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Immobilization of RGD peptide on HA coating through a chemical bonding approach

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Abstract In this work, Arg-Gly-Asp (RGD) sequence containing peptide was immobilized on hydroxyapatite (HA) coatings through a chemical bonding approach in two steps, surface modification with 3-aminopropyltriethoxysilane (APTES) and RGD immobilization. The results indicate that RGD has been successfully immobilized on HA coatings. Comparing with physical adsorption coatings, the chemically bonded RGD on the coatings shows much better anti-wash-out ability. Since RGD is able to recognize cell-membrane integrins on biointerfaces, the present method will be an effective way to favor interaction of cells with HA coatings.

1 Introduction

Hydroxyapatite (HA) as a coating material has been widely investigated due to its biological and chemical similarity to the inorganic phases of bones and teeth [1]. HA coatings on Ti result in enhanced bone formation and apposition [2], and greatly improve fixation to adjacent bone in comparison to uncoated Ti [3]. As implantable biomaterials, the coatings are primarily required to facilitate cell adhesion, promote cell proliferation and allow the retention of differentiated cell functions. Although HA is bioactive, it has no functional groups as biological signals, HA coatings can not well interact with cells [4].

More recently, the mechanisms of interaction between proteins from the extracellular matrix (ECM) and cell membrane receptors were reported [5]. A number of the biomolecules (native proteins and synthetic peptides) are acknowledged to control cell adhesion and growth. Cell–ECM interactions are considered as a significant step for the osteointegration which is much sensitive to the surface composition and feature of implantable biomaterials [6]. Surface modification is a powerful way to enhance positive cell–ECM interactions and promote bone biomechanical stability [7]. Substances including poly(L-lysine), collagen, and cell adhesive proteins such as fibronectin, laminin, vitronectin, or peptide sequences have been adsorbed onto the surface of biomaterial matrix to promote cell attachment, the modified biomaterial surfaces can be functionalized to modulate cell–ECM interactions [8–11].

Many peptide sequences involved in cellular interactions by receptor binding have been identified, including RGD, IKVAV, and YIGSR [12]. Among these, the RGD sequence, which was first discovered in fibronectin on 1984 [13], is probably one of the best known for use as integrin-binding domains. The surface immobilization of RGD has several advantages: higher stability against conformational change, easy controllability of surface density, and orientation more favorable for ligand–receptor interaction and cell adhesion. It is also beneficial for minimizing immune responses and infection [10–12]. Many materials have been modified with RGD to improve the interaction with cells [11, 14]. However, these reports just limit on the materials in powders and porous bulks, and the methods in simply physical absorption.

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The existing stability of RGD on the surface, especially on coating surface, determines whether its function works or not [15, 16]. Immobilization of RGD onto implantable biomaterial surface by covalent bond can be a much more effective strategy, resulting in a durable modified surface and protecting RGD from being washed out by blood and body fluid [17].

In this work, firstly, 3-aminopropyltriethoxysilane (APTES) was used to create amine groups on HA coatings; then, RGD was covalently coupled to the surface amino groups on HA coatings. As a comparison, HA coatings were directly soaked in the RGD-containing PBS buffer solution, RGD was physically adsorbed on the coatings. The different modified HA coatings were characterized by means of FTIR-ATR, contact angle and XPS techniques, and their differences were discussed.

2 Materials and methods

2.1 Materials

HA coatings were prepared on 30 mm × 20 mm × 1 mm Ti substrate following a sol–gel method [18]. Arginine-glycine-aspartic acid (Arg-Gly-Asp) sequence of fibronectin (RGD; FW = 346.3 g/gmol, NeoMPS PolyPeptide laboratories), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride($C_8H_{17}N_3 \cdot HCl$, EDC.HCl, Acros), 3-aminopropyltriethoxysilane($SiO_3C_9H_{23}N$, APTES, Acros), 2-(*N*-morpholino)-ethanesulfonic acid($C_6H_{13}O_4NS$ H_2O, MES buffer, Shanghai Major Bio Technologies Co., Ltd), phosphate buffer saline(PBS, Dycent Biotech shanghai CO., Ltd), ethanol (C_2H_5OH, Sinopharm Chemical Reagent CO., Ltd) were used.

2.2 Methods

RGD was chemically immobilized on HA coatings (C-RGD-HA) through the following 3 steps: (1) HA coatings were immersed into APTES ethanol solution (20 mmol/L) at room temperature for 4 h with rigorously stirring, washed by ethanol for three times, and dried in a vacuum oven at 120°C for 24 h to be APTES-modified HA coating. (2) The modified coatings were again immersed in MES buffer solution with 0.3% (w/v) 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride at designed temperature for 6 h with rigorously stirring, washed with the MES buffer solution for three times. (3) the MES buffer solution treated coatings were put into RGD-containing PBS buffer solution (300 μg/ml) and settled for 4 h at room temperature, then, the coatings were rinsed with PBS for three times and lyophilized for 2 days.

As a comparison, the physically immobilized RGD on HA coatings (P-RGD-HA) were prepared by immersing the coatings directly into RGD-containing PBS buffer solution (300 μg/ml) as the above step 3.

To evaluate the stability of RGD on the modified HA coatings, both C-RGD-HA and P-RGD-HA samples in ethanol solution were further treated by ultrasonic irradiation for two minutes at room temperature.

Fourier transform infrared-attenuated total reflectance-spectra (FTIR-ATR, Thermo, NICOLET830), water contact angles (OCA20, Dataphysics) were recorded for the modified coatings before and after ultrasonic irradiation. X-ray Photoelectron Spectroscopy analysis (XPS, Thermo ESCALAB 250 system) with focused monochromatic Al K. X-ray source (1486.6 eV) was used, C1s peak at 284.8 eV was used for calibration.

3 Results and discussions

3.1 FTIR spectra

Figure 1 gives the FTIR-ATR spectra of HA coating and RGD-modified HA coatings, the results show that both P-RGD-HA coating and C-RGD-HA coating have a strong absorption at 1655 cm^{-1} and at 1557 cm^{-1}, these bands are characteristic of Amide I and Amide II of RGD molecule, respectively. Obviously, RGD has existed on the HA coatings obtained by both physical adsorption and chemical bonding approaches.

Figure 2 gives the FTIR-ATR spectra of HA coating and RGD-modified HA coatings after ultrasonic irradiation.

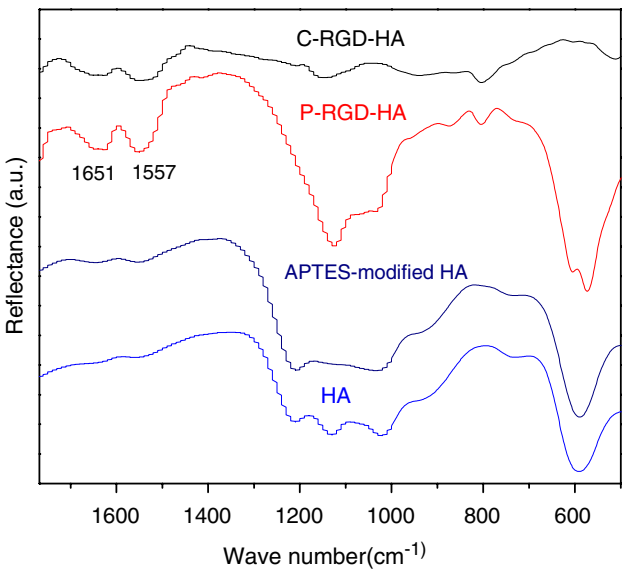

Fig. 1 FTIR-ATR spectra of HA coating and RGD-modified HA coating

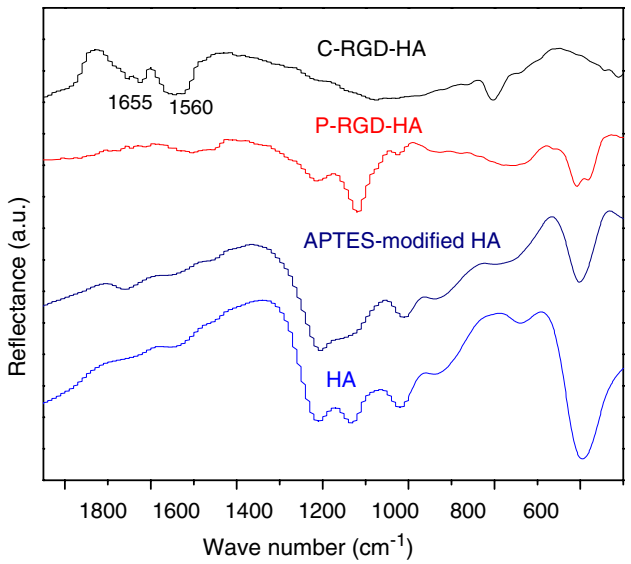

Fig. 2 FTIR-ATR spectra of HA coating and RGD-modified HA coating after ultrasonic irradiation

The strong absorptions at 1655 cm^{-1} and at 1557 cm^{-1} still exist in C-RGD-HA coating but disappear in P-RGD-HA coating. It is demonstrated that the immobilization of RGD by physical adsorption is unstable, and the immobilization by chemical bonding shows good stability, and can provide a better cell adhesive stratum.

3.2 Water contact angle measurement

In this work, the water contact angle of HA coating is 51.5°. As tabulated in Table 1, the contact angle of APTES-modified HA coating (75.1°) is higher than that of HA coating because siloxane in APTES has low-surface-energy and shows hydrophobicity. After RGD modification, the water contact angle of P-RGD-HA decreases due to hydrophilic amino and carboxyl groups existing in RGD. RGD is suggested to be covalently coupled by carbodiimide mediated condensation between carboxyl groups present in the RGD and amino groups of the HA coatings surface. It can be showed that water contact angle of C-RGD-HA decreases obviously compared with APTES-modified HA coating. While it is noteworthy that the water contact angle of C-RGD-HA coating remains almost unchanged after ultrasonic irridiation, it is again proved that the surface of C-RGD-HA coating is stable, i.e., RGD exists stably. As for P-RGD-HA, after ultrasonic irradiation, the water contact angle increases obviously. That means that most of physical adsorbed RGD have been washed out, and physical adsorbed surface is unstable. The change in contact angle is in good agreement with those in its FTIR-ATR spectra.

3.3 XPS analysis

In Fig. 3, the high-resolution XPS spectra of N1s region recorded from the ultrasonic irridiated surface further confirms the preservation of RGD on C-RGD-HA after ultrasonic treatment. Besides the peak at 400.0 eV from N in APTES, a shoulder peak at 401.8 eV is observed. This peak is attributed to N in RGD [19, 20]. Calculated from the areas of deconvolved N1s curve, there is actually about 38% N1s signal coming from chemically bonded RGD molecules. That again coincides well with the FTIR result: for C-RGD-HA, much RGD peptides still exists even after ultrasonic irridiation.

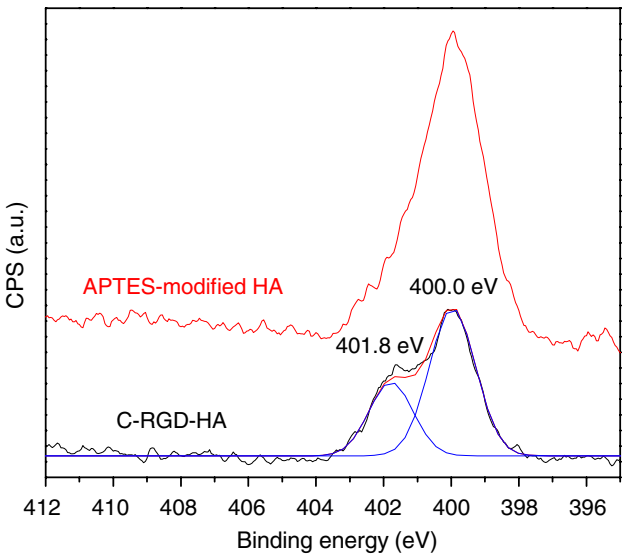

Fig. 3 High-resolution XPS spectrum of N1s region of APTES-modified HA coating and C-RGD-HA

Table 1 Water contact angle measurement of APTES-modified HA coating and RGD-modified HA coating

P-RGD-HA		C-RGD-HA	
Before RGD modification (Bare HA coating)	51.5°	Before RGD modification (APTES-modified HA)	75.1°
After RGD modification	22.4°	After RGD modification	48.2°
After ultrasonic irradiation	36.0°	After ultrasonic irradiation	47.9°

4 Conclusions

In this study, RGD peptide is successfully immobilized onto the surface of HA coatings by both physical adsorption and chemical bonding. Through ultrasonic irradiation test, it is proved that RGD in C-RGD-HA is more stable than that in P-RGD-HA. The present work also shows that immobilization of RGD onto implantable biomaterial surface by covalent bond could be an effective strategy, it will protect RGD from being influenced by blood and body fluid. It can favor RGD sequences to act as cellular adhesion ligands to respond interactions between cell-membrane integrins and extracellular matrix proteins.

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