Fabrication and in vitro evaluation of the collagen/hyaluronic acid PEM coating crosslinked with functionalized RGD peptide on titanium

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ABSTRACT

Surface modification of titanium (Ti) using biomolecules has attracted much attention recently. In this study, a new strategy has been employed to construct a stable and bioactive coating on Ti. To this end, a derivative of hyaluronic acid (HA), i.e. HA-GRGDSPC-(SH), was synthesized. The disulfide-crosslinked Arg-Gly-Asp (RGD)-containing collagen/hyaluronic acid polyelectrolyte membrane (PEM) coating was then fabricated on Ti through the alternate deposition of collagen and HA-GRGDSPC-(SH) with five assembly cycles and subsequent crosslinking via converting free sulphydryl groups into disulfide linkages (RGD–CHC-Ti group). The assembly processes for PEM coating and the physicochemical properties of the coating were carefully characterized. The stability of PEM coating in phosphate-buffered saline solution could be adjusted by the crosslinking degree, while its degradation behaviors in the presence of glutathione were glutathione concentration dependent. The adhesion and proliferation of MC3T3-E1 cells were significantly enhanced in the RGD–CHC-Ti group. Up-regulated bone specific genes, enhanced alkaline phosphatase activity and osteocalcin production, the increased areas of mineralization were also observed in the RGD–CHC-Ti group. These results indicate that the strategy employed herein may function as an effective way to construct stable, RGD-containing bioactive coatings on Ti.

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1. Introduction

Commercial pure titanium (cp Ti) and its alloys have been extensively used in the dental [1] and orthopedic fields for manufacturing medical devices, such as dental implants or hip-joint replacement devices, mainly due to their excellent resistance to corrosion and good biological performances [2,3]. However, Ti-based implants are only passively integrated with bone; they cannot directly bind to it [4]. In order to speed up the tissue–implant integration to shorten the clinical healing time and improve the clinical healing results, surface modification of Ti-based implants has attracted worldwide attention.

Getting inspiration from tissue repairing, during which the type and amount of rapidly adsorbed biomolecules on an implant surface immediately after insertion determine the subsequent cellular events, including the recruitment, proliferation and differentiation of osteogenic cells, researchers have introduced biomolecules with clear biological functions onto the implant, with the aim of tailoring the critical process of protein adsorption after implantation. Consequently, by using selected patterns of signals, the process of tissue–implant integration can be accelerated considerably [5].

Among the bioactive substances used, the peptide Arg-Gly-Asp (RGD) has been extensively employed to biologically functionalize the Ti-based implant surface. It is well known that the RGD motif is related to cell adhesion, and many biomolecules present this motif [6]. Previous reports showed that the introduction of RGD sequences could promote the cell attachment and differentiation of osteogenic cells on Ti [7–9]. Moreover, the bone formation could even be accelerated by immobilizing RGD on the Ti-based implant surface [10–12].

Numerous methods have been developed so far to introduce RGD peptides onto the surface of biomaterials, including physical adhesion [13], chemical binding using spacer reagents with bifunctional terminal groups [14–16] and electrochemical deposition [17,18]. However, the physically adsorbed RGD peptide is unstable on Ti in physiological conditions. RGD that is freely dissolved in cell culture medium can in fact partially inhibit host cell attachment onto fibronectin-coated Ti in a competitive, dose-dependent manner [19]. The burst release of the physically adsorbed RGD inevitably leads to high concentration of free RGD in the local domain during the initial stage of tissue repair, which would partially counteract the experimental expectation. RGD chemically bound to Ti using spacer reagents can improve cell attachment. However, the effect of the spacer reagents on the long-term osteointegration between Ti and the newly formed bone after the degradation of RGD peptides is uncertain.

Consequently, seeking effective strategies for RGD decoration has become a real challenge for potential applications in consideration of the limitations of the currently available methods. It is well known that the disulfide bond is necessary to maintain the stability and functions of many proteins. The reversible changes in the redox state of thiol/disulfide couples can also serve as a "sulfur switch" to control the structure and activities of a broad range of proteins under biological conditions [20,21].

Inspired by the conversion of thiol/disulfide couples, a novel strategy was employed in this study to fabricate a disulfide-crosslinked RGD-containing coating on cp Ti. First, a special type of peptide, with the sequence of GRGDSPC-(S-S)-CPSDGRG, was synthesized and introduced into hyaluronic acid (HA) molecules via chemical grafting in the presence of 1-ethyl-3,3-dimethylaminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) [22,23]. The disulfide linkage of this peptide was cleaved with dithiothreitol (DTT) to obtain HA-GRGDSPC-(SH). Collagen and HA-GRGDSPC-(SH) were used to fabricate a collagen/HA-GRGDSPC-(SH) coating on the surface of cp Ti via the layer by layer (Lbl) technique. Eventually, the collagen/HA-GRGDSPC-(SH) polyelectrolyte membrane (PEM) coating was crosslinked by the disulfide linkage via the oxidation of free mercapto groups, forming a semi-interpenetrating network (semi-IPN) gel coating. In addition to introducing RGD into the coating, the disulfide bonds gave the coating better stability and tunable biodegradability. The purpose of this study was to evaluate the effectiveness of this strategy by investigating the physicochemical properties and degradation behaviors of the disulfide-crosslinked RGD-containing PEM coating and its effect on the in vitro behaviors and functions of preosteoblasts.

2. Materials and methods

2.1. Synthesis of HA-GRGDSPC-(SH) and HA-GRGDSPC-(SH0)

The peptide GRGDSPC-(S-S)-CPSDGRG was synthesized by Beijing Scilight Biotechnology Ltd., Co. For this, 1 mg ml⁻¹ HA (Furuida Biomedical Company, Zhejiang, PR China) was reacted with 0.12 mg ml⁻¹ GRGDSPC-(S-S)-CPSDGRG (the mole ratio of carboxylic groups in HA to GRGDSPC-(S-S)-CPSDGRG was about 20:1) in a solution containing 40 mM EDC and 10 mM NHS at 4 °C for 48 h, followed by dialysis against deionized water for 2 weeks. The HA-GRGDSPC-(S-S)-CPSDGRG-HA was then obtained by lyophilization. To reduce the disulfide linkages to mercapto groups, HA-GRGDSPC-(S-S)-CPSDGRG-HA (1 mg ml⁻¹) was reacted with 1 M DTT at room temperature for 4 h, followed by dialysis against deionized water for 1 week. HA-GRGDSPC-(SH) was then harvested by lyophilization. Through the same method, 1 mg ml⁻¹ HA and 0.2 mg ml⁻¹ GRGDSPC-(S-S)-CPSDGRG were used to synthesize HA-GRGDSPC-(SH0) (the molar ratio of carboxylic groups in HA to GRGDSPC-(S-S)-CPSDGRG was about 20:1.8).

2.2. Surface modification

Titanium discs (cp, 30 mm in diameter and 1 mm in thickness) were kindly supplied by Zhejiang Guangci Biomedical Instrument Company (Zhejiang, PR China). They were polished first with SiC papers with various grain sizes (from #320 to #4000) and then with a diamond suspension. The discs were cleaned for 15 min by sonication cycles in acetone, ethanol and deionized water, respectively, then dried in air. These thoroughly polished Ti discs were then thoroughly rinsed with deionized water and dried under an N₂ atmosphere.

Col/HA coating group (CHC-Ti): collagen (Col, type I, from calf skin) was purchased from Sigma-Aldrich, USA. A 1 mg ml⁻¹ collagen solution was prepared by dissolving collagen in 0.1% HCl while HA was directly dissolved in pure water at a concentration of 0.5 mg ml⁻¹. The isoelectric points of collagen and HA are 5.5 [24] and 2.9 [25], respectively. When they were dissolved in a solution at pH 4, the collagen can carry positive charges while the HA can carry negative charges. Accordingly, the pH value of these two solutions was adjusted to about 4.0 with 0.1 M HCl. The Col/HA PEM coating was constructed by dipping the AE-Ti discs alternately into the collagen and HA solutions for 15 min, and each dipping process was followed by rinsing with deionized water three times. A collagen deposition plus a HA deposition was considered as one assembly cycle. The Col/HA PEM-coated Ti discs that had undergone five assembly cycles were blow-dried with N₂.

Disulfide-crosslinked RGD-containing Col/Ha coating group (RGD–CHC-Ti): in this group, HA was replaced with HA-GRGDSPC-SH) to form the Col/HA-GRGDSPC-(SH) coating on AE-Ti discs. The other experimental conditions were the same as those for the CHC-Ti group. The Ti discs with the Col/HA-GRGDSPC-SH) PEM coating were immersed in 10 mM 2-(N-morpholino)-ethanesulfonic acid (Mes) buffer solution (pH 6) containing 2 mM chloramine T (Cat) at room temperature for 1 min [26]. The discs were then taken out of the Mes/Cat solution and thoroughly rinsed with 10 mM phosphate-buffered saline (PBS, pH 6.5) and deionized water. Eventually, the discs with disulfide-crosslinked RGD-containing Col/Ha PEM coating were dried under an N₂ blower.

It should be noted that all the samples were sterilized via ultraviolet (UV) irradiation for further experiments.

2.3. Characterization

Fourier Transform infrared (FTIR) spectra were obtained from samples in KBr pellets using a Thermo Nicolet Avator 370 FTIR spectrometer (USA). Scanning force microscopy (SFM) images were recorded under the tapping mode in air at 20–25 °C using a NanoScope III multimode scanning force microscope (Digital Instruments, Santa Barbara, CA). Contact angle measurements were performed using a dynamic contact angle system (GBX Instruments, Valence, France) at ambient temperature employing the sessile-drop measuring method. A 4 µl water droplet was dispensed onto the substrate investigated, and five different positions were then measured to get an average contact angle.

A quartz crystal microbalance (QCM) was used to measure the mass or thickness of each absorbed layer. A CH1215A quartz crystal with a fundamental frequency of 8 MHz was obtained from CHI (USA). The crystal (15 mm in diameter) was coated on both sides with mirror-like polished gold electrodes (5 mm in diameter). A similar procedure to that mentioned above was used to fabricate PE multilayer films on the QCM electrodes. The electrodes were put into a special apparatus to only allow the front side of the electrode to come into contact with the solutions (about 0.5 ml). The frequency shift of the quartz crystal, Δf, was measured for each layer using a commercial QCM (QTEZ; Resonance Probe GmbH, Goslarn, Germany). The area mass density of each layer, Δm, could be calculated by Sauerbrey’s equation [27]:

\[
-\Delta f = 14.5 \times 10^6 \Delta m
\]

(1)

The nominal thickness of each layer, \(d\) (nm), can also be estimated as [28]:

\[
d = (-6.8 \times 10^{-7}) - \Delta f / \rho (\text{nm})
\]

(2)
and HA, the density was about 1.2 g cm\(^{-3}\). For collagen and HA, the density was about 1.2 g cm\(^{-3}\).

2.4. Degradation behaviors of the different PEM coatings

The glass slides (1 cm \(\times\) 2 cm) were rinsed in turn with acetone, ethanol and deionized water, then dried with the N\(_2\) blower. The rinsed glass slides were immersed in piranha solution (a mixture of H\(_2\)SO\(_4\) and H\(_2\)O\(_2\), H\(_2\)SO\(_4\):H\(_2\)O\(_2\) = 7:3) at 60 °C for 2 h, then rinsed with deionized water. The rinsed glass slides were dried with the N\(_2\) blower for further experiments. Collagen (1 mg ml\(^{-1}\)) were labeled with 1 mg ml\(^{-1}\) fluorescein isothiocyanate (FITC; Sigma) at 4 °C for 48 h, followed by dialysis against deionized water for 4 weeks. The dry Col-FITC was obtained by lyophilization. One group of the clean glass slides was coated with (Col-FITC/HA)_5 PEM coatings via the same assembly methods described in Section 2.2. The other two groups of the clean glass slides were deposited with Col-FITC/HA-GRGDSPC-(SH) and Col-FITC/HA-GRGDSPC-(SH)_0 multilayers of five deposition cycles, respectively. Eventually, two RGD-containing PEM coatings were crosslinked with Mes/CaO oxidation, recorded as RGD-crosslinked Col-FITC/HA PEM coating and RGD-crosslinked Col-FITC/HA PEM coating.

Five glass slides covered with the Col-FITC/HA PEM, five glass slides covered with the RGD-crosslinked Col-FITC/HA PEM coatings and five glass slides covered with the RGD-crosslinked Col-FITC/HA PEM coating were immersed in PBS solution (pH 7.2). Each of the different PEM coatings vs. the corresponding immersion days.

For this, 10 \(\mu\)l of both forward and reverse primer, 7.2 \(\mu\)l of distiller water and 2 \(\mu\l\) of the cDNA samples. The plate was covered and centrifuged briefly to remove air bubbles, followed by the PCR quantification using cycling parameters: 95 °C for 10 s and then 60 °C for 34 s, for 40 cycles. All samples were analyzed in triplicate. The comparative Ct-value method was used to calculate the relative quantity of Runx2, Oss, alkaline phosphatase-2 (Alp-2), osteocalcin (OC), collagen type I alpha 1 (Col1a1) and GAPDH genes of mouse are given in Table 1. Amplification reactions were performed with a SYBR Premix EB Taq was added to each well of an optical 96-well plate, together with 0.4 \(\mu\l\) of both forward and reverse primer, 7.2 \(\mu\l\) of distilled water and 2 \(\mu\l\) of the cDNA samples. The plate was covered and centrifuged briefly to remove air bubbles, followed by the PCR quantification using cycling parameters: 95 °C for 10 s and then 60 °C for 34 s, for 40 cycles. All samples were analyzed in triplicate. The comparative Ct-value method was used to calculate the relative quantity of Runx2, Oss, alkaline phosphatase (ALP), OC and Col1a1. Expression of the housekeeping gene GAPDH was used as internal control to normalize results.

2.7. Characterization of differentiation

2.7.1. Real-time polymerase chain reaction (PCR) analysis of bone-specific genes

Total RNA was isolated from MC3T3-E1 cells on the Ti discs with an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The RNA concentration was determined by a spectrophotometer and a total of 1.5 \(\mu\l\) of the RNA solution was immediately used as the template in CDNA synthesis. Quantitative real-time PCR was performed with an ABI Prism 7500 sequence detection system (PerkinElmer). The sequences of primers for Runx2, Osterix (Oss), alkaline phosphatase-2 (Alp-2), osteocalcin (OC), collagen type I alpha 1 (Col1a1) and GAPDH genes of mouse are given in Table 1. Expression of the housekeeping gene GAPDH was used as internal control to normalize results.

2.7.2. Alkaline phosphatase activity assay

The activity of intracellular ALP was measured with a commercial phosphatase substrate kit (Wako, Japan). A cell-seeded Ti disc was rinsed with DPBS (Sigma, St. Louis, MO, USA) and recovered by cell lysis buffer (CellyticTM M, Sigma), then incubated for 15 min on a shaker at 4 °C. The lysate was collected and centrifuged for 15 min to pellet the cellular debris, and finally the supernatant was stored at −80 °C until assay. Aliquots of 20 \(\mu\l\) of the supernatant were mixed with 80 \(\mu\l\) of p-nitrophenylphosphate disodium in

$$\text{where } \rho \text{ is the density of the film in unit of (g cm}^{-3}\).$$

and 3 days in \(\alpha\)-MEM supplemented with 10% FBS. The number of cells cultured on the Ti discs was determined by fluorometric quantification of the amount of cellular DNA. Each Ti disc with cultured cells was rinsed with PBS, recovered using sodium citrate buffer solution containing 50 mM sodium citrate and 100 mM NaCl, and stored at −80 °C until assay. After thawing, the cells were lysed in the sodium citrate solution with sonication. Then 20 \(\mu\l\) of cell lysate was mixed with 80 \(\mu\l\) of DNA-binding fluorescent dye solution. The fluorescent intensity of the mixed solution was measured on a fluorescence spectrometer (Sunrise-Basic TECAN, Austria), with excitation and emission wavelengths of 480 and 520 nm, respectively.

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Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Amplicon (bp)</th>
<th>5’–3’ Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC</td>
<td>178</td>
<td>F 5’–AGCTGGTCTCCAGGATCCACCTAGCTAC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–TACAGGCAGTTCGTGGTACATAC-3’</td>
</tr>
<tr>
<td>AKP-2</td>
<td>164</td>
<td>F 5’–GCTGCTGGTGCTGGGAGCTTAA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–TTATTCTCTTCTCCAGAATAC-3’</td>
</tr>
<tr>
<td>Col1a1</td>
<td>153</td>
<td>F 5’–CTTAGGCGCTGCAAGACTGATCAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–TCCAGGGGTTCGACATTCCTAGC-3’</td>
</tr>
<tr>
<td>Runx2</td>
<td>144</td>
<td>F 5’–TCTACAAAGCTGACGCCAGATTCA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–TGTCTGCTGTTGGGAGCTTCTCTC-3’</td>
</tr>
<tr>
<td>Oss</td>
<td>144</td>
<td>F 5’–GCTTCTCAGCAGTGAGGATTC3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–GCTTCTGCTGTTGGGAGCTTCTCTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>150</td>
<td>F 5’–GCTTCTGCTGTTGGGAGCTTCTCTC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5’–GCTTCTGCTGTTGGGAGCTTCTCTC-3’</td>
</tr>
</tbody>
</table>

OC, osteocalcin; AKP-2, alkaline phosphatase 2; Col1a1, collagen type I alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

microplates. Mixtures were then shaken for 1 min by a plate mixer and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 80 μl of 0.2 mol l⁻¹ sodium hydroxide solution to each well. The mixtures were shaken for 1 min again and the resulting optical densities were measured at 405 nm with a spectrophotometer (Sunrise-Basic TECAN). Measurements were compared to p-nitrophenol standards and normalized with total protein content. The total cellular protein content was determined with a BCA protein assay kit (Beyotime, China). Briefly, 20 μl aliquots of cell lysate were mixed with 200 μl of BCA working reagent containing cupric sulfate and bicinchoninic acid in microplates and incubated at 60 °C for 30 min. The resulting optical densities were measured at 562 nm with a spectrophotometer. Bovine serum albumin was used to generate a standard curve.

2.7.3. OC release
OC released to the cell culture medium was measured with a Mouse Osteocalcin EIA Kit (Biomedical Technologies Inc., USA) according to the manufacturer’s protocol. Briefly, 25 μl of the cell culture medium and 100 μl of osteocalcin antiserum were placed in a 96-well EIA plate and incubated at 4 °C for 18–24 h. Each well was washed with PBS and 100 μl of streptavidin–horseradish peroxidase reagent was added and incubated at room temperature for 30 min. To this was added 50 μl of tetramethyl benzidine solution and hydrogen peroxide solution, before incubating at room temperature for 15 min. After adding 100 μl of the stop solution, absorbance was measured at 450 nm on a spectrophotometer. The results were normalized with total cellular protein content.

Scheme 1. Synthesis of HA derivatives. GRGDSPC-(S-S)-CPDGRG was chemically conjugated to HA in the presence of EDC/NHS to form HA-GRGDSPC-(S-S)-CPDGRG-HA. DTT was used to cleave the disulfide linkage in HA-GRGDSPC-(S-S)-CPDGRG-HA to obtain HA derivatives (HA-GRGDSPC-(SH)).
2.7.4. Detection of mineralization

The mineralization of MC3T3-E1 cells on different specimens was evaluated by alizarin red S staining. After differentiation had been induced for 28 days, cells on the specimen were fixed with 4% paraformaldehyde for 30 min. The fixed cells were washed with deionized water and then stained with 2% alizarin red S aqueous solution (pH 4.1–4.3) for 5 min at room temperature. The alizarin red S solution was removed and the remaining dye washed out with deionized water. The Ti discs with the stained cells were air-dried and photographed.

2.8. Statistical analysis

Statistical analysis was carried out using an SPSS statistical software package (V11, SPSS, Inc., Chicago, IL, USA). All the results are expressed as mean ± standard deviation (SD) and tested for statistical significance with a one-way analysis of variance test. p < 0.05 was considered to be statistically significant.

3. Results

3.1. Synthesis of HA-GRGDSPC-(SH)

The synthesis of HA derivatives is illustrated in Scheme 1. FTIR was used to characterize the various molecules, including HA, HA-GRGDSPC-(SH) and HA-GRGDSPC-(SH0). As shown in Fig. 1, compared with the FTIR spectrum of HA, three new peaks at 1530, 1600 and 3030 cm⁻¹ appeared in the spectra of HA-GRGDSPC-(SH) and HA-GRGDSPC-(SH0). However, due to the extremely weak absorbance of mercapto groups, there was no difference between the absorption curves of HA-GRGDSPC-(SH) and HA-GRGDSPC-(SH0).

3.2. Morphology characterization

Unlike scanning electron microscopy images, SFM micrographs may show the real surface morphology, because the samples do not need to be coated in gold. The AE-Ti surface exhibited some groove-like structures that are characteristic features from the mechanically grinding process (the left panel of Fig. 2A). The high-resolution image further revealed that the acid etching produced a succession of honeycomb-like pits at the nanoscale (the left panel of Fig. 2A). Compared with the AE-Ti surface, the depth of parallel grooves decreased on the CHC-Ti and RGD–CHC-Ti surfaces (the left panels of Fig. 2B and C). As shown in the right panel of Fig. 2B, after the assembly with the Col/HA multilayer, the successive honeycomb-like nanopits were enshrouded with a novel uniform structure at the nanoscale. When compared with the CHC-Ti group, another fabrication structure was seen on the surface of the RGD–CHC-Ti group, as shown in the left panel of Fig. 2C. The difference in surface morphology between the CHC-Ti group and the RGD–CHC-Ti group can be mainly attributed to the crosslinking.

3.3. Contact angle measurement

The contact angle measurement was used to follow the real-time assembling process of the CHC-Ti and RGD–CHC-Ti groups. The contact angle of SM-Ti was 52°. As shown in Fig. 3, the contact angle of the AE-Ti decreased to 24.5° after it was treated with H₂SO₄/H₂O₂ mixture. For the tested Col/HA samples, after the first collagen layer and the second HA layer were deposited, the contact angle of the resultant Ti discs decreased to 16.8 and 15.1°, respectively. HA is more hydrophilic than collagen. As a result, the contact angle of the odd layer was larger than the corresponding even layer. Similarly, for the Col/HA-GRGDSPC-SH samples, the changing trend of the contact angle was similar to that of the Col/HA samples, and the contact angle of the odd layer was also larger than the corresponding even layer. However, the contact angle of the crosslinked Col/HA-GRGDSPC-SH coating was slightly increased, from 22 to 23.2°. The contact angle measurement clearly suggests the successful construction of the PEM coatings via the LBL technique.

3.4. Measurement of the thickness of different PEM coatings

The results shown in Fig. 4 reveal that the thickness of Col/HA PEM and Col/HA-GRGDSPC-SH PEM increased almost linearly with the deposition number, which is one of the two elementary fabrication patterns according to the growth behaviors of the thickness of the two-dimensional PEM, i.e. linear fabrication [29] and exponential fabrication [30]. The thickness of a single Col/HA-GRGDSPC-SH bilayer was slightly greater than that of a single Col/HA bilayer. Each bilayer was more than 5 nm, a typical thickness for a number of PEM ultrafilms [31]. The thickness of the dry Col/HA coating and that of the Col/HA-GRGDSPC-SH coating with five assembly cycles was 27.2 and 30 nm, respectively. After the crosslinking, however, the thickness of the crosslinked Col/HA-GRGDSPC-SH PEM coating decreased from 30 to 27.1 nm.

3.5. The degradation behaviors of the Col/HA coating and the RGD-crosslinked Col/HA coating

Since it was difficult to directly quantify the degradation behaviors of the coatings on the CHC-Ti and RGD–CHC-Ti discs, we studied the degradation profiles of these PEM coatings labeled fluorescently on glass slides. The results showed that the degradation of the Col/HA PEM coating was fairly rapid, as shown in Fig. 5A. On the first day, about 25% of multilayer was degraded. Within 5 days, more than 90% of the Col/HA PEM coating was degraded. However, the RGD-crosslinked coatings displayed a slower degradation rate. For the HA-GRGDSPC-(SH) of the RGD-crosslinked Col/HA group, the molar ratio of the carboxylic groups of the HA to GRGDSPC-(SH) was 10:1.1, while for the RGD-crosslinked Col/HA group the molar ratio was 10:1.8. The different graft ratios resulted in the different degrees of crosslinking for the corresponding PEM coatings, which in turn led to different degradation behaviors. As a result, in contrast to the degradation behavior of the Col/HA PEM coating, about 16% and 10% of the
Coatings were degraded on the first day for the RGD-crosslinked Col/HA group and the RGD-crosslinked Col/HA₀ group, respectively. The degradation periods of the RGD-crosslinked Col/HA coating and the RGD-crosslinked Col/HA₀ coating were 14 and 19 days, respectively.

Based on the functions of the resultant coating, its degradation rate should be adjusted to match the stages of peri-implant bone tissue regeneration. The major role of RGD is to promote host cell adhesion on an implant. Consequently, the RGD-crosslinked PEM coating should exert its influence during the early phase of bone regeneration. As documented [32], newly formed bone may appear on the inorganic implant surface after 1 week of insertion, with more appearing after 2 weeks. Therefore, the coating was assumed to exist for no more than 2 weeks. Herein, the RGD-crosslinked Col/HA PEM coating was selected for further detection and in vitro biological evaluation.

Since the GSH concentrations in the cellular interior and extracellular surroundings are 0.5–10 mM [33] and 2–10 μM [34], respectively, the degradation behaviors of the RGD-crosslinked Col/HA PEM coating were separately studied in 5 mM and 10 μM GSH solutions. As shown in Fig. 5B, the degradation profiles of the coating were dramatically different in the different GSH concentrations. In the 5 mM GSH solution, the degradation curve was similar to that of the Col/HA PEM coating in PBS solution. During the first 5 days, more than 90% of the coating was degraded. In the 10 μM GSH solution, however, the degradation of the RGD-
crosslinked Col/HA coating was relatively slow, and the time for total degradation was about 10 days.

3.6. Adhesion and proliferation behaviors of MC3T3-E1 cells

The number of cells attached to Ti discs was determined by fluorometrically quantifying the amount of cellular DNA (ng disc⁻¹), because the cellular DNA content of each cell is constant in the same cell line. As shown in Fig. 6, the DNA content of the RGD–CHC-Ti and CHC-Ti groups was higher than that of the AE-Ti group after 1 h of cell seeding (p < 0.05). Moreover, the DNA content of the RGD–CHC-Ti group was the highest among the three groups (p < 0.05). After 3 days of culture, the change in the proliferation of preosteoblasts of the three groups showed almost the same tendency. The cellular DNA content of the CHC-Ti group and the RGD–CHC-Ti group was higher compared with that of the AE-Ti group (p < 0.05), and the cellular DNA content of the RGD–CHC-Ti group remained the highest (p < 0.05).

3.7. mRNA expression of bone-specific genes

The mRNA levels of AKP-2 (Fig. 7A), Runx2 (Fig. 7B), Osx (Fig. 7C), Col1a1 (Fig. 7D) and OC (Fig. 7E) were detected at days
4, 7 and 14. From Fig. 7, one can observe that the mRNA expression levels of AKP-2, Runx2, Osx, Col1a1 and OC increased with the culture time no matter what the substrate was. Moreover, the mRNA expression levels of all five genes were significantly higher in the CHC-Ti and RGD–CHC-Ti groups than that in the AE-Ti group \( (p < 0.05) \) at each time point. The highest expression level was found in the RGD–CHC-Ti group \( (p < 0.05 \text{ or } p < 0.01) \). The mRNA expression of AKP-2 at week 2 (Fig. 7A), Runx2 at week 1 (Fig. 7B), Col1a1 at week 1 (Fig. 7D) and OC at days 4 and 7 in the RGD–CHC-Ti group were almost twice as high as those of the other two groups at the same time point.

3.8. ALP activity and OC release

As shown in Fig. 8A, ALP activity of MC3T3-E1 cells increased with culture time independent of the substrate used, and approached a maximum on day 14. At day 4, the ALP activity of the RGD–CHC-Ti group was the highest among the three groups.
(p < 0.05). Moreover, the ALP activity of the AE-Ti group was lower than that of the CHC-Ti group (p < 0.05). The same tendency was found at days 7 and 14.

OC is regarded as a late marker of preosteoblast differentiation. As shown in Fig. 8B, after 2 weeks of cell growth, the RGD–CHC-Ti group tended to promote MC3T3-E1 differentiation, as the OC production of this group was obviously higher than that on the CHC-Ti group or the AE-Ti group (p < 0.05). Also, OC production in the CHC-Ti group was higher than that of the AE-Ti group (p < 0.05).

3.9. Detection of mineralization

As shown in Fig. 9, the red area indicated the mineralized nodules of the different groups. The red areas of the RGD–CHC-Ti and CHC-Ti groups were much larger than that of the AE-Ti group. In particular, the mineralization area on the RGD–CHC-Ti specimen was the largest among the three groups. Moreover, because the MC3T3-E1 cells used in this study were heterogeneous, the calcified deposits were nonuniform and clustered in certain locations. A similar phenomenon has been reported previously [35].

4. Discussion

Ideally, two essential requirements should be fulfilled for the construction of bioactive coatings on implantable devices. One is that the activity of the introduced bioactive molecules should be maintained; the other is that the coating formed by bioactive molecules should be stable. Often, however, there is a dilemma between maintaining the stability of the bioactive coating and conserving the activity of the bioactive molecules. Physical adsorption can only produce unstable coatings, which limits the applications of the resulting coatings in practice. Whereas chemical crosslinking can produce stable yet bioactive coatings.

In this study, we developed a novel strategy to construct a disulfide-crosslinked RGD-containing coating, taking advantage of the reversible translation between mercapto groups and disulfide linkage. In such a coating, collagen molecules were physically intertwined within a three-dimensional HA network to form semi-IPNs, as shown in Scheme 2. SFM images (Fig. 2) and the contact angle measurement (Fig. 3) evidenced that the Col/HA coating and the crosslinked Col/HA-GRGDSPC-SH PEM coating were successfully introduced onto the AE-Ti surface. Compared with the hydrophilicity of the AE-Ti discs, the hydrophilicity of the CHC-Ti and RGD–CHC-Ti discs was increased further (Fig. 3). The surface wettability of biomaterials plays an important role in cell–surface interactions. It has been proved that the increased hydrophilicity of biomaterials is beneficial for the biological behaviors of the cells involved [36]. Moreover, the crosslinking procedure led to a more compact PEM coating, which could account for the thickness of the Col[HA-GRGDSPC-SH]3 PEM coating being decreased from 30 to 27.1 nm after being crosslinked (Fig. 4).

From the results shown in Fig. 5A, one can see that the cross-linking of disulfide bonds could significantly improve the stability of the coating. Furthermore, a higher graft ratio of HA-GRGDSPC-(SH0) molecules leads to a higher degree of crosslinking, which in turn results in a longer degradation period. Since the graft ratio of HA-GRGDSPC-(SH) can be readily adjusted, the crosslinking
degree and therefore the degradation behaviors of the disulfide-crosslinked RGD-containing Col/HA coatings may be easily tailored.

GSH exists extensively in living organisms, and can influence the stability of disulfide linkage by converting this bond into sulfhydryl groups. Fig. 5B shows that the degradation behavior of the disulfide-crosslinked coating in the 5 mM GSH solution was similar to that of the Col/HA coating in PBS solutions, and the coating could be almost completely degraded within 5 days. In the 10 μM GSH solution, however, the crosslinked coating did not degrade completely within 9 days. The degradation behaviors of the disulfide-crosslinked coatings would thus appear to be GSH concentration dependent: the higher the GSH concentration, the faster the cleavage of disulfide bond. It has been demonstrated that the cleavage of disulfide bonds can be accomplished within several hours in the 5 mM GSH solution [26], which could account for the similar degradation behaviors of the disulfide-crosslinked Col/HA PEM coating in the 5 mM GSH solution and the Col/HA PEM coating in PBS. A low concentration of GSH could slow the cleavage of disulfide bonds, leading to the slower degradation rate and prolonged existence of the crosslinked PEM coating. In other words, the introduction of disulfide bonds can not only improve the stability of the coating, but may also render the disulfide-crosslinked PEM coating biodegradable in a physiological environment.

It seems that the incorporation of RGD peptides is of primary importance in the establishment of cell-surface recognition on the Ti-based implant surface, as the DNA content of the RGD-CHC-Ti group was the highest at hour 1 (Fig. 6). Similar results have been reported by other researchers [37,38]. This shows that the employed strategy to introduce RGD peptides is effective and
that the bioactivity of the RGD peptide is maintained well. A number of studies have suggested that cell attachment plays a dominant role in the subsequent proliferation and differentiation [9,39,40]. From Fig. 6, we can see better cell attachment behaviors in the RGD–CHC-Ti group, and a subsequently increased capacity for proliferation.

Osteoblasts, the cells responsible for bone formation, are generally differentiated from preosteoblasts. Runx2 and Oxsx, expressed selectively and at high levels in osteoblasts, are two transcription factors involved in regulating the multistep molecular pathway of preosteoblast differentiation. Generally speaking, the up-regulated expression level of Runx2 and Oxsx can increase the expression of osteoblast marker genes [41], such as AKP-2 and OC. In this study, the mRNA expression levels of transcription factors (such as Runx2 and Oxsx) and osteoblast-specific genes (like ALP, OC and Col1a1) were obviously increased in the RGD–CHC–Ti group (Fig. 7). ALP and OC are indicators of fully functionalized osteoblasts and participate in bone formation and mineralization [39,42–44]. The changing trends of ALP activity and OC production were consistent with the corresponding mRNA expression levels in the three groups at the predetermined time points (Fig. 8). These results revealed the superiority of the disulfide-crosslinked RGD-containing PEM coating on promoting preosteoblast differentiation.

Osteoblasts not only synthesize the bone matrix, they also mineralize it [45,46]. Alizarin red S histochemical staining [46] is a method for analyzing mineralized nodule formation via selective binding to calcium salts in vitro, which can visually reflect the capacity of different groups on the matrix mineralization. The results of Fig. 9 clearly indicate that the strongest capacity for matrix mineralization is in the RGD–CHC–Ti group.

Taken together, the results of the mRNA expression levels, ALP activity and OC production as well as alizarin red S staining reveal that the RGD–CHC–Ti group exhibited a stronger influence on the cell differentiation of preosteoblasts than the other two groups. Overall, the in vitro evaluation indicates the increased cell responses of preosteoblasts to the disulfide-crosslinked RGD-containing Col/HA PEM coating on Ti.

5. Conclusions

In conclusion, this study has developed a new method to fabricate disulfide-crosslinked RGD-containing Col/HA PEM coatings onto Ti via the LBL technique. In addition to introducing RGD into the Col/HA PEM coating, the crosslinking procedure provided the obtained PEM coatings with several special traits. First, the formation of the disulfide bonds promoted the stability of the PEM coating and made the length of time the PEM coating existed in PBS solution adjustable. Secondly, the presence of disulfide bonds gave the crosslinked PEM coating a tunable biodegradability. Thirdly, the specific transformation of sulphydryl groups into disulfide bonds had virtually no influence on the activity of the RGD sequence and the other components of the PEM coating. In vitro evaluation showed the crosslinked RGD-containing PEM coatings could markedly promote the adhesion, proliferation and differentiation of preosteoblasts. Moreover, such disulfide-crosslinked bioactive coatings with a semi-IPN structure can be further optimized by integrating other peptides, proteins or drugs into the semi-IPN architecture. The strategy used herein may find general applications where controlling either molecular or cell responses at biomaterial–tissue interfaces is important.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2 and 9, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.10.020.

References


