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Competitive adsorption of bovine serum albumin and lysozyme on characterized calcium phosphates by polyacrylamide gel electrophoresis method

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Abstract Characterizations of hydroxyapatite (HA), biphasic calcium phosphate (BCP) and beta tricalcium phosphate (β-TCP) ceramic particles were carried out using X-ray diffusion (XRD), Scanning electron micrograph (SEM), Particle Sizer and Zeta potential analyzer. Competitive adsorption of bovine serum albumin (BSA) and lysozyme (LSZ) on the three calcium phosphates were investigated by polyacrylamide gel electrophoresis (PAGE) method. The results showed that HA, BCP and β-TCP ceramic particles with irregular shapes and similar size distributions all had negative surface net charges in pH7.4 phosphate buffered saline (PBS) solution and exhibited alike behaviors of BSA and LSZ adsorption. LSZ had higher affinity for calcium phosphate ceramics than BSA and its adsorption on them didn't be almost influenced by the increasing of BSA concentration in the solution. Electrostatic interaction played an important role on the competitive adsorption of BSA and LSZ on the surface of calcium phosphate ceramic particles.

Introduction

Calcium phosphates are biologically familiar and had been widely used in a variety of biomedical applications. Besides biocompatibility and bioactivity, the osteoinduction of calcium phosphates also had been confirmed by some recent researches [1–8]. It is known that a biomaterial will firstly evoke the adsorption of proteins from the surround body fluids upon implantation [9–11]. The monolayer of adsorbed protein will affect the subsequent cellular interaction with the implant surface [12–14], so the investigation of protein adsorption behavior on the implant surface has been considered as one of the means to evaluate the biocompatibility of a biomaterial. Protein adsorption is a quite complex process and mainly influenced by the surface chemistry and topography of the implant, as well as the kind and structure of protein [15, 16]. Thus far, most of the work on this in vitro concerned the study on single protein adsorption behavior [17–25]. As we know, the body fluids, such as blood, contain a large number of proteins. So the competitive adsorption of the proteins on the surface of a biomaterial is more close to the actual conditions in vivo. Moreover, the preferential adsorption of proteins, especially the growth factors, on the implant surface has been considered as one of the key factors related to the bioactivity and osteoinduction of calcium phosphates [3, 26].

When a biomaterial is implanted into the body, various kinds of proteins from body fluids will adsorb to the substrate surface. Albumin is the most abundant protein in blood. On the other hand, the proteins relating with bone regeneration are some growth factors, such as BMP, TGF-β etc., which have lower molecular weight and are quite rare in amount in body fluids. So it is very interest thing for us to investigate the competitive adsorption of the two kinds of proteins on calcium phosphates with good biological properties. As a high sensitive analytical technology, PAGE allows the detection and quantitatively analysis of different proteins, and it had been used to analyze the adsorption pattern of proteins on some biomaterials [27–29]. In this study, BSA was used as the analog of albumin, and LSZ was

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that of low amount of growth factors because of its low cost and similar properties with the latter. Their single and competitive adsorption on HA, BCP and β-TCP ceramics, which are the three calcium phosphates often used as bone repair materials in clinic, was investigated using the conventional protein quantitative analysis and PAGE methods, respectively. The adsorption affinities of BSA and LSZ for the three ceramic particles were also evaluated and the mechanism was discussed in detail.

Materials and methods

Preparation and characterizations of HA, BCP and TCP ceramic particles

HA, BCP and TCP precursor powders prepared by a wet precipitation method in our center were sintered at 1,100 °C and their phase compositions were measured by XRD (Philips X'Pert Pro MPD). The sintered ceramic powders were crushed and sieved for zeta potential tests and protein adsorption experiments. Zeta potentials were analyzed with a Nano ZS90 zetasizer system (Malvern Co., UK), which measures the electrophoretic mobility of the particles and automatically calculates zeta potential by the Henry equation. Morphologies of the ceramic particles were observed by SEM (JSM-5900 OL) and the size analysis of them was performed in a Mastersizer Micro instrument (Malvern Co., UK), which is a laser scattering based particles sizer. The specific surface area of the particles used for protein adsorption experiments were determined by BET method (SA3100 Surface Area Analyzer, BECKMAN COULTER).

Proteins

BSA and LSZ purchased from Ameresco were used without further purification. All other chemicals of analytical grade were from domestic companies. BSA and LSZ have significantly different molecular weights and physiochemical properties, and some of their properties were summarized in Table 1.The protein solutions were prepared by directly dissolving proteins into PBS solution with pH7.4.

The protein adsorption experiments

Adsorption experiments were carried out in 1.5 mL of capacity polypropylene centrifuge tubes in which 1 mL of the protein solutions were added to disperse the ceramic particles (0.1 g), according to the conventional method described elsewhere [20]. The initial concentrations of BSA or LSZ solutions were both 0.5 mg/mL, and the binary BSA/LSZ solutions had three different total concentrations in which LSZ kept a constant of 0.5 mg/mL, but BSA was respectively 0.5, 1.0 and 1.5 mg/mL. The tubes were incubated at room temperature for 1 h with continual agitation to the suspending particles. After centrifugation, the protein concentrations in the supernatants were determined by BCA method [32] (BCA Protein Assay Kit, Pierce) using the absorbance values at the wavelength of 570 nm against a PBS blank.

The protein desorption and SDS-PAGE analysis

For the binary BSA/LSZ system, the protein-covered particles were washed three times by deionized water for removing those loose-binding proteins after above adsorption experiments. Thereafter, the particles were suspended again using 200 μL of 2% (w/v) sodium dodecyl sulphate (SDS) solution, which is a denaturant and can desorb the proteins attached to the surface. After sharply stirring, the suspensions were centrifuged and the supernatants containing the desorbed proteins were collected for SDS-PAGE analysis, which was performed in a Mini-PROTEAN 3 system (BIO-RAD, USA) according to the method of Laemmli [33]. In brief, 10 μL of 2 × loading buffer was added to each 10 μL of sample containing the desorbed proteins, and then the mixtures were loaded to each lane of the gel (10% separating gel and 3.9% stacking gel) for electrophoresis after boiling for 5 min. After running under constant voltage conditions of 80 V for 20 min at the stacking gel and 120 V for about 1 h at the separating gel, the gels were stained with Coomassie Brilliant Blue R-250 and quantitatively analyzed using Quantity One® 1-D Analysis Software (BIO-RAD, USA).

Results and discussions

Characterizations of HA, BCP and TCP ceramic particles

The phase compositions of HA, BCP and TCP ceramics were confirmed by XRD patterns shown in Fig. 1. HA and TCP were made up of pure HA and β-TCP phase,

Table 1 Some physiochemical properties of BSA [30] and LSZ [31]

Proteins	BSA	LSZ
Molecular weight KDa	67	14.3
Isoelectric points	4.7	11.1
Dimensions	Heart shaped of ~8 nm of side and ~3 nm depth	$4.5 \times 3.0 \times 3.0$ nm^3

respectively. According to the relative heights of the specific peaks for HA and β-TCP that were observed at 31.81° for HA and 31.07° for β-TCP, BCP was a bi-phasic ceramic in which the ratio of HA to β-TCP were about 70/30. The typical SEM photomicrographs of the three ceramic particles were shown in Fig. 2. The three kinds of crushed ceramic particles all showed the irregular shapes. Moreover, the particle sizes of them exhibited wide distributions. The sintering neck shrinkages could be clearly seen from the 10,000 × photos, indicating that HA, BCP and TCP particles all became ceramic well under 1100 °C sintering temperature. It should be noted that the grains composing HA and β-TCP particles had similar sizes ranging from 1.0 to 2.0 μm, but that of BCP showed smaller one.

In accordance with sequent protein adsorption experiments, zeta potentials tests of the three ceramic particles were performed using pH7.4 PBS solution as dispersant. The size measurements of them were carried out using deionized water as dispersant. The obtained size distribution graphs were shown in Fig. 3. The average zeta potentials, particle diameters and the specific surface area of the three ceramic particles were summarized in Table 2. All of the three ceramic particles had negative zeta potentials in pH7.4 PBS solution, only the magnitude of zeta potential of HA was a few higher than BCP and β-TCP, as meant that HA had a few higher surface net charge than the other two ceramic particles. In addition, they all had wide and similar size distributions. The average diameter of BCP ceramic particles was a few smaller than that of HA and β-TCP, as could be the reason that BCP particles had the highest specific surface area.

The single BSA and LSZ adsorption behavior

Heretofore, adsorption kinetics and equilibrium adsorption isotherms of a variety of proteins have been widely investigated. Though an equilibrium state may be reached after the initially rapid adsorption during the first several minutes, the long-time behavior of protein adsorption is different from the short-time one, and the final amount of adsorbed protein as well as the surface coverage is related to the initial amount of protein introduced into the system [34, 35]. The adsorption isotherms of single protein on various calcium phosphates or the other materials mostly show the Langmuir type [18, 20, 21, 36, 37]. That is to say that the amount of adsorbed protein will increase with the increase in the initial protein concentration and tends to reach a saturated value in the end. Based on that, we here investigate the difference of HA, BCP and β-TCP ceramic particles in adsorbing BSA and LSZ molecules under 0.5 mg/mL protein concentration conditions. The results were shown in Fig. 4. From this histogram, it could be observed that BCP showed the higher the ability to bind BSA molecules than HA and β-TCP. However, there was not too much difference in the amounts of adsorbed LSZ on the three ceramic particles, and HA adsorbed a few more LSZ molecules. Except for BCP, HA and β-TCP both adsorbed more LSZ than BSA.

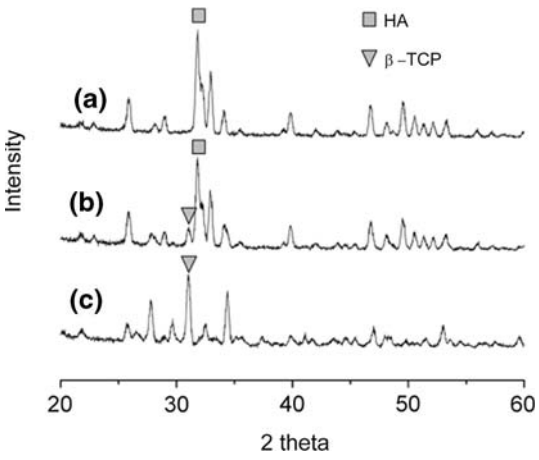

Fig. 1 XRD patterns of HA (**a**), BCP (**b**) and TCP (**c**) ceramic particles

On the basis of their atomic composition, HA, BCP and β-TCP have the same surface reactive points, i.e. the positive C-Sites formed by calcium ions and the negative P-Sites come from six oxygen atoms of phosphate ions [38, 39], indicating that the electrostatic interaction would play a important role on protein adsorption on them. In pH7.4 PBS solution, BSA has negative net charge but LSZ has positive one; HA, BCP and β-TCP all have negative surface zeta potentials and thus have negatively surface net charges. Higher LSZ adsorption but lower BSA adsorption on HA and β-TCP can be explained by the electrostatic interaction. The electrostatic attraction between LSZ and the ceramic particles can promote the adsorption of LSZ on the surface, but the repulsive forces between BSA and the particles hold back the BSA adsorption. Under given concentration condition, as we know, the equilibrium amount of adsorbed proteins is determined by their spatial orientation and distribution, lateral interactions and conformational alterations [30, 31]. Assuming the formation of a close-packed monolayer of unperturbed protein molecules on the substrate surface, the theoretical saturated amounts of adsorbed BSA and LSZ are about 4.0 [30] and 1.8 mg/m^2 [40], respectively. Obviously, the surface coverage of BSA or LSZ on HA, BCP and β-TCP surfaces are all rather low according to the experimental results. This meant that there were a large number of surface reactive points on them that were unoccupied, as confirmed that the difference of the three ceramics in Ca/P molar ratio, resulting in the different surface C-Sites and P-Sites, could not be the primary

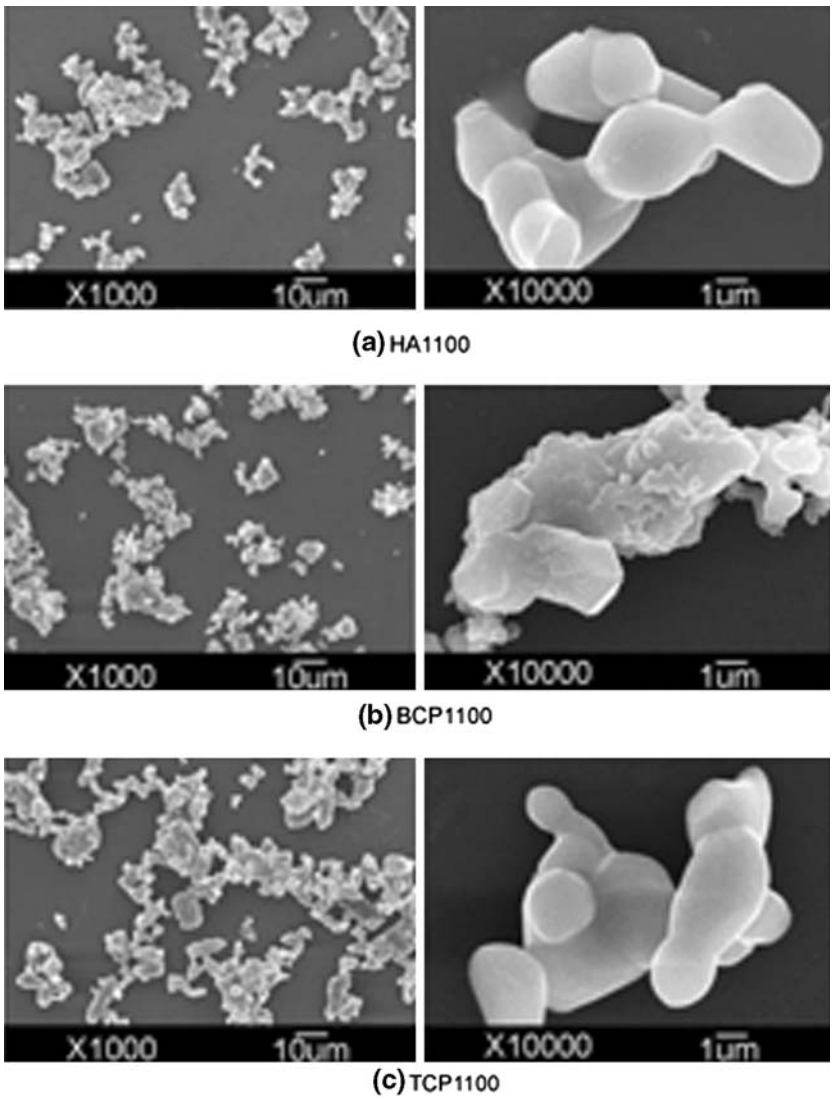

Fig. 2 SEM photographs of HA (**a**), BCP (**b**) and TCP (**c**) ceramic particles for protein adsorption

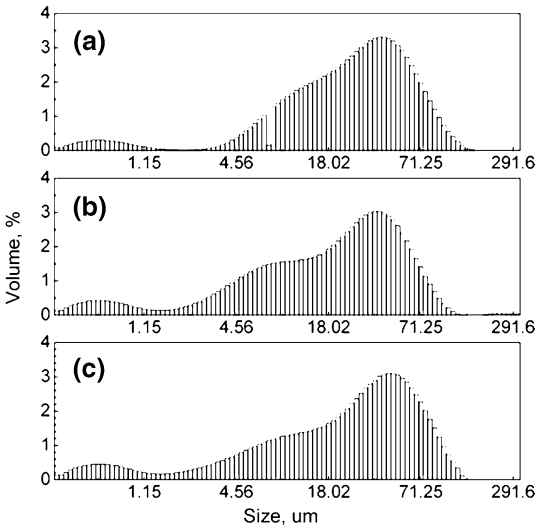

Fig. 3 The size distribution graphs of HA (**a**), BCP (**b**) and TCP (**c**) ceramic particles for protein adsorption

factor determining their protein adsorption ability. Comparing with BCP and β-TCP, the higher absolute value of zeta potential of HA particles would lead to the minor electrostatic repulsion between BSA and HA and the higher electrostatic attraction between LSZ and them. This might be the major reason that HA had the higher ability to adsorb LSZ but the lower one to bind BSA. However, the electrostatic interaction could not be used to explain the BSA adsorption behavior on BCP particles. The higher BSA adsorption on BCP might be explained by its minor grain size and bi-phasic structure. It had been reported [36] that the texture of HA particles, i.e. the exposed crystal surface was another important factor determining BSA adsorption. The minor grain size and the occurrence of second β-TCP in BCP particles could make the more crystal faces, which are favorable for BSA adsorption, expose to the surface of the particles, resulting more BSA molecules adsorption on the surface.

Table 2 The properties of HA, BCP and TCP ceramic particles

Material	HA1100	BCP1100	TCP1100
Zeta potential, mV	–24.0 ± 1.4	–20.9 ± 1.5	–19.3 ± 1.7
Average diameter, μm	28.23	22.12	28.35
Specific surface area, m^2/g	1.366	2.455	2.162

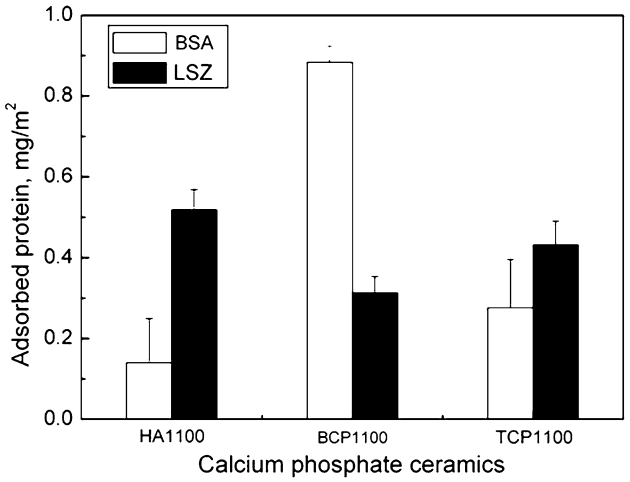

Fig. 4 Single BSA and LSZ adsorption on HA, BCP and TCP (the initial protein concentration of 0.5 mg/mL)

The BSA/LSZ competitive adsorption behavior

Figure 5 showed the pattern of the separation gel after electrophoresis and staining by Coomassie Brilliant Blue, on which the BSA and LSZ bands were obviously observed, confirming the co-adsorption of the two protein molecules on the three calcium phosphate ceramic particles. The results of semi-quantitative analysis for the detected protein bands on the stained gel were summarized in Fig. 6. The adsorbed BSA and total proteins on the three calcium phosphates increased with the increasing of initial protein concentration, as were in accordance with the adsorption behavior of single protein on them. Obviously, the surface coverage of proteins on the surface was also rather low, liking the single BSA or LSZ adsorption. Under each concentration condition, HA showed higher LSZ adsorption and thus higher the amount of adsorbed total proteins than BCP and β-TCP. However, there was not notable difference in BSA adsorption on the three ceramic particles, as was different from the above results about the single BSA adsorption. On the other hand, it should be noted that the amount of adsorbed LSZ on the three ceramic particles didn't almost alter with the increase in BSA concentration, and it was always higher than that of adsorbed BSA. The experimental results proved that the adsorption behavior of protein on the single protein solution could be different from that on multicomponent solution.

In general, the driving forces of protein adsorption come from the electrostatic and hydrophobic interactions between the protein and the substrate surface, as well as the structural rearrangement of the absorbed protein [15, 16]. BSA is a "soft" protein and will more or less alter its conformation during adsorption on either hydrophilic or hydrophobic surface [30, 41, 42]. However, LSZ has a relative rigid structure [31, 40] and cannot change its conformation as easy as BSA. The recent investigations [43] on the wettability of calcium phosphates confirmed that HA and β-TCP are both moderately hydrophobic ceramics. BCP should exhibit an intermediate behavior between HA and β-TCP because of its bi-phasic structure.

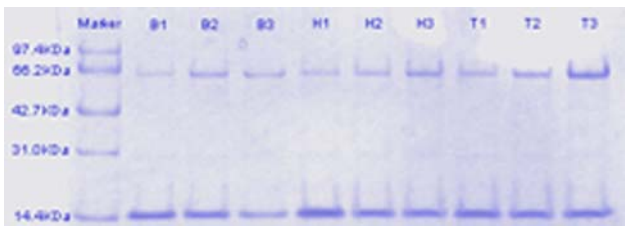

Fig. 5 The pattern of the gel stained by Coomassie blue R-250 (B—BCP1100; H—HA1100; T—TCP1100; 1–0.5 mg/mL BSA/0.5 mg/mL LSZ initial concentration; 2–1.0 mg/mL BSA/0.5 mg/mL LSZ initial concentration; 3–1.5 mg/mL BSA/0.5 mg/mL LSZ initial concentration)

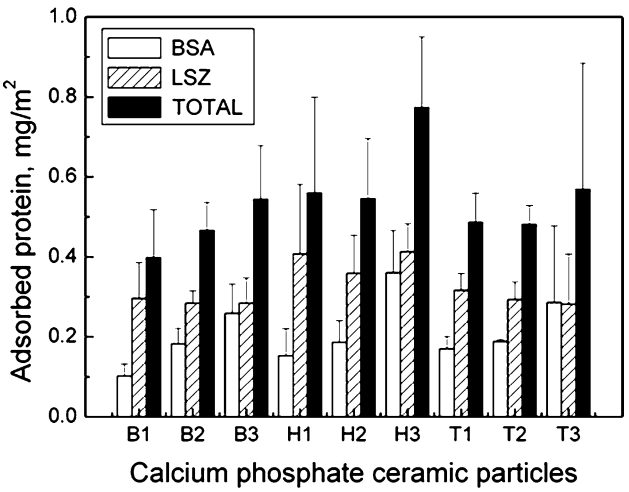

Fig. 6 The amount of adsorbed BSA and LSZ in pH7.4 PBS solution with different protein concentrations (B—BCP1100; H—HA1100; T—TCP1100; 1–0.5 mg/mL BSA/0.5 mg/mL LSZ initial concentration; 2–1.0 mg/mL BSA/0.5 mg/mL LSZ initial concentration; 3–1.5 mg/mL BSA/0.5 mg/mL LSZ initial concentration)

So the adsorption of negatively charged BSA on HA, BCP and β-TCP with the same negative surface net charge could be ascribed to the alteration of BSA conformation and the hydrophobic interaction, as was discussed in detail in our previous study [20]. The role of electrostatic interaction on LSZ adsorption had also been widely investigated [31, 40]. It is believed that LSZ adsorption should be primarily driven by the electrostatic attraction between the oppositely charged LSZ molecules and calcium phosphate ceramic particles. According to "the Vroman effect", when a solid surface is exposed to a multi-proteins solution, the different protein molecules will compete for the binding sites on the substrate surface, and there occurs homomolecular as well as heteromolecular exchange during the adsorption process, until a steady state is reached [15, 30]. The affinity and kinetic factors will be important for determining the profile of protein molecules on the surface [15]. From the above investigation on single BSA or LSZ adsorption, it could be found that LSZ showed higher affinity for calcium phosphate ceramic particles than BSA. When the calcium phosphate ceramic particles were dispersed into the BSA/LSZ binary solution, BSA and LSZ would competitively bind to the surface. Considering a simple diffuse-controlled situation, because the initial LSZ concentration was a constant of 0.5 mg/mL under the three experimental conditions, the competitive adsorption process would vary with the increasing of BSA concentration in the solutions. When BSA concentration was also 0.5 mg/mL, LSZ molecules with lower molecular weight would first arrive and tightly bind to the surface due to their high affinity for calcium phosphates. In multi-proteins solution, there happens intermolecular attraction between the proteins with opposite surface charge, as is different from the single protein system in which only intermolecular repulsion occurs. Besides, there are some unoccupied sites on the surface when the low surface coverage of protein molecules is reached. Thus, the larger BSA molecules with relatively low affinity could also adsorb to the surface because of the intermolecular attraction between negatively charged BSA and positively charged LSZ molecules, only the adsorbed amounts was much lower than that of LSZ. On the contrary, when its concentration was 1.0 or 1.5 mg/mL, BSA would tend to dominate the initial adsorption process. As above mentioned, BSA adsorption is mostly driven by its conformation alteration. At high bulk concentration, the less unfolding of BSA would occur because of the adsorbate-adsorbate interactions [15], leading to the decline in the binding forces between BSA and the surface. One hand, LSZ molecules arriving from the bulk solution could bind to the residual sites on the surface due to the attractions between LSZ and BSA as well as the surface. On the other hand, the previously adsorbed BSA could also be replaced by LSZ molecules with relatively higher affinity for the surface of calcium phosphate ceramic particles. Thus, the amounts of adsorbed LSZ couldn't be almost influenced by the increasing of BSA concentration in the solution, and it was always higher than that of BSA.

Conclusions

HA, BCP and β-TCP ceramic particles crushed mechanically all had irregular shape and wide size distributions. BCP had larger specific surface area due to its smaller grain sizes. The three calcium phosphate particles all had negatively surface zeta potentials after being dispersed into pH7.4 PBS solution. HA had larger absolute value of zeta potential and thus higher surface net charge.

BSA, LSZ and their competitive adsorption on the three ceramic particles were investigated using the conventional protein quantitative analysis and sensitive PAGE methods. It has been found that either single BSA and LSZ or their co-adsorption under experimental conditions, the surface coverage was always rather low. LSZ showed higher affinity for the three calcium phosphate ceramics than BSA. Electrostatic interaction played an important role in the adsorption process. HA with higher surface net charge exhibited higher LSZ adsorption due to the stronger electrostatic attraction between them, but less BSA adsorption for the stronger electrostatic repulsion. Though the amount of adsorbed total proteins increased with the increasing of the initial protein concentration, the competitive adsorption behavior of BSA and LSZ still differed from their single adsorption. The competitive adsorption behaviors of BSA and LSZ on HA, BCP and β-TCP were similar. There was not markedly difference in the amount of adsorbed BSA under each condition, LSZ adsorption was always higher than BSA and not be almost influenced by the increasing of BSA concentration in the solutions. From this result, it could be inferred that those bone growth factors with similar properties as LSZ might preferentially bind to the surface when calcium phosphate ceramics being implanted into the body, as was undoubtedly important for exploring the mechanism of their osteoinduction.

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