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ORIGINAL CONTRIBUTION

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Preparation and characterization of calcium phosphate–albumin colloidal particles by high ultrasonic irradiation

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Abstract Using high intensity ultrasonic irradiation, we prepared calcium phosphate–albumin colloidal particles from aqueous solutions of $Ca(H_2PO_4)_2$ and $Ca(OH)_2$ in the presence of bovine serum albumin (BSA). The effect of concentration of BSA (2–5 g/L) properties of the colloidal particles was studied at constant temperature. The effect of a resting period on the size distribution of the colloidal particles was also investigated. Morphology, phase composition, average diameter, size distribution and zeta potential were obtained by transmission electron microscopy, X-ray diffraction, particle size determination by PCS and electrokinetic measurements.

Keywords Calcium phosphate · Bovine serum albumin · Ultrasonic irradiation

Introduction

Inorganic nanoparticles such as calcium phosphate (CaP) [1], silica [2] and gold [3] have an interesting potential as genes carrier systems because of the ease in preparation and reduced risk of immune response compared to viral vectors [4], low toxicity compared to organic polymers [5] and resistibility to bile salts and lipases in contrast to liposomes [6]. It might become more advantageous to use CaP nanoparticles as genes carrier systems due to their excellent biocompatibility and biodegradable properties compared to inert inorganic nanoparticles [1–3]. Albumins such as bovine serum albumin (BSA) or human serum albumin (HSA) are biodegradable, non-toxic and non-antigenic. Because of their defined primary structure and high content of charged amino acids, the albumin-based nanoparticles could allow direct electrostatic adsorption of positively or negatively charged molecules without addition of other compounds. Albumin nanoparticles have been used as excellent drug delivery carriers [7–10]. CaP possesses a high affinity for albumin, and it was found that calcium complexation by albumin plays a key role in the initial states of mineralization of CaP [11–14]. Therefore, colloidal CaP/BSA might become excellent carriers for drugs or genes.

An efficient and versatile drug carrier system has to fulfill the following requirements: (1) particle sizes in the submicrometer range; (2) possibility of surface modification; (3) high drug loading capacity; (4) colloidal stability in biological media; (5) the lack of toxic side effects induced by additives [15, 16]. The method of preparation of the nanoparticles is very important to fulfill the above requirements. Coacervation, heat denaturation and desolvation yielded protein micro- or nanospheres with short storage stability, and high toxicity [10, 17, 18]. Ultrasonic irradiation is a very unique and effective method. It has been successfully utilized to prepare various nanosize materials [19, 20]. The chemical effects of ultrasound irradiation are derived primarily from acoustic cavitation, that is, formation, growth and implosive collapse of bubbles in a liquid. Bubble collapse in liquids results in an enormous concentration of energy—from the conversion of the kinetic energy of the liquid motion into heating of

the contents of the bubble [21, 22]. Currently, the sonochemical method is also being used to prepare albumin microspheres or microcapsules [18, 23, 24]. Consequently, we selected ultrasonic irradiation method to prepare CaP/BSA colloidal particles.

The CaP/BSA colloidal particles were easily obtained and in a short time from aqueous solutions of $Ca(H_2PO_4)_2$ and $Ca(OH)_2$ in the presence of BSA by high intense ultrasonic irradiation. The effect of concentration of BSA on the properties the colloidal particles was studied. The samples were characterized by transmission electron microscopy (TEM), X-ray diffraction (XRD), particle size measurements and zeta potential determination.

Experimental

Materials

Bovine serum albumin consisting of 96–99% albumin (Sigma Chemicals) was used without purification; calcium dihydrogen phosphate (A.R., Shanghai, China) was purified by filtration and re-crystallization (at 100 °C removing crystal water). A saturated solution of calcium hydroxide was prepared by dissolution and filtration of calcium hydroxide (A.R., Tianjin, China).

Preparation of CaP/BSA colloidal particles

Calcium dihydrogen phosphate of 0.226 g (0.0009658 mol) was dissolved in 120 mL distilled water to give about 7.473 mmol/L solution. Then 101 mL saturated calcium hydroxide solution (20 °C, solubility is 1.65 g/L) was rapidly added under stirring. This yielded a Ca/P ratio of about 1.67. Different amounts of BSA were added (2–5 g/L). The turbid dispersion was intensely stirred for a few minutes before it was irradiated for 8 min at an acoustic power of about 100 W cm^{-2} (high-intensity ultrasonic probe, Institute of Acoustics, China). A transparent dispersion of CaP/BSA particles was obtained.

Transmission electron microscopy

A HITACH-600 scanning transmission electron microscope was used to determine the morphology and the size of the colloidal particles. A drop of the colloidal dispersion was placed on a copper grid and dried on air.

X-ray diffraction

A RIGAKU X-ray diffraction instrument was used to determine the crystallinity of the freeze-dried colloidal particles.

Measurement of the size distribution and zeta potential

The Malvern Zetasizer 3000HS was used to determine the size distribution and zeta potential of the colloidal particles at 25 °C. For the measurements, the samples were not diluted; the ionic strength and pH value of the colloid dispersing medium (distilled water) were not changed by the addition of a buffer. The size distribution obtained from photon correlation spectroscopy (PCS) is based on the intensity of scattered light. The size distribution will therefore differ from the values obtained by other techniques, e.g., electron microscopy. The *intensity* distributions can be converted to *volume* and *number* distributions if the refractive index and absorbance of the particles are known. The dependence of the *intensity* distribution, *volume* distribution and *number* distribution on the particle diameter are d^6 (*intensity*), d^3 (*volume*), and d(*number*).

Results and discussion

Figure 1 shows the morphology of the CaP/BSA colloidal particles with 4 g/L BSA. A regular network of many irregular shape colloidal particles was observed. The size of the colloidal particles was ~20–80 nm. A central region was surrounded by a dark layer. In the central region of some colloidal particles, black dots of ~10–30 nm were recognized. Black materials were also attached to the shell of the colloidal particles forming the black layers and are considered the CaP nanoparticles.

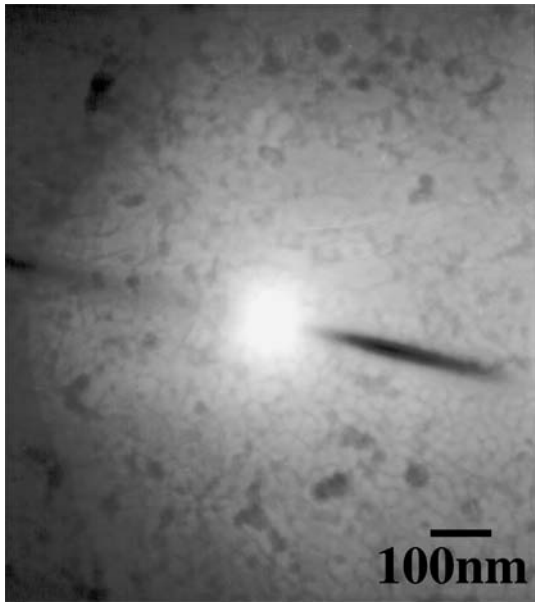

Fig. 1 Transmission electron microscopy (TEM) image of CaP/BSA colloidal particles with 4 g/L BSA

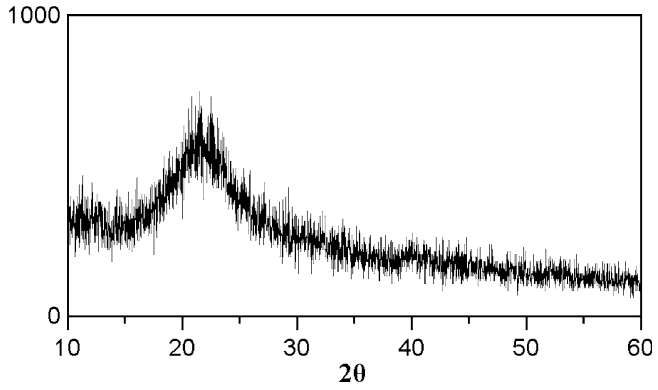

Fig. 2 X-ray diffraction (XRD) pattern of freeze dried CaP/BSA particles with 4 g/L BSA

The XRD patterns of the colloidal particles did not show reflections of crystalline calcium phosphate; the CaP nanoparticles were amorphous (Fig. 2).

The formation mechanism of CaP/BSA colloidal particles seems to be similar to the mechanism of sonochemical formation of albumin and haemoglobin microspheres [25]. According to this mechanism, the nanospheres are held together by disulfide bonds between protein cysteine residues and that HO_2 radicals, sonochemically produced by acoustic cavitation, act as the cross-linking agent (Fig. 3). H· and OH· radicals are formed [26] from water molecules by the absorption of the ultrasound energy and recombine to H_2 and H_2O_2 [27]. H_2O_2 may react further with OH· radicals to form HO_2· radicals [25]. The –SH groups in the cysteine residues of BSA are easily oxidized by the HO_2· radicals yielding disulfide bonds which link the BSA molecules. This chemical cross-linking is responsible for the formation of the netlike morphology of CaP/BSA colloidal particles shown in Fig. 1.

Table 1 shows the *intensity* size distribution of the particles with 4 g/L BSA with an *intensity*-averaged particle size of 87 nm and a distribution width of 98 nm. The corresponding *volume*- and *number*-averaged particle size were 41 nm and 20 nm, with the distribution widths of 48 nm and 22 nm respectively.

As reported in Table 1, the average size of the particles was affected by the presence of BSA. Without BSA, the dispersion was extremely unstable and the particles after a few seconds. At a BSA concentration of 1 g/L, the dispersion was stable a few minutes. Between 2 g/L and 5 g/L BSA the dispersion became more stable and the size distribution and the averaged particle size

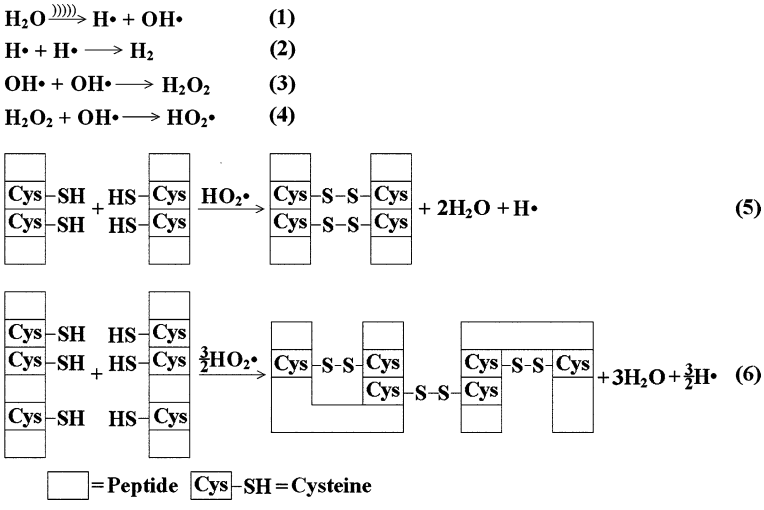

Fig. 3 Cross linking of BSA by ultrasonic irradiation

Table 1 The effect of concentration of BSA on the size distribution of CaP/BSA colloidal particles

Concentration of BSA (g/L)	Intensity		Volume		Number	
	Mean (nm)	Width (nm)	Mean (nm)	Width (nm)	Mean (nm)	Width (nm)
0	Extremely unstable					
1	Unstable					
2	104.0	107.5	57.1	61.0	30.2	31.4
3	91.2	97.5	47.4	52.2	24.0	26.0
4	87.3	98.2	41.8	48.1	19.7	22.1
5	88.3	94.5	45.8	50.5	23.2	25.2

Table 2 The effect of BSA concentration on the zeta potential of CaP/BSA particles

Concentration of BSA (g/L)	2	3	4	5
Zeta potential mean (mV)	−19.7	−22.3	−25.5	−19.3

Table 3 The effect of the resting period on the size distribution of CaP/BSA particles with 4 g/L BSA

Resting period	Intensity		Volume		Number	
	Mean (nm)	Width (nm)	Mean (nm)	Width (nm)	Mean (nm)	Width (nm)
0 h	87.3	98.2	41.8	48.1	19.7	22.1
1 h	89.0	95.5	45.9	50.8	23.2	25.2
2 h	90.1	96.8	46.4	51.4	23.4	25.4
7 days	188.4	239.9	115.2	108.3	26.7	27.7

were slightly influenced by the concentration of BSA. With increasing BSA concentration from 2 g/L to 4 g/L, the average particle size decreased and increased between 4 g/L and 5 g/L.

The average zeta potential of the CaP/BSA particles was −25.5 mV (Table 2). It was only slightly affected by the concentration of BSA. A weak minimum appeared at the concentration of 4 g/L.

Based on the above discussions, we selected the concentration of 4 g/L as the optimal concentration of BSA and studied the effect of the resting period on the size distribution of the CaP/BSA colloidal particles (Table 3). At short resting period of 1 h or 2 h distinct changes in the size were not observed, but the average particle size increased within 7 days. However, the stability of the colloidal particles was reduced and the size distribution was broadened.

Conclusion

CaP/BSA colloidal particles were prepared from aqueous solutions of $Ca(H_2PO_4)_2$ and $Ca(OH)_2$ containing BSA by high intensity ultrasonic irradiation. The presence of BSA was the key factor in preparing the CaP/BSA hybrid particles. The CaP nanoparticles were amorphous. The concentration of BSA slightly influenced the size distribution and the zeta potential of colloidal particles. With the increase in the resting period, the stability of the particles reached a maximum and decreased after several days.

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