Calcium Phosphate Cement Reinforced by Polypeptide Copolymers

Jiaping Lin, Suning Zhang, Tao Chen, Changshen Liu, Shaoliang Lin, Xiaohui Tian

Key Laboratory for Ultrafine Materials of Ministry of Education, School of Materials Science and Engineering, East China University of Science and Technology, Shanghai 200237, People's Republic of China

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Abstract: Water-based calcium phosphate with bone repairing capability was reinforced by polypeptide graft copolymers and micelles that were formed by polypeptide copolymers. The mechanical studies showed that the compression strength and fracture energy of the calcium phosphate cement (CPC)/polypeptide composites are appreciably higher than those of CPC. The molecular structure of the polypeptide graft copolymers and the association form of the polypeptide copolymers exhibit a marked effect on the mechanical properties of CPC/polypeptide composites. The polypeptide copolymers with more hydrophilic side chains and with core-shell micelle forms have more effective reinforcement effect. The morphological studies based on the scanning electron microscope (SEM) observations revealed that both polypeptide graft copolymers and polypeptide copolymer micelles are well dispersed in CPC matrix. According to the obtained experimental results, reinforcement mechanism was suggested.
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Keywords: calcium phosphate cement; polypeptide copolymer; micelle; composite

INTRODUCTION

Calcium phosphate cement (CPC) is a promising biomaterial which can be used in numerous medical procedures for hard tissue repairing because of its excellent osteoconductivity, bone repairing capability, and random molding characteristic.[1–4] CPC is made of particulate mixture of tetracalcium phosphate (TECP) ($Ca_4(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA) ($CaHPO_4$), which can react in aqueous environment to self-set and form hydroxyapatite.[5–7] The final hydration product has similar chemical and crystallographic structures to the carbonated apatite found in bones.[8] When CPC is used as a filling material for bone defects, it can be slowly absorbed and replaced by new-formed bone. However, the low compression strength of CPC and its brittleness restrict its use in many supported defects and stress-bearing locations.[9]

A common method to overcome this weakness is to incorporate fibers to improve the compression strength and the fracture resistance of CPC materials. Santos and colleagues used polyamide fibers to reinforce the mechanical properties of CPC.[10] It was found that the compression strength was increased when the fibers were incorporated into CPC. A study carried out by Xu and colleagues showed that the CPC could be considerably strengthened and toughened via fiber reinforcement.[11,12] Both the fiber volume fraction and the fiber length have effects on the mechanical properties of CPC composites. The composite ultimate strength increases linearly with the strength of the reinforced fibers. Use of fiber mesh to reinforce CPC has also been reported.[13] The obtained results demonstrated that a flat fiber mesh placed on the tensile surface of a CPC specimen results in an increase in work-of-fracture. The reinforcement is anisotropic, as the thin fiber mesh is limited to the tensile side of the samples.

However, most fibers used in the reinforcement are not biodegradable, biocompatible, and some are even toxic to human body. Synthetic polypeptide, with similar basic structure to natural protein, is a promising candidate to reinforce CPC. Because the polypeptides have a similar basic structure to natural protein, they exhibit good biocompatibility.[14] The polymer chains with a number of CO—NH bonds can also be degraded by enzymes.[15] The degradation rates are molecular structure dependent,[16] and the degradation products, mainly amino acid and their derivatives, can be easily absorbed without inflammation. But synthetic polypeptides such as poly (γ-benzyl L-glutamate) (PBLG), poly (γ-ethyl L-glutamate) (PELG), and poly (γ-methyl L-glutamate) (PMLG) are usually hydrophobic, which could hinder their miscibility

Correspondence to: J. Lin (e-mail:jplinlab@online.sh.cn)
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TABLE I. Characteristics of Polypeptide Graft Copolymers

Sample	PBLG-*g*-PEG350	PBLG-*g*-PEG1308	PBLG-*g*-PEG2000	PELG-*g*-PEG350	PMLG-*g*-PEG350
Original polypeptide	PBLG	PBLG	PBLG	PELG	PMLG
M_v of original polypeptide	146,000	146,000	146,000	266,000	25,000
M_w of PEG side chain	350	1308	2000	350	350
Grafting rate (%)	31.8	12.1	5.0	28.2	56.3

with CPC in the aqueous condition before self-setting. One way to improve the compatibility is to introduce hydrophilic side chains onto the polypeptide backbone. The grafting can be achieved through an ester exchange reaction. After grafting the water-soluble side chain, the polypeptides may have a good compatibility with CPC paste before self-setting and have fine dispersion in CPC matrix of the composites. On the other hand, under certain conditions, polypeptide copolymers with amphiphilic characteristics can form micelles with a core-shell structure.[17,18] The water-soluble shells make the micelles compatible with CPC paste, and such micelles can also be used for reinforcing CPC.

In this study, for the first time polypeptide graft copolymers and micelles formed by polypeptide graft copolymers and polypeptide block copolymer were used to reinforce the self-setting CPC. Enhanced mechanical properties of CPC were demonstrated after introduction of either polypeptide graft copolymers or micelles. Effects of molecular structure of polypeptide graft copolymer and shapes of micelles were examined.

MATERIALS AND METHODS

CPC Powder

The CPC powder used in the experiments was a mixture composed of equimolar tetracalcium phosphate (TECP) and dicalcium phosphate anhydrous (DCPA). All the calcium phosphates were prepared in our laboratory. The details of the preparation method are available in Liu and colleagues.[19] TECP was synthesized by a solid-state reaction between calcium phosphate and calcium carbonate at 1500°C for 10 h where the mole ratio of Ca to P was 2.0. DCPA was obtained by removing the crystallization water in dicalcium phosphate dihydrate (DCPD) at 120°C, and DCPD was prepared from $(NH_4)_2HPO_4$ and $Ca(NO_3)_2$ in an acidic environment. The best temperature for the heat treatment was 130°C.

Syntheses of Polypeptides and Polypeptide Copolymers

Poly ethylene glycol mono-methyl ether (PEG) (the molecular weights, M_w, are 350, 1308, and 2000, respectively) and methoxypolyethylene glycol amine (M_w is 5000) were purchased from Sigma. All polymers were used without further purification. Hexane, tetrahydrofuran (THF), and 1,4-dioxane are of analytical grade and dried with sodium to remove water before use. All other solvents are of analytical grade and used without further purification.

The polypeptides, such as PBLG, PELG, and PMLG were prepared by a standard *N*-carboxyanhydride (NCA) method as adopted in our previous works.[20,21] Briefly, NCA was prepared first. Polypeptide was then obtained by the ring-opening polymerization of NCA initiated by triethylamine in 1,4-dioxane at room temperature. The reaction mixture was poured into a large volume of anhydrous ethanol. The precipitated product was dried under vacuum and then purified twice by repeated precipitation from a chloroform solution into a large volume of anhydrous methanol. The molecular weight of polypeptide was estimated from the intrinsic viscosity measured in dichloroacetic acid (DCA).

Polypeptide graft copolymers such as PBLG-*g*-PEG, PELG-*g*-PEG, and PMLG-*g*-PEG were prepared by the ester exchange reaction of PBLG, PELG, and PMLG with PEG in 1,2-dichloroethane with *p*-toluenesulfonic acid as a catalyst according to the method described in our previous work.[17] In all the cases, the mixture reacted at 55°C for 72 h and then was precipitated into a large volume of anhydrous ethanol. The resulting product was purified twice from a chloroform solution in a large volume of anhydrous methanol and dried under vacuum. The characteristics of the obtained polypeptide graft copolymers are shown in Table I, and a schematic representation of polypeptide graft copolymer is given in Figure 1(a). PBLG-*b*-PEG block copolymer, as shown schematically in Figure 1(b), was obtained by the ring-opening polymerization of *N*-carboxyl-γ-benzyl-L-glutamate anhydride (γ-BLG NCA) using methoxypolyethylene glycol amine (M_w = 5000) as an initiator. The molecular weights of the block copolymer and the grafting rate of the graft copolymers were estimated by nuclear magnetic resonance (NMR) measurements (Avance 550, Bruker Co., Switzerland). It was calculated by the peak intensities of the methylene proton signal (5.1ppm) of polypeptide and the ethylene proton signal (3.6 ppm) of PEG in the ^1H NMR spectrum. The molecular weight of the block copolymer, according to the NMR analysis, is 50,000.

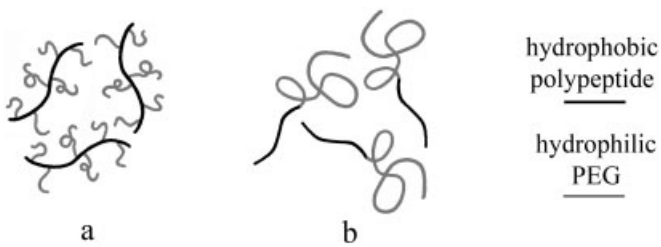

Figure 1. Schematic representation of the polypeptide graft copolymer (a) and polypeptide block copolymer (b).

Specimen Fabrication

The obtained polypeptide graft copolymers were dissolved into water and sonicated until homogenous solution was obtained. On the other hand, the polypeptide micelle solutions were also prepared according to the method reported in our previous work.[17] Briefly, PBLG-*g*-PEG or PBLG-*b*-PEG was dissolved in *N,N*-dimethylformamide (DMF). The solution was stirred at room temperature and solubilized completely. Then the solution was dialyzed against distilled water using dialysis membrane (3500 molecular weight cut-off) to remove the organic solvent for about 48 h at room temperature. The obtained micelle solution was further concentrated through vaporing the water.

A prescribed amount of CPC powder was mixed manually with polypeptide graft copolymer solution or polypeptide micelle solution by spatulation into paste. The mass ratio of CPC to solution was 3:1. The paste was loaded into a stainless-steel mold with a diameter of 6 mm and a height of 12 mm with periodic packing by means of a 5.6 mm diameter stainless-steel rod. The force applied to the rod during packing was 19.6 *N*, corresponding to a pressure of 8.0×10^5 Pa, the same conditions as those used by Fukase and collagues.[22] Then the specimen was demolded and placed in a glass tube (8 mm D $\times$ 20 mm H) which was then sealed with plastic film and stored at 37°C in a 100% relative humidity box for 24 h, at which the hydration reaction was completed. The hardened specimens were polished on both sides and ready for testing. Five parallel experiments were carried out for the data of each sample.

Testing

The compression strength, fracture energy, and elastic modulus were measured with a universal testing machine (AG-2000A, Shimadzu Autograph, Shimadzu Co., Ltd., Japan). The loading rate was 1 mm/min.

The morphologies of the micelles were observed by TEM (JEM-1200-EXII). Drops of micelle solution were placed on a carbon film coated on a copper grid, and then were dried at room temperature. The TEM bright-field imaging was performed with 120 kV accelerating voltage.

Selected specimen fracture surfaces were examined with a scanning electron microscope (SEM) (JSM-6360LV, JEOL Ltd., Japan). For studying the dispersion of the polypeptide micelles in CPC matrix, the specimen was immerged into DMF for 24 h to remove the micelles as the DMF can dissolve the polypeptide copolymers.

RESULTS

Figure 2 shows compression strength, fracture energy, and elastic modulus versus polymer content for CPC composites containing polypeptide graft copolymer with various molecular structures. The mean deviation of the experimental data was calculated by:

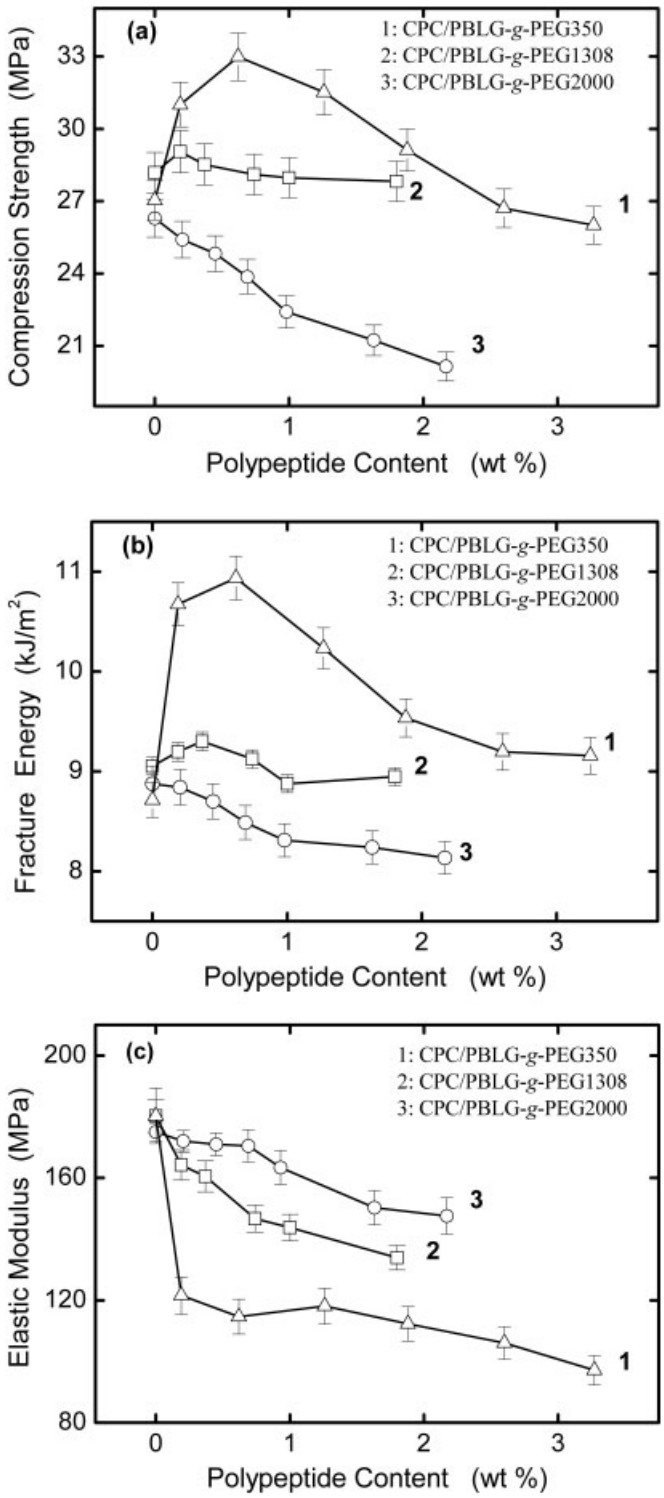

Figure 2. Compression strength (a), fracture energy (b), and elastic modulus (c) versus polypeptide content for CPC reinforced by PBLG-*g*-PEG350 (1), PBLG-*g*-PEG1308 (2), and PBLG-*g*-PEG2000 (3).

$$\theta = \frac{\sum_{i=1}^{n} |X_i - \bar{X}|}{n} \quad (1)$$

where n is the number of specimens for each material, X_i is the obtained result for the specimen (i), and $\bar{X}$ is the mean value for n specimens. In the present work, five specimens were tested for each material. $\bar{X}$ was plotted and θ was set as error bar in all figures.

Illustrated in Figure 2(a) are the obtained results of compression strength. A negative effect on the compression strength of the CPC was observed when PBLG-*g*-PEG2000 was added. Slight increase in compression strength is shown for CPC/PBLG-*g*-PEG1308 systems when small amount of copolymer was introduced into CPC matrix. The enhanced effect becomes evident for CPC/PBLG-*g*-PEG350 systems where PEG chain is short and the grafting rate is high. The compression strength increases from 27.0 MPa to 33.0 MPa when the content of copolymer increases from 0 to 0.55 wt %, being 22.3% higher compared with the pure CPC materials.

Shown in Figure 2(b) are the dependence of the fracture energy on the copolymer content in the composites corresponding to those in Figure 2(a). For the composites of CPC/PBLG-*g*-PEG1308 and CPC/PBLG-*g*-PEG350, with increasing copolymer content the fracture energy is increased first, and then followed by a decrease. Such a result suggests that the introduction of the polypeptide graft copolymer could increase the toughness of CPC.

The elastic moduli of the corresponding CPC/PBLG-*g*-PEG composites are given in Figure 2(c). For all the systems examined, the elastic modulus decreases as the polymer content increases, indicating the brittleness of CPC/PBLG-*g*-PEG composites becomes less with respect to CPC.

For the ester exchange reaction, under the same condition employed higher grafting rate can be achieved when the molecular weight of PEG is lower. The grafting rates of PBLG-PEG2000, PBLG-PEG1308, and PBLG-PEG350 are 5.0%, 12.1%, and 31.8%, respectively. The higher grafting rate of the PBLG-PEG350 samples could give rise to a higher compatibility between the polypeptide copolymer and CPC paste, and resulting in an evident enhancement of the mechanical properties.

Mechanical properties of the composites containing polypeptide graft copolymer other than PBLG-*g*-PEG have also been studied. Figure 3 gives the results obtained for CPC/PELG-*g*-PEG350 and CPC/PMLG-*g*-PEG350 composites. As shown by curve (1) in Figure 3 (a,b), when the PELG-*g*-PEG350 polymer content is increased both the compression strength and the fracture energy increase first, and then followed by a decrease. The highest values of the compression strength and the fracture energy are 46.2 MPa and 15.26 kJ/m^2, respectively, which are considerably larger than those of CPC/PBLG-*g*-PEG350 composites. Effectively enhanced effect is also shown for CPC/PMLG-g-PEG350 systems [curve (2) of Figure 3(a,b)]. Both compression strength and fracture energy increase with increasing the polymer content, reaching a maximum value of 50.9 MPa and 14.02 kJ/m^2, respectively, and then decreasing gradually. Because PELG and PMLG side chain do not have hydrophobic benzyl substitutes, the compatibility between PELG-*g*-PEG, PMLG-*g*-PEG, and CPC paste could be better than that between

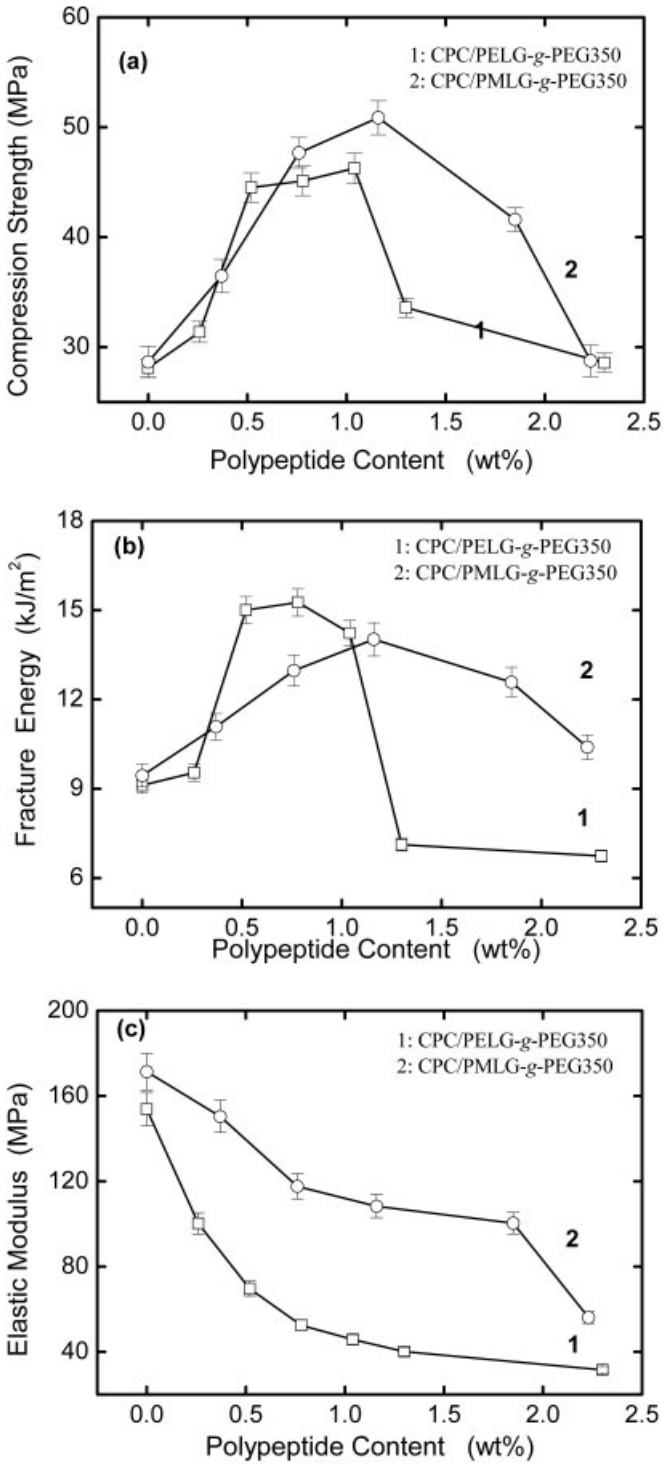

Figure 3. Compression strength (a), fracture energy (b), and elastic modulus (c) versus polypeptide content for CPC reinforced by PELG-*g*-PEG350 (1) and PMLG-*g*-PEG1308 (2).

PBLG-*g*-PEG and CPC paste. Therefore, a better mechanical property enhancement was obtained for CPC/PELG-*g*-PEG and CPC/PMLG-*g*-PEG composites. The elastic modulus values of the corresponding composites are shown in Figure 3(c). Similar to the results observed for CPC/PBLG-*g*-PEG350 composites, the elastic moduli were observed to decrease with increasing the polymer content.

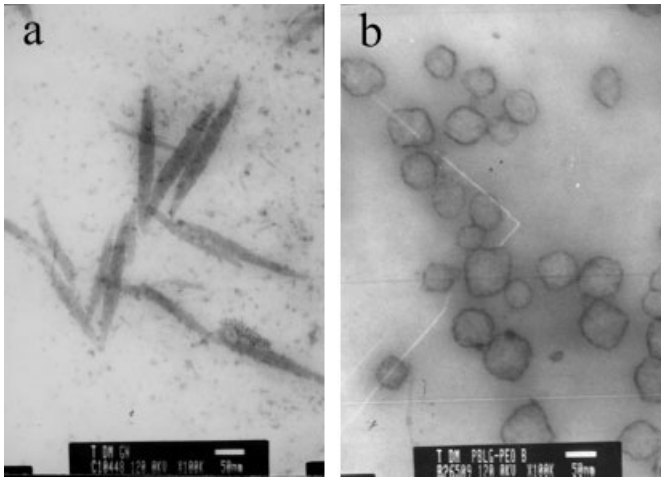

Figure 4. TEM photographs of the micelles formed by PBLG-*g*-PEG350 (a) and PBLG-*b*-PEG (b).

Under certain conditions, PBLG-*g*-PEG or PBLG-*b*-PEG copolymer can self-assemble to form micelles with a core-shell structure. Figure 4 gives a typical example of the morphologies for the micelles formed by PBLG graft and block copolymers. As it can be seen, difference in molecular structure has remarkable effect on the shape of the formed micelles. Spindly shaped micelles are showed for PBLG-*g*-PEG350 [Figure 4(a)], while the micelles formed by PBLG-*b*-PEG exhibit spherical morphologies [Figure 4(b)]. For these formed micelles, PBLG chain segments are restricted within cores and PEG chains form water-soluble shells of the micelles.[17,18] Studies on PBLG-*g*-PEG and PBLG-*b*-PEG copolymer micelles reinforcement of CPC have also been carried out. The effects of the micelle content and the micelle type on the mechanical properties of CPC composites are illustrated in Figure 5. As shown by curve (1) in Figure 5(a), the compression strength increases to a maximum value of 43.7 MPa at the micelle content of 0.83% for graft copolymer micelle composites, then decreases when the micelle content further increases. The dependence of the fracture energy on the micelle content is demonstrated by curve (1) in Figure 5(b). The maximum value of the fracture energy for CPC/PBLG-*g*-PEG micelle composites is 15.26 kJ/m^2 at the micelle content of 1.10 wt %. As for the elastic modulus, it is shown to be decreased with increasing the micelle content [curve (1) in Figure 5(c)]. Effect of the introduction of PBLG-*b*-PEG micelles into CPC is shown by curve (2) in Figure 5(a–c). Both the compression strength and the fracture energy are increased with increasing the micelle content first and then decreased. The highest values of the compression strength and the fracture energy are 59.7 MPa and 15.67 kJ/m^2, respectively. The elastic modulus remains almost unchanged when the micelles are introduced. As it can be seen from Figure 5, PBLG-*b*-PEG micelles exhibit more evident reinforcing effect than PBLG-*g*-PEG micelles. The anisotropic form of PBLG-*g*-PEG micelles, that is, spindlelike shape as shown by Figure 4(a), could make the reinforcement less effective.

Figure 6 shows SEM micrographs of the fracture surfaces of CPC reinforced either by polypeptide graft copolymers or micelles. Shown in Figure 6(a–c) are the results obtained for CPC composites containing various amount of polypeptide graft copolymers. No aggregation of the copolymer in form

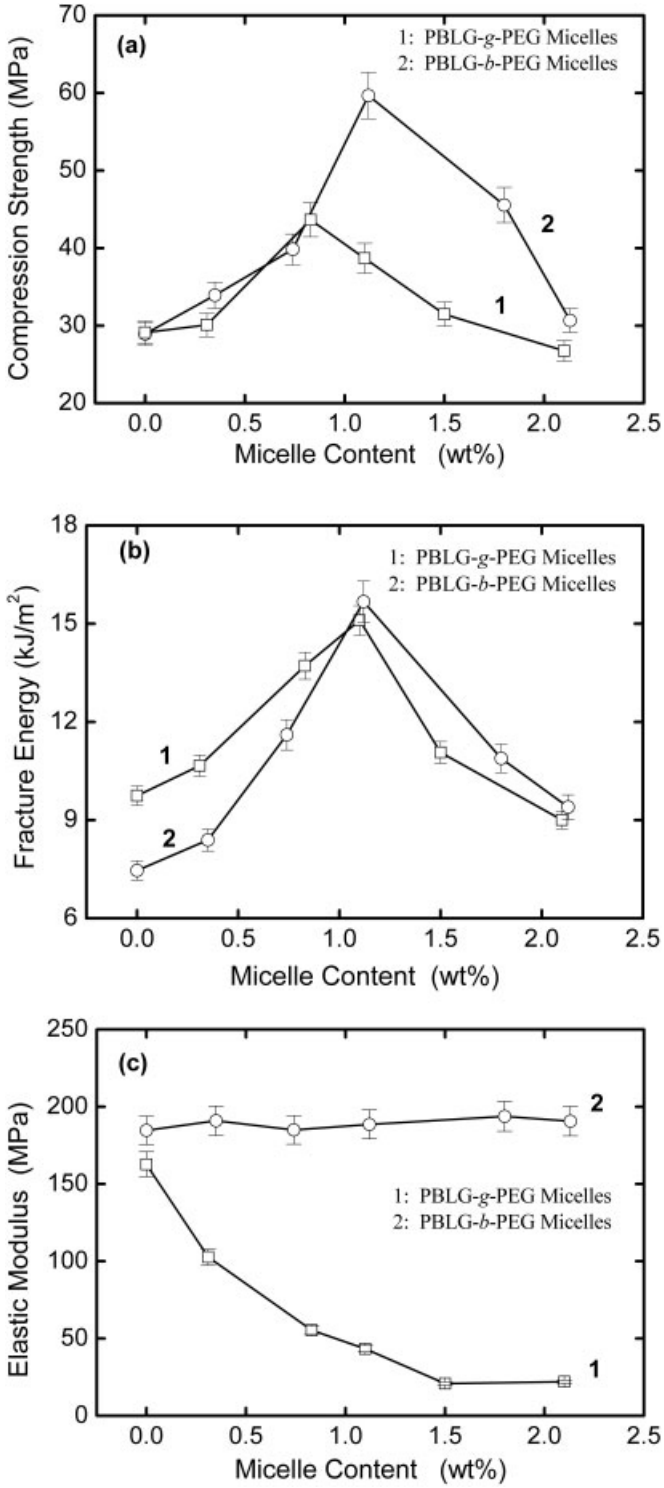

Figure 5. Compression strength (a), fracture energy (b), and elastic modulus (c) versus micelle content for CPC reinforced by micelles formed by PBLG-*g*-PEG350 (1) and PBLG-*b*-PEG (2).

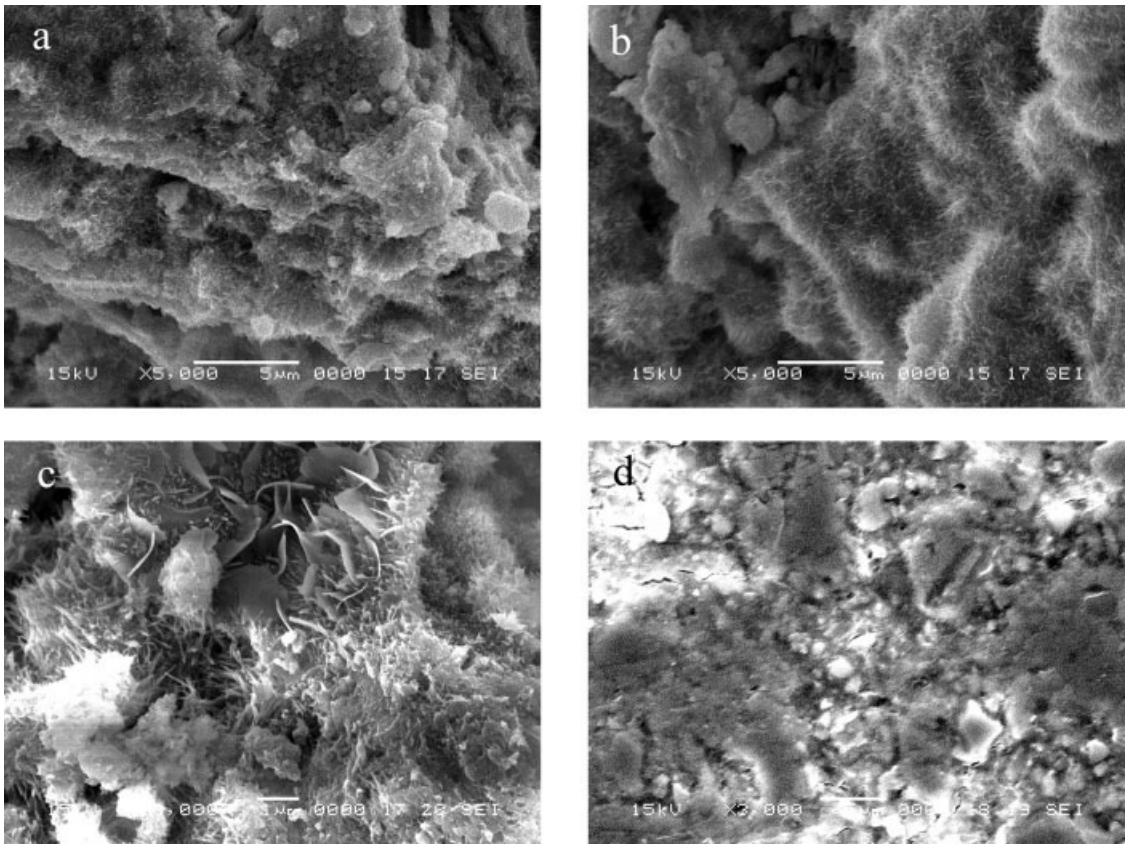

Figure 6. SEM micrographs of fracture surfaces of CPC composites with polypeptide graft copolymer content of 0 wt % (a), 1.0 wt % (b), and 5.0 wt % (c). Shown in (d) is the surface of CPC/PBLG-*b*-PEG micelle composite etched by DMF. The micelle content was 2.0 wt %.

of filler, domain, etc., is shown, suggesting that PBLG molecules with hydrophilic PEG side chains could be well dispersed in CPC matrix. It is also noted that the introduction of the polypeptide copolymer has an effect on the shape of the developed hydroxyapatite crystallites. For virgin CPC, small needlelike crystals are observed. As the polypeptide graft copolymer is added, the formed needlelike crystals become larger and well developed. Further increase in the content of the copolymer results in a flakelike crystal shape as shown in Figure 6(c). SEM observations for the surface of CPC/micelles composites etched by DMF were also carried out, and a typical result is shown in Figure 6(d). After removing the organic micelles through etching by organic solvent DMF, small holes remain in CPC matrix. The distributions of these small holes are homogenous.

DISCUSSION

CPC has excellent bone repairing properties, and has been suggested for use in a number of medical and dental procedures, including the reconstruction of frontal sinus and augmentation of craniofacial skeletal defects, endodontics, and the repair of periodontal bone defects and tooth defects. But its brittleness and low compression strength have severely limited its usage to only non–load-bearing applications. A common way to overcome this weakness is to use various types of fibers to reinforce CPC. Improved strength and toughness were reported for CPC/fiber composites. The fibers used include carbon fibers,[11,12] aramide fibers,[9,12] nylon fibers,[10] and glass fibers.[12] However, those fibers are usually not biocompatible and biodegradable, which could hider the applications of these composites in the medical procedures. In the present work, a concept of a novel CPC composite reinforced by polypeptide copolymers has been proposed. In addition, the good biocompatibility and proper biodegradation,[23] the polypeptide copolymers also have excellent mechanical properties. For example, the adhering strength of polypeptide-reinforced fibrin can be increased about 2-fold with respect to fibrin.[24] The polypeptides with hydrophilic PEG side chain and polypeptide micelles with water-soluble shells can be well mixed with CPC powder in the aqueous environment to harden and from hydroxyaptites. The compression strength and fracture energy of CPC were increased considerably after the introduction of certain polypeptide graft copolymers or micelles. Concomitantly with the increase in the compression strength and fracture energy, the elastic modulus of CPC tends to be decreased suggesting that the brittleness of CPC is reduced. The micelles show more effective reinforcement effect than the graft copolymers as

can be seen from the comparison between curve (1) and (2) of Figure 5 and curve (1) and (2) of Figure 2. This could be due to the water-soluble shells of the micelles allowing them to disperse better in CPC paste than polypeptide graft copolymers. As a result, the formed CPC/micelle composites exhibit better mechanical properties.

The presence of the polypeptide graft copolymer can also affect the morphology of the formed hydroxyapitite crystallites. With increasing copolymer content, the shape of the crystals changes from needle to flake. The formation process of CPC hydroxyapatite crystallites is a dissolve-aggregation process. When local supersaturating concentration of Ca^{2+} and HPO_4^{2-} near CPC grain surface is high enough to get the nucleation threshold value, the nuclei form and the crystallites grow subsequently.[25] When CPC powder is mixed with a polypeptide aqueous solution instead of pure water, the viscosity around CPC grain surface could be increased substantially due to the existence of the polymer. It becomes slow for Ca^{2+} and HPO_4^{2-} to diffuse under such a condition.[26] The process of the nucleation and crystallite growth thus tend to slow down. Consequently, well-developed CPC crystallites in the form of needle or flakelike structures can be formed.

A well-formed needlelike crystal at the copolymer content at 1 wt % is shown in Figure 4(b). These well-developed needlelike crystals could be attributed greatly to the improved mechanical properties of CPC/polypeptide graft copolymer composites. On the other hand, when the matrix CPC cracks under stress, the well-dispersed polypeptide copolymers may also bridge the cracks to resist its further opening. Such an effect could also contribute to the strength and toughness of CPC/polypeptide graft copolymer composites. As for CPC/micelle composites, the reinforcement mechanism could be that the embodied micelles bridge the cracks and consume the external energy. An increased fracture resistance of the composites is thus resulted.

CONCLUSIONS

Self-setting calcium phosphate cement was strengthened via polypeptide copolymer reinforcement. The molecular structure of the polypeptide graft copolymer has significant effects on the mechanical properties of CPC/polypeptide graft copolymer composites. The polypeptides with more PEG side chains, that is, higher grafting rate, have more effective reinforcement effect. Better mechanical properties have also shown for CPC enhanced by certain polypeptide copolymers without stronger hydrophobic side chain substitutes, such as PELG and PMLG. Compared with the polypeptide graft copolymer reinforcement, the form of polypeptide copolymer micelles has more efficient effect. Both polypeptide graft copolymers and polypeptide micelles were found to be well dispersed in CPC matrix. This could be helpful for the increased fracture resistance of CPC/polypeptide composites. As for CPC reinforced by the polypeptide graft copolymers, the shape of the developed hydroxyapatite crystallites was also observed to be dependent on the polypeptide graft copolymer content. A well-formed needlelike crystal is good for improving the mechanical properties. The results obtained in the present work may help to extend the medical and dental applications of CPC to the repair of larger defects and use in stress-bearing locations.

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