Grafting RGD containing peptides onto hydroxyapatite to promote osteoblastic cells adhesion

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Ceramics possess osteoconductive properties but exhibit no intrinsic osteoinductive capacity. Consequently, they are unable to induce new bone formation in extra osseous sites. In order to develop bone substitutes with osteogenic properties, one promising approach consists of creating hybrid materials by associating *in vitro* biomaterials with osteoprogenitor cells. With this aim, we have developed a novel strategy of biomimetic modification to enhance osseointegration of hydroxyapatite (HA) implants. RGD-containing peptides displaying different conformations (linear GRGDSPC and cyclo-DfKRG) were grafted onto HA surface by means of a three-step reaction procedure: silanisation with APTES, cross-linking with N-succinimidyl-3-maleimidopropionate and finally immobilisation of peptides thanks to thiol bonding. Whole process was performed in anhydrous conditions to ensure the reproducibility of the chemical functionalisation. The three-step reaction procedure was characterised by high resolution X-ray photoelectron spectroscopy. Efficiency of this biomimetic modification was finally demonstrated by measuring the adhesion of osteoprogenitor cells isolated from HBMSC onto HA surface.

Introduction

With advances in understanding tissue-materials interactions, the application of calcium phosphate materials as a bone substitute has recently received considerable attention. Since these ceramics have good biocompatibility and exhibit osteoconduction properties [1, 2], a larger number of calcium phosphate biomaterials have been proposed as artificial bone fillers in repairing bone defects during the last decade. Bioactive ceramics (BAC), for example Bioglass®, sintered hydroxyapatite (HA), sintered β-tricalcium phosphate (TCP) and glass-ceramic A–W [3–7], are the only materials that provide biological tissue-integration in bone defects. In dense and porous bulk materials, particulates, grouts and coatings, BACs have been important biomedical materials in clinical hard tissue repairs and replacements [3, 4, 7]. Nevertheless, in clinical applications, complaints have been related to ceramics performances, which mostly implies inadequacies of bulk properties in load-bearing physical functions and regenerative biological functions. In addition, many troubles have been reported considering filling of large bone defects. Henceforth, it would be useful to improve these properties in particular modifying the material to interact selectively with a specific cell type through biomolecular recognition events.

This later approach implies the biomimetic modification of the material in which peptides containing the adhesions domains of the extracellular matrix (ECM) are attached to the base material. The central hypothesis of biomimetic surface engineering is that peptides that mimic part of the ECM affect cell attachment to the material, and that surfaces or 3-D matrices modified with these peptides can induce tissue formation conforming to the cell type seeded on the material. Therefore extensive research over the last decade has been performed on the incorporation of adhesion promoting oligopeptides into biomaterial surfaces [8–11]. Since identification of the

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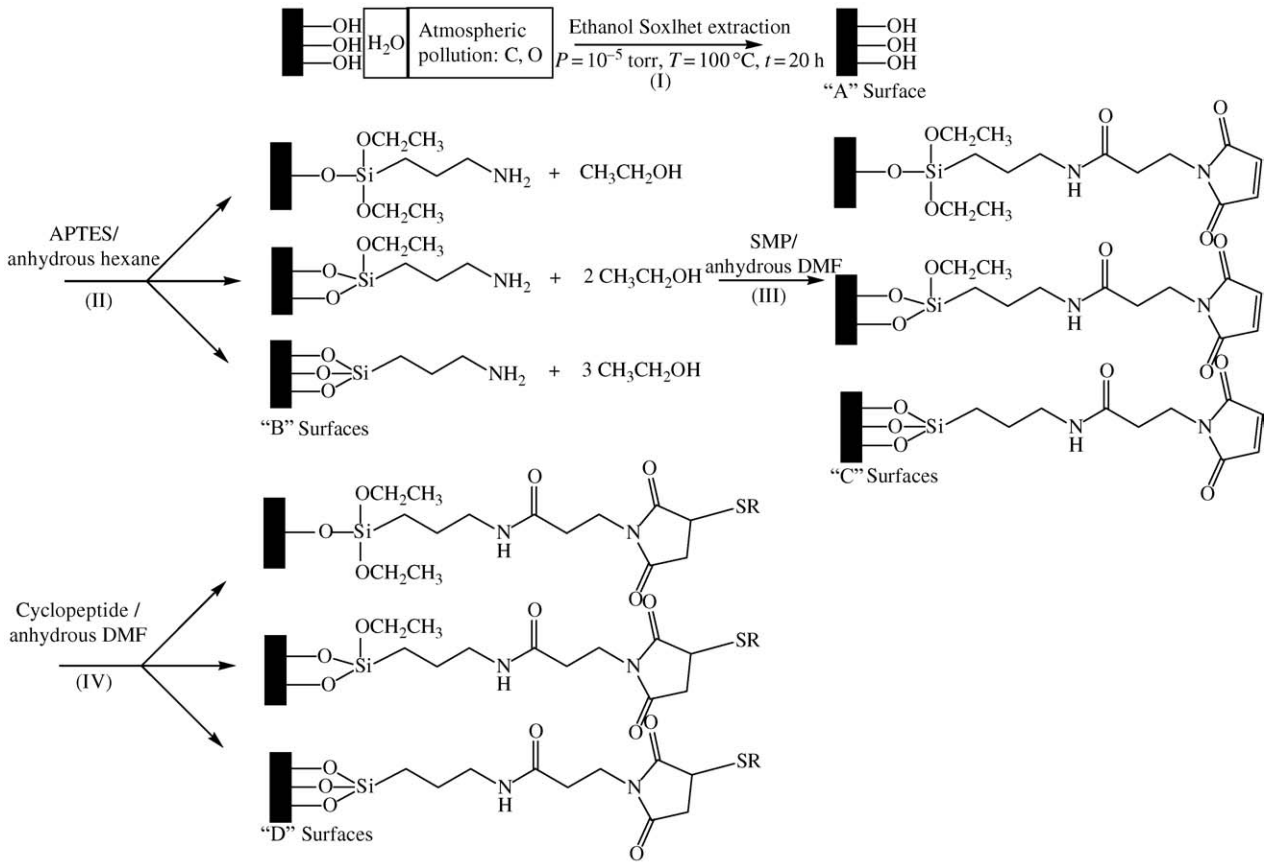

Figure 1 Sequence of the HA surface modification procedure: (I) cleaning and annealing of HA [''A'' Material]; (II) APTES grafting (three ways) [''B'' Material]; (III) reaction to the hetero-bifunctional cross-linker (SMP) [''C'' Material]; (IV) immobilisation of RGD containing peptide though thiol bounding [''D'' Material].

RGD (Arg–Gly–Asp) sequence as mediating the attachment of cells through ligand–receptor interactions to several plasma and ECM proteins (fibronectin, vitronectin, collagen, laminin,) [12], researchers have been depositing RGD-containing peptides on biomaterials to promote cell attachment [13].

For porous materials, one desirable objective would be the biomimetic modification over the three dimensions [14] to ensure cell colonisation in the depth of the material. In this context, the present paper focuses on the covalent immobilisation of two RGD-containing peptides displaying a different conformation (linear GRGDSPC and cyclo-DfKRG) onto macroporous HA (Fig. 1) [15–17]. In a first part of this paper, we describe a new method to immobilise bioactive molecules onto HA by attaching silane – derivatised spacer arms to its surface as an intermediary for the covalent linkage of RGD-containing peptides. The reaction procedure was characterised by high resolution X-ray photoelectron spectroscopy (XPS). Finally, the influence of these different RGD-containing peptides on osteoprogenitor cells adhesion is discussed. These data suggest that, by controlling the preparation of HA, bone substitutes with intrinsic osteoinductive property can be developed from HA.

Materials and Methods

Materials

3-aminopropyltriethoxysilane (APTES) was obtained from ABCR-Roth Sochiel, France. Hexane, Dimethylformamide (DMF) and 3-Succinimidyl-3-MaleimidoPropionate (SMP) were purchased from Aldrich, France. HA and cyclic peptides (cyclo-(Asp–Dphe–Lys (mercaptopropionyl)–Arg–Gly)) (cyclo-DfKRG) [18] were produced by Biomet-Merck Biomaterials, Darmstadt, Germany. GRGDSPC peptides were obtained from Bachem, France. HA cylinders (length: 10 mm, diameter: 6 mm, porosity: $75 \pm 10\%$, pore size: $550 \pm 50\,\mu m$; interconnexion size: $150 \pm 50\,\mu m$) were obtained from Biocetis Society, Berck sur Mer, France.

Surface preparation

HA have been cleaned using a Soxlhet extractor (with ethanol) during 24 h.

Biomimetic modification

Biomimetic modification was carried out in a dry and air-free chamber in order to avoid surface contamination by water and carbon compounds from surrounding atmosphere and hence to ensure reproducibility and stability of the biomolecules covering. The strategy of peptide immobilisation (Fig. 1) involves (i) grafting of an aminofunctional organosilane (APTES) onto the surface of HA, (ii) substitution of the terminal amine for a hetero-bifunctional cross-linker SMP in order to (iii) react the ''outer'' maleimide group with a peptide, thanks to the thiol group present in the terminal cysteine for the GRGDSPC or in the mercapto group for cyclo-

DfKRG. Experimentally, the HA surface modification has been performed following this procedure, also presented in Fig. 1.

1. HA was outgassed at 100 °C under vacuum (10^{-5} torr) for 20 h (surface A).
2. Silanisation of HA surface was performed by immersing the substrate in a solution of APTES (1×10^{-2} M) in anhydrous hexane under Ar atmosphere for 2 h under stirring.
3. Samples were cleaned under Ar atmosphere by three rinsings under stirring and sonication for 30 min (both the steps have been performed using anhydrous hexane).
4. Samples were outgassed at 70 °C under vacuum (10^{-5} torr) for 4 h (surface B).
5. Silanised surfaces were coupled with the hetero-bifunctional cross-linker SMP (2×10^{-3} M) in DMF for 2 h under Ar atmosphere (surface C).
6. Samples were cleaned under Ar atmosphere by three rinsings under stirring and sonication for 30 min (both the steps have been performed using anhydrous hexane).
7. Samples were outgassed at 70 °C under vacuum (10^{-5} torr) for 4 h.
8. RGD-containing peptides were immobilised on HA samples in anhydrous DMF (1 mM) (surface D).
9. Samples were cleaned under Ar atmosphere by two rinsings under stirring and sonication for 30 min in anhydrous DMF.

Surface characterisation

X-ray photoelectron spectroscopy has been applied to control reactions at each step of the procedure. XPS spectra have been recorded with a VG 220i-XL Escalab spectrometer on HA substrates at each step of the RGD peptides grafting. Power of the non-monochromatised MgKα source was 200 W with an investigated area of about 250 µm. A flood gun was used for charge compensation. Acquisition of high-resolution spectra was done at constant pass energy of 20 eV. Fitting was then realized with software provided by VG Scientific, each spectrum being referenced to carbon pollution at 284.8 eV. Binding energy (BE) values are given at $\pm$ 0.2 eV.

Cell culture

Osteoprogenitor cells were isolated from human bone marrow stroma cells (HBMSC) according to Vilamitjana *et al*. [19]. Briefly, human bone marrow was obtained by aspiration from the iliac crest of healthy donors (20–50 years) undergoing hip prosthesis surgery after traumatic shock. Cells were separated into single suspension by sequentially passing the suspension through syringes fitted with 16, 18 and 21 gauge needles. After centrifugation for 15 min at 800 g, the pellet was resuspended in Iscove modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% (v/v) foetal calf serum (FCS, Gibco) and 10^{-8} M dexamethasone (Sigma). Cells were then plated into 75 cm^2 cell culture flasks (Nunc) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Five days later, the medium was removed, replaced two times with the complete medium supplemented with 10^{-8} M dexamethasone, and then every three days with IMDM containing 10% FCS (v/v). Subculturing was performed using 0.2% (w/v) trypsin, 5 mM EDTA. Cell differenciation was followed by the measurement of alkaline phosphatase activity and osteocalcin synthesis as described previously [19].

Cell attachment

Cell attachment was measured by a modified colorimetric method: measurement of the activity of the lysosomial enzyme, N-acetyl β-D hexosaminidase as described by Majeska [20] with some modifications [16]. At first, 24-wells tissue culture plates (NUNC) had been coated with 2% (w/v) agarose gel. The positive and negative controls of cells adhesion were the tissue culture polystyrene (TCPS) and TCPS with 2% (w/v) agarose gel. Second, the samples with and without peptide (HA, HA with GRGDSPC peptide, HA with cyclo-DfKRG peptide) grafting were preadsorbed with iscove modified Dulbecco's medium (IMDM (Gibco)) containing 0.5% BSA (w/v) for 2 h in order to block non specific cell attachment on the samples [21]. The osteoprogenitor cells were seeded at a density of 2×10^4 cell/cm^2 on each substrate and allowed to attach for 1, 3 or 24 h at 37 °C. Then, the samples were placed in other wells to avoid any contribution of attached cells on agarose coating. After that, 500 µl of chromogenic substrate solution (7.5 mM chromogenic substrate (p-nitrophenyl N-acetyl β-D glucosaminide); 0.1 M Na citrate; pH 5.5% (v/v) Triton-X100) was added for 2 h at 37 °C. in a humidified atmosphere. The reaction was stopped with EDTA/ Glycine buffer (respectively 5 and 50 mM at pH 10.4). The resulting chromophore was measured spectrophotometrically at 405 nm. Data were obtained from three experiments and for each experiment, we used osteoprogenitor cells pooled.

Results

Biofunctionalisation procedure

XPS has been used to monitor each reaction step as it can provide information on chemical bondings and atomic concentrations. Table I gives the change in the atomic proportions on the top surfaces. Figs. 2 and 3 show successively the C1s and N1s spectra obtained after each step of the treatment. The as-mentioned BEs are comparable with BEs found in the literature (Table II). In the following discussion, solely the XPS characterisation of the cyclo-DfKRG peptide grafting procedure will be presented, the GRGDSPC grafting procedure leading to similar results.

The main elements present in HA surface are Ca, P, C and O. The expected theoretical ratio Ca : P is about 1.7. The experimental ratios obtained are successively 1.8, 1.3, 1.15, 1.6 for HA, HA + APTES, HA + SMP and HA + cyclopeptide, respectively. After grafting of the surface, Ca and P detections are more difficult since electrons must succeed in crossing over the layer of grafting.

TABLE I XPS atomic composition in percentages of HA at each step of the treatment

	Ca	P	C	O	Si	N	S	N/Si
HA	13.3	7.4	30.0	49.3	—	—	—	—
HA + APTES	7.0	5.5	45.5	34.0	4.0	4.0	—	1
HA + APTES + SMP	4.6	4.0	55.7	26.7	3.3	5.7	—	1.7
HA + APTES + SMP + Cyclo-peptide	6.8	4.2	44.2	35.5	2.8	5.7	0.8	2.0

TABLE II BEs (eV) assigned to specific nitrogen–carbon and silicium containing functional groups in agreement with litterature data

	C1s	Si2p	N1s
SiO_3C	283.9	102.7 [27]	
CH_x	284.8 [28]		
C–CO	285.3 [29, 28]		
C–NH_2, C–O	285.9–286.1 [28]		398.9 [30]
N–C=O	286.4 [28]		399.7–400.8 [29]
Imide, Maleimide, guanidine	287.2–288 [28]		399.7–400.7 [9, 31]
COOH	288.5 [28]		

XPS analysis of HA treated with APTES confirms that silicon and nitrogen are detected in addition to Ca, P, O and C that are usually found at the HA surface. Our experimental measurements (Table II) lead to a N : Si ratio close to the expected ratio (4.0 : 4.0 vs. 1 : 1). In addition to CH_x bondings emerging at 284.8 eV (Fig. 2(b)), carbon contributions with smaller oxidised components (at 285.4 eV) may be related to residual ethoxy groups belonging to silane molecules. As compared to the ''A'' C 1s spectrum (Fig. 2(a)), a new contribution arises at 283.9 eV. This latter peak is attributed to SiO_3C groups (Table II) in accordance with the peak emerging at 102.5 eV in the Si 2p spectrum (Fig. 4). At the same time, the N 1s spectrum (Fig. 3(a)) reveals two components: one of low energy characteristic of C–NH_2 groups (398.9 eV) and two others (at 401.7 and 400.2 eV) which can be attributed to nitrogen involved in oxidised environments. This latter contribution may be due to some interactions between the terminal amino group and oxygen groups near the surface [22–24]. From all these observations, it is obvious that the –CH_2–CH_2–NH_2 chains are well grafted on the surface.

At the SMP step (surface ''C''), the experimental value gives 5.7 : 3.3 (about 1.7) (Table I) while the expected N : Si ratio is 2 : 1. In our opinion, the SMP grafting occurs without a systematic bonding between SMP and APTES molecules. This may be due to (i) the inaccessibility of few amino groups (nitrogen involved in oxidised environments) or else (ii) the over-size of the cross-linker molecules. Similarly in spectra obtained after APTES grafting, the C1s and the Si2p spectra exhibit peaks at 283.9 and 102.5 eV, respectively. In addition, the corresponding peak areas are very similar, which is consistent with the presence of the SiO_3C groups and also the retention of this bond during the SMP grafting process. As compared to ''B'' surface, some oxidized components of C1s appear in the 285.4–289.0 eV range (Fig. 2(c)). They may be related to the C–C=O, C(=O)N groups present in the SMP molecules as well as to the imide groups present in the maleimide group (Table II). Concerning the N1s spectrum (Fig. 3(b)), the component at 398.6 eV decreases and two components (at 399.7 and 401.1 eV) increase featuring the changing of the amine groups to amide or imide groups (Table II, Fig. 1).

After peptide grafting (surface ''D''), XPS analyses confirm the increase of the nitrogen content and the presence of sulphur that comes from the mercapto group of cyclo-DfKRG (Table I). As shown in Fig. 2(d), a C1s component increases at 288 eV, which can be partly attributed to guanidine [NH–C(=NH)–NH_2] carbons. The N1s spectrum (Fig. 3(c)) shows a maximum at 399.9 eV probably due to the amide species (Table II). The experimental N : Si and N : S ratios become 2.0 and 7.2 respectively. These values are very far from the expected ratios (i.e. N : Si=N : S=11) corresponding to one cyclo-DfKRG peptide grafted onto each silicon atom.

Human osteoprogenitor cells adhesion

The potential of these GRGDSPC and cyclo-DfKRG peptides to promote the cells adhesion to biomaterials was investigated using osteoprogenitor cells isolated from HBMSC (Fig. 5). Quantitative results obtained in this study reveal that both cyclic and linear peptides constitute good ligands to increase cell adhesion at times (3 and 24 h) with respect of virgin materials.

Quantitative results show that the cyclo-DfKRG grafting induced a significant increase of cell adhesion in a short time (3 h) with respect to the linear GRGDSPC grafting. However, at 24 h, results show that the amount of attached cells is more important in the case of HA grafted with linear peptides with respect to cyclic peptides.

Discussion

The macroporous structure of HA has been processed to provide a support for the tissue ingrowth and a mechanical scaffold for the developing tissue. The scaffold can be either seeded with cells prior to implantation or designed for invasion by host cells following implantation. The goal of our approach has been to immobilise RGD-containing peptides on HA for the purpose of inducing specific cell and tissue responses. To control exposure and concentration of peptides and hence the necessary homogeneity of the peptides spatial distribution, we have developed a novel strategy of biomimetic modification using covalent immobilisation of peptides.

The major advantage of small peptides with respect of large peptides or proteins is their resistance to proteolysis and their ability to bind with higher affinities to integrin

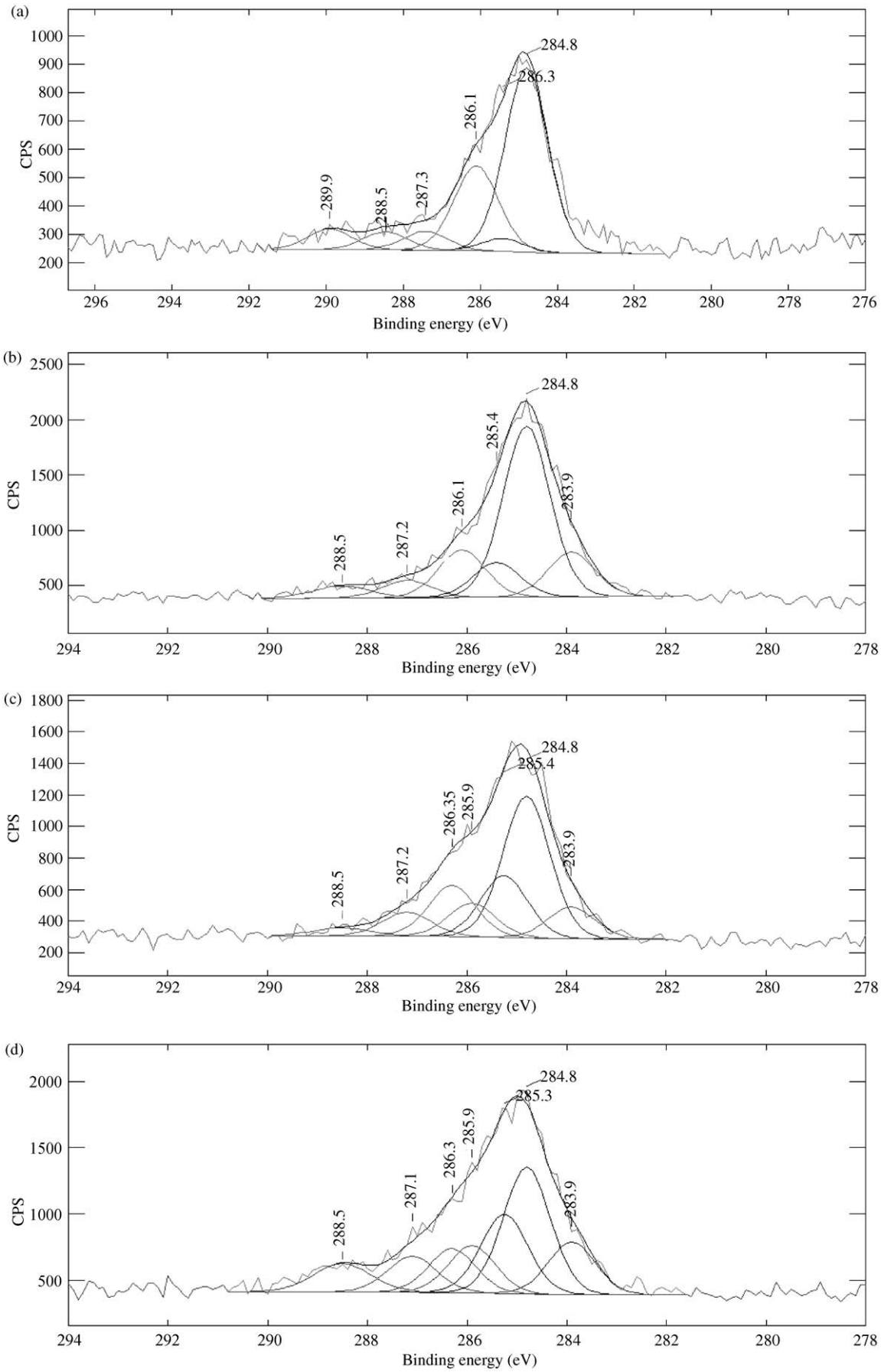

Figure 2 C1s XPS spectra for materials ''A'', ''B'', ''C'' and ''D'' (a, b, c, d, respectively).

receptors. As already shown, with the coating of proteins and peptides onto titanium alloy (Ti–6Al–4V), incorporation of this RGD sequence into cyclic peptides can improve cell attachment [16, 23]. Moreover, with respect to proteins, peptides benefit from a lower immunogenic activity as well as from the ability to be synthesised and handled.

Concerning RGD peptides, highly active and integrin

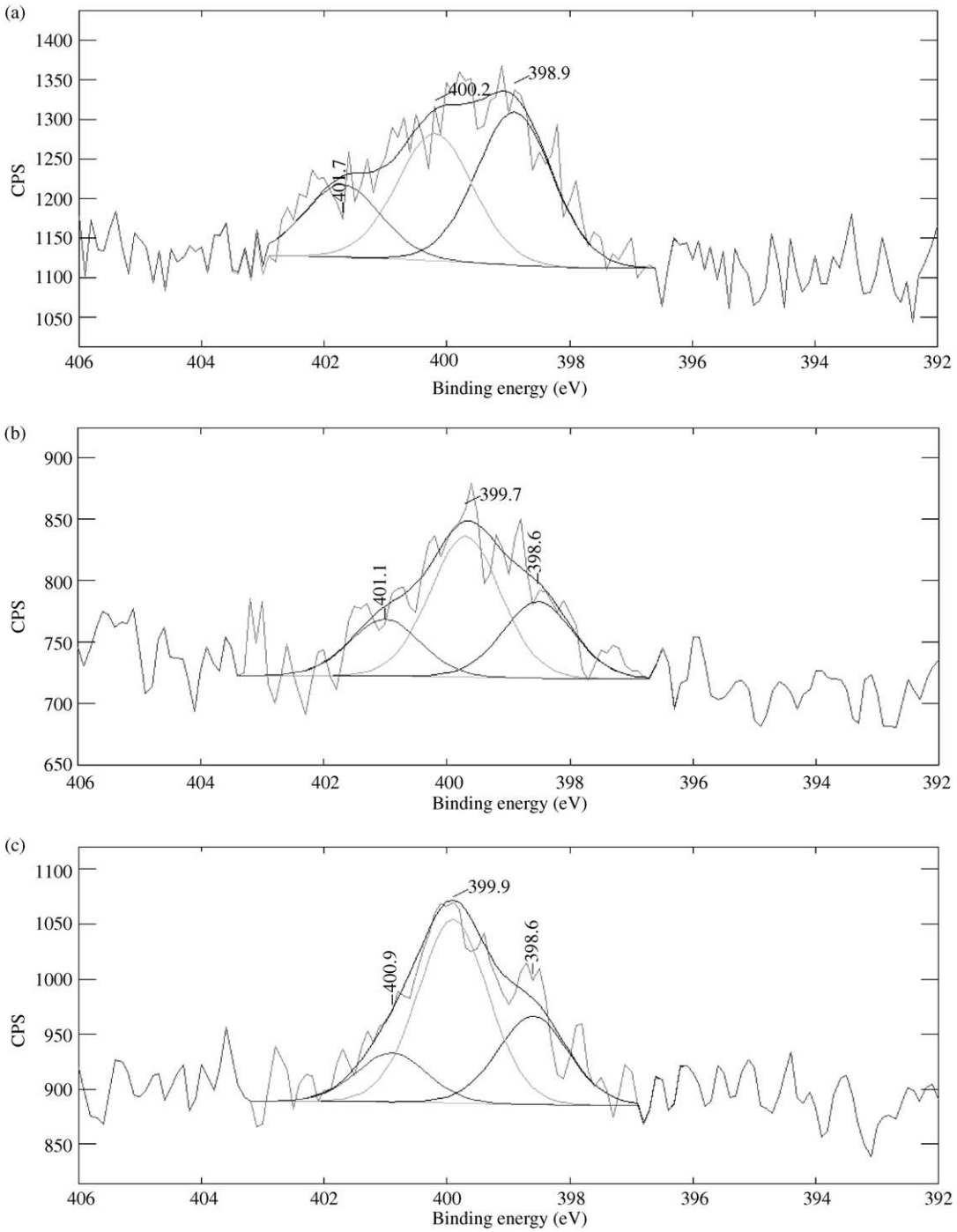

Figure 3 N1s XPS spectra for materials ''B'', ''C'' and ''D'' (a, b, c, respectively).

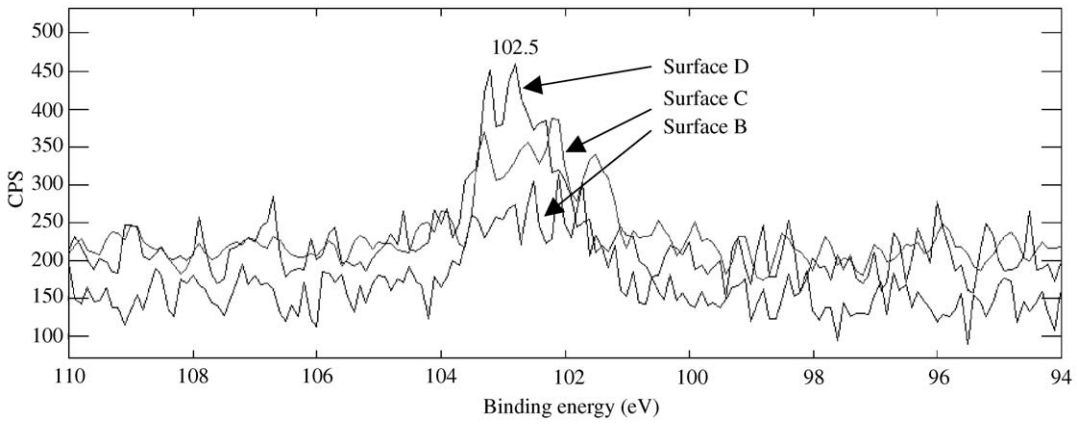

Figure 4 Overlap of XPS Si2p spectra for materials ''B'', ''C'' and ''D''.

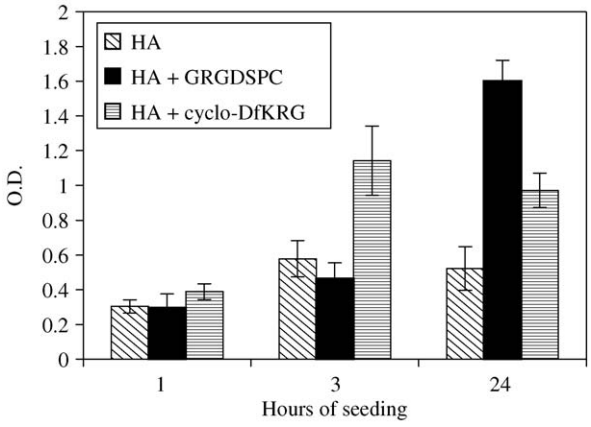

Figure 5 Osteoprogenitor cells attachment to HA, HA grafted with GRGDSPC, or cyclo-DfKRG.

($\alpha v\beta 3$- and $\alpha v\beta 5$) selective cyclic pentapeptide ligands such as cyclo(-RGDfX) have been developed over the last decade [18]. In this context, it was demonstrated that in addition to the RGD binding sequence, a D-amino acid, especially D-Phe following the Asp residue in the cyclus, is essential for high activities and αv selectivity. Cyclic pentapeptides with D-amino acid in other positions and/or a non-hydrophobic amino acid following Asp as well as linear peptides were shown to have lower activity and are less selective towards αv integrins.

Our results clarify that osteoprogenitor cells exhibit a differential binding to RGD peptides displaying a specific conformation (linear GRGDSPC and cyclo-DfKRG). Such behaviour is probably due (i) to the cell expression of different integrin subunits and (ii) to the respective accessibility of peptides by integrin receptors. Both cyclo-DfKRG and linear GRGDSPC appear to be good candidates for promoting bone substitute integration by activating adhesion of human osteogenic cells arising from bone marrow. However, the concept of hybrid biomaterials (that plans to seed human osseoprogenitor cells isolated from fresh bone marrow patient's cells onto bioactive-ceramic implants for the formation of tissue matrix) involves the introduction of implants as quickly as possible after the bone marrow cells collect, the cell attachement has to be strongly increased in the first hours. In these conditions, although cell adhesion increased after 24 h of seeding on GRGDSPC linear peptide, the cyclo RGD peptide is more attractive since this latter peptide favours cell attachment in short incubation times and promotes cell differentiation also [25].

Conclusion

A three-step reaction procedure was developed to attach RGD-containing peptides onto macroporous HA. First, cleaned HA was silanised with APTES in anhydrous conditions, resulting in a well-adherent silane layer. Second, the free primary amino groups were linked to the hetero-cross-linker: N-sucinimidyl-3-maleimidopropionate (SMP). Finally, cell-adhesive peptides (cyclo-DfKRG and linear GRGDSPC) were immobilised onto the resulting terminal-maleimide surface by means of covalent thiol bondings. Obviously, surface pre-treatment (which means controlling cleaning agents, pressure, temperature and atmosphere) was an essential prerequisite for reproducible silanisation; this latter step being critical as regards subsequent reproducibility of the chemical functionalisation. The efficiency of this new route for biomimetic modification of macroporous HA was demonstrated by measuring the adhesion between 1 and 24 h of osteoprogenitor cells isolated from human bone marrow stroma cells. Considering the design of hybrid materials, the cyclo RGD peptide was found more attractive than GRGDSPC peptide since it favours cell attachment in the short incubation times.

Moreover, since bone growth and remodelling depend on complex interactions occurring between osteoblasts and other cells present, particularly vascular endothelial cells that may be pivotal members of a complex interactive communication network in the bone [26], future prospects would deal with identifying RGD peptides propitious to endothelial cells recruitment. As a result, by developing a co-grafting procedure, we should be able to enhance HA implant osseointegration strongly.

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