next step, BSA is chosen as a packing biopolymer comparable to other positively charged biomaterials. After sufficient washing, the resultant BMP particles were observed under scanning electron microscopy. Fig.1a shows that most of the particles were well dispersed and separated from each other, resulting particle diameters of around 40 nm except for few ones with larger size (~100 nm). But there were some bigger clusters produced, probably caused by the self-agglomerate of BSA. Experiments showed that the dispersion methods had significant effect on the BMP particle size. Fig.1b is the graph of particle size distribution of BMP particles prepared by magnetically stirred dispersing. The diameters of the BMP particles varied from 100

to 500 nm, but the particle size distribution was very narrow. About 72% of the particles ranged from 160 to 250 nm, while only less than 5% of the particles were larger than 300 nm. Therefore, ultrasonication dispersion was adopted for the following studies.

We observed again that the pH value of the buffer solution affected the storage stability of the BMP dispersion. When the pH of the buffer solution was 5, the dispersion was stable after storage for 3–4 weeks at 4 °C. However, when the pH was higher or lower than this value, precipitation occurred from the BMP dispersion in 1 or 2 weeks. Hence one can conclude that BMP nanospheres can be prepared under the stabilizing influence of BSA and phosphatidylcholine. The shorter storage period should be caused by the lack of strong interaction between BSA and BMP, leading to release of BSA and phosphatidylcholine to the bulk solution. Hence, the stability of the BMP dispersion is expected to improve through increasing the molecular interaction between BMP and the packing materials. It is well known that electrostatic attraction is stronger than the intermolecular force. Thus, positively changed biopolymers would be preferable for obtaining a more stable BMP dispersion through the opposite charge interaction.

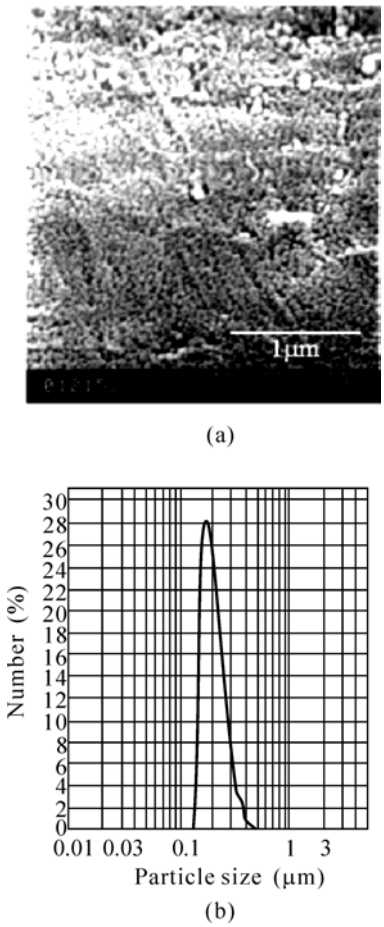

Fig.1 (a) SEM image of BSA-packed BMP particles prepared under ultrasonication, (b) The size distribution diagram of BSA-packed particle with magnetically mechanical dispersion

For this purpose, alkali-treated collagen, a weakly positively charged protein with Ip of 8, was then chosen as a packing material. Collagen matrices for delivery of BMPs were mentioned by Kirker-Head (2000). Different shape of matrices including membrane film, thread, sponge and tube forms have been used for implantation loaded with BMP. From AFM observation, the diameters of particles are almost uniform and about 200 nanometers, which is larger than albumin-BMP-lecithin particles. Due to the strong interaction among collagen, the nuclei formed were bigger than those packed with BSA. Therefore, the BMP particles obtained were bigger; and the storage stability was extended a month at 4 °C. A biopolymer with better emulsion ability is necessary for obtaining smaller particles with retained storage stability.

Thus, diethylaminoethyl dextran (DEAE-dextran), instead of BSA, a positively charged polysaccharide, was used as a packing material due to its strong charge nature. When the composite solution of BMP and DEAE-dextran was added to a pH 7 phosphate buffer solution, the negatively charged BMP tended to adsorb to the positively charged DEAE-dextran, forming microparticles under electrostatic stabilization and auxiliary emulsification of phosphatidylcholine. The SEM image of DEAE-dextran-packed BMP dispersion clearly showed that the resultant particles were comparatively uniform and well dispersed (Fig.2a). The diameters of the particles were about 70 nm. Fig.2b is the AFM phase graph of one particle. The particle surface was smooth. The AFM image showed that the average size of the particles was more than one hundred nanometers, while the

height was about 30−60 nm. This would mean that the particles collapsed in the drying process. Although the size of the resultant BMP particles was in the same range as in the case of BSA, the stabilization during storage was apparently improved. Continuous observation showed that the dispersion was stable up to one and a half months at ambient condition.

Fig.3 shows the morphology of the particles during storage. The particles produced were highly uniform, well separated, and had a regular spherical shape (Fig.3a). There was comparative change after 60 day's storage (Fig.3b). Most of them were obviously agglomerations of two or three particles and formed a united mass. This implies that BMP particles collided and agglomerated together in the solution during storage, and that as a result precipitation occurred.

A schematic presentation to illustrate the forming process and the mechanism of the co-precipitation method is given in Fig.4. In an urea solution at acidic condition, positively charged biopolymers and BMPs are homogeneously dissolved. At Ip of 5.0, BMP is also positively charged. After subjecting the above composite solution to neutral PBS solution that is a poor solvent for BMPs, BMPs immediately precipitated from the solution to form tiny particles following its charge reversal. Because of the electrostatic interaction, the positively charged biopolymer spontaneously wraps the negatively charged particles to form hydrophilic shells to decrease the interfacial tension. Therefore, the newly formed tiny BMP cores are stabilized instantaneously without further growth and self-accumulation. Moreover, phosphatidylcholine

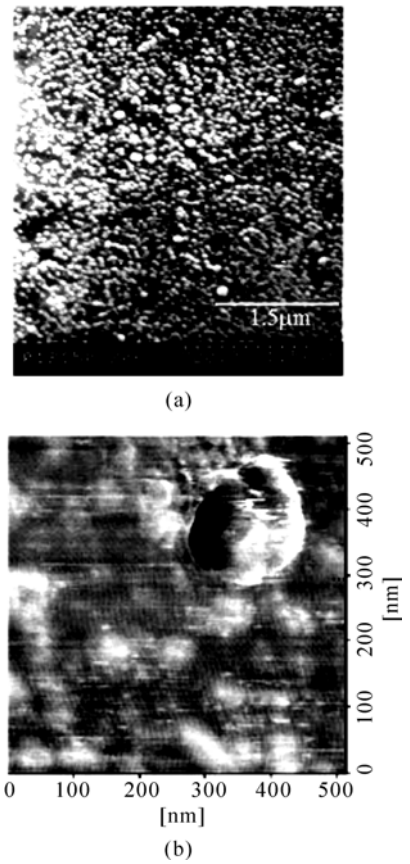

Fig.2 (a) SEM photo of DEAE Dextran-packed BMP particles (BMP/DEAE Dextran=1/5); (b) AFM photo of a DEAE Dextran-packed BMP particle

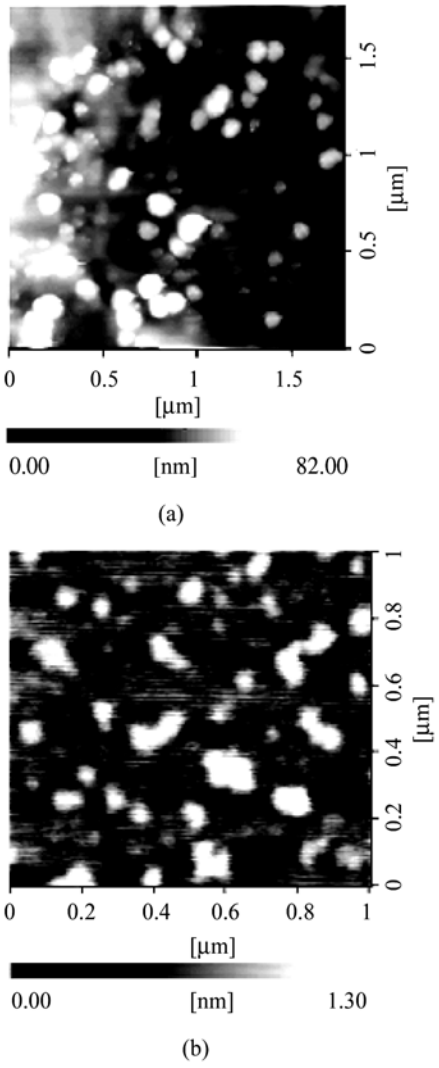

Fig.3 AFM photo of DEAE Dextran-packed BMP particles stored for (a) 1 day; (b) 60 days

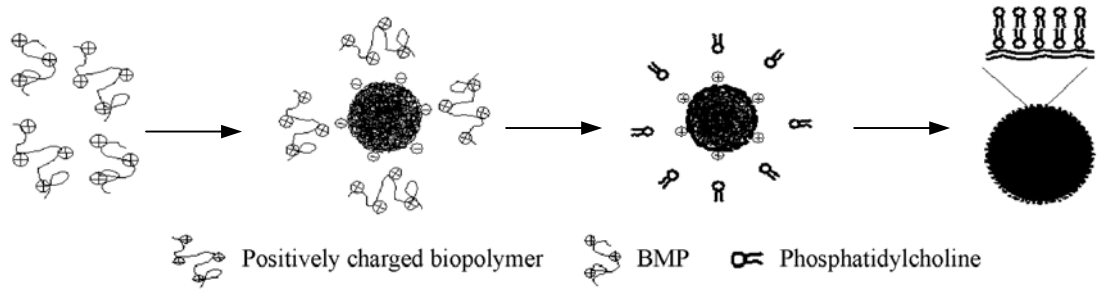

Fig.4 Schematic presentation to show the fabrication process and microstructure of BMP nanoparticles packed with DEAE Dextran and phosphatidylcholine

can adhere on the positively charged biopolymer-wrapped nanoparticles, forming lipid bilayers to delay the biopolymer release and the aggregation of the nanoparticles. Hence BMP nanoparticles with improved stability are produced.

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