Carbonate hydroxyapatite functionalization: a comparative study towards (bio)molecules fixation

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Different methods for the functionalization of carbonate hydroxyapatite granules with free amine groups by reaction with (3-aminopropyl)triethoxysilane (APTES) have been compared in order to improve the potential for tethering of bioactive molecules to bioceramics. The combined use of tetraethoxyorthosilicate and APTES with acid catalysis resulted in an evident increase in amine surface grafting.

1. Introduction

Since the late 1960s, there has been growing interest in the use of bioceramics prepared from calcium orthophosphates as biomaterials [1]. Hydroxyapatite-based biomaterials are used in a number of different tissue-engineering applications, including dental implants, percutaneous devices in periodontal treatment, reconstruction of bone and cranio-maxillofacial defects, fractures, total joint replacement, spinal surgery and otolaryngology [2]. Owing to their ion-exchange ability, adsorption capacity and acidic properties, such materials can also be used as effective ion exchangers [3], sorbents [4] and carriers for proteins [5] or peptides [6–8]. In addition, modified hydroxyapatite particles have been suggested as carriers for drugs, such as interferon alpha, testosterone enanthate and cyclosporin A [9–11]. To date, the main effort towards the covalent functionalization of bioceramics has focused on the introduction of specific organic groups [12–15], biodecoration with whole proteins [16–18], short peptide epitopes [19–22] and carbohydrates [23,24].

One of the most exploited methods for covalent functionalization of bioceramics with bioactive molecules is the reaction of surface hydroxyl groups with substituted alkoxysilanes [12,16–18,25–28]. Silanol groups from hydrolysed silicon alkoxides are able to condense with the hydroxyl groups of the orthophosphate scaffold, while the alkyl chain bears the functional group that can be exploited for further functionalization. Amine functional groups are extensively used for surface functionalization because they are quite versatile and particularly suited for condensation reaction to the corresponding amides; in this respect, (3-aminopropyl)triethoxysilane (APTES) is one of the most widely used reagents. The silanol groups at one end of hydrolysed APTES can form a binding with OH groups exposed on the material surface, while its amine group can be exploited for further covalent derivatization with organic (bio)molecules [29,30]. While the mechanism of the silanol condensation is pretty well established [31,32], no detailed comparative studies on the efficacy of aminosilane grafting on bioceramics in different reaction conditions have been performed to date. In this work, we propose and compare
three different grafting methods for the modification of carbonate hydroxyapatite microparticles (CHA) with amino groups.

2. Material and methods

All reagents were supplied by Sigma Aldrich, Italy, unless otherwise specified. Solvents were dried over molecular sieves, for at least 24 h prior to use.

2.1. Carbonate hydroxyapatite granules

Carbonate-substituted apatite powder with a specific surface area value of 36 m² g⁻¹ was synthesized through a wet synthesis based on dropwise addition of a phosphoric acid solution containing 88.8 g H₃PO₄ (Aldrich, 85 wt% pure) in 600 ml of distilled water and, simultaneously, of a sodium hydrogen carbonate solution containing 13.3 g NaHCO₃ (Merck-Shuchardt, 99.7% pure) in 400 ml of distilled water to a stirred calcium hydroxide dispersion containing 100 g of Ca(OH)₂ (Aldrich, 99.7% pure) in 700 ml of distilled water. The amounts of reagents were chosen in order to respect the Ca/P molar ratio 1.67 of the stoichiometric hydroxyapatite but, for the presence of sodium hydrogen carbonate, competition for entering in the apatitic cell structure between phosphate and carbonate groups was set up. During the reaction process (taking 3–4 h), the temperature was kept at 40°C under mechanical stirring. Once the reaction was completed, the suspension was maintained at the same temperature of 40°C and stirred for 24 h by hand, and finally aged for 24 h. The precipitate was washed three times with deionized water, freeze-dried and finally sieved at 150 µm. The obtained powder was processed into granules having dimensions in the range of 400–600 µm, to obtain a material usable as bone filler for dental and orthopaedic applications (similar granules made of stoichiometric hydroxyapatite are already commercialized by Finceramica Faenza Spa, Italy). The inductively coupled plasma (ICP) analysis gave a Ca/P molar ratio of 1.87, which is higher than the stoichiometric value (1.67), which proves that carbonate ions entered the hydroxyapatite lattice replacing phosphate ions leading to the so-called B-type carbonation. In fact, A-type carbonation (occurring when carbonate ions replace hydroxyl ions) does not change the Ca/P molar ratio in comparison with stoichiometric hydroxyapatite. At higher temperatures, carbonate ions decompose causing weight loss due to CO₂ elimination, which allows estimation of the starting carbonation of the hydroxyapatite granules as about 5.5 wt%. This value, which is in agreement with those indirectly found by the other analysis, is in the range of the contents of the biological apatite (2–8 wt%).

2.2. Carbonate hydroxyapatite microparticles

Silanization of hydroxyapatite was carried out by immersing 0.400 g of granules in a 1 M solution of APTES in dry hexane (1 ml of APTES solution per 0.1 g of CHA) for 3 h while stirring. The resulting suspension was thoroughly washed with ethyl acetate and hexane, and then the granules were dried under vacuum for 24 h.

2.3. Carbonate hydroxyapatite microparticles

A total of 0.312 ml of tetraethyl orthosilicate (TEOS, 1 equiv.) was dissolved in deionized H₂O (0.101 ml, 1 equiv. with respect to TEOS) and the pH was adjusted to 2 with 2 N aq. HCl (0.108 ml). The solution was stirred for 1 h to allow hydrolysis of alkoxy groups, then 0.352 ml of APTES (1 equiv. with respect to TEOS) and 0.500 g of CHA granules in 2 ml of water were added and the suspension was stirred for 1 h. The mixture was then transferred into screw top Teflon moulds (3 cm diameter) for gelling and ageing. The gels were aged (3 days at 40°C), then dried (3 days at 60°C).

2.4. Carbonate hydroxyapatite microparticles

2.4.1. First step

A total of 0.500 g of CHA was suspended in 3 ml of dry hexane, then a solution containing 0.312 ml of TEOS (1 equiv.) in deionized H₂O (0.101 ml, 1 equiv. in respect to TEOS) at pH 2 (by addition of 2 N aq. HCl, 0.108 ml) was added. The suspension was stirred for 2 h, filtered, then the granules were washed with hexane several times and dried under vacuum for 24 h, and finally dried for 1 day at 40°C.

2.4.2. Second step

TEOS-modified CHA granules (0.500 g) were suspended in 3 ml of dry hexane, 0.352 ml of APTES (1 equiv. with respect to TEOS) were added and the pH was adjusted to 2 with 2 N aq. HCl (0.153 ml). The solution was stirred for 2 h, filtered, then the granules were washed with hexane several times and dried under vacuum for 24 h, and finally dried for 1 day at 40°C.

2.5. General procedure for the dansylation reaction of CHA samples

A total of 0.100 g of granules were suspended in a 0.25 M solution of dansyl chloride in dry tetrahydrofuran containing 5% of triethylamine (1 ml) and stirred for 4 h. The resulting suspension was washed with ethyl acetate and hexane several times, and then granules were dried under vacuum for 24 h.

2.6. Material characterization

2.6.1. Fourier transform infrared/ATR analysis

Fourier transform infrared (FTIR) spectra were measured in attenuated total reflectance (ATR)-IR mode at RT in the range of 800–2000 cm⁻¹ with a micro-FTIR Nicolet iN10, equipped with a micro-ATR germanium tip, under nitrogen flux, with a spectral resolution of 2 cm⁻¹, 25 kHz scan speed, 512 scan coadditions and triangular apodization. The ATR/FTIR spectra were reported after background subtraction, baseline correction and binomial smoothing (11 points).

2.6.2. Inductively coupled plasma analysis

ICP–atomic emission spectrometry analysis (Liberty 200, Varian, Clayton South, Australia) was used to assess the percentage content of Si ion in the functionalized materials. The samples were previously prepared as follows: 10 mg of powder was dissolved in 1 ml of HNO₃ (Aldrich, 65 wt% pure) and the solution volume was increased up to 50 ml with deionized water. The obtained values were expressed in terms of Si/CHA wt%.

2.6.3. Nuclear magnetic resonance

Solid-state nuclear magnetic resonance (NMR) experiments were acquired on a Bruker AV 400 WB spectrometer equipped with a 4 mm multi-nuclear (¹⁵N–³¹P) triple-channel (BL4 X/Y/¹H) cross polarized magic angle spinning (CPMAS) probe. CPMAS experiments were performed using a 10 kHz spinning rate and a 3.5 ms contact time. All the experiments were performed overnight (16 h). Additional spectra (65 h) were acquired for the sample obtained after CHA treatment following scheme 2d (vide infra).
3. Results and discussion

The silanization through APTES is widely used for the functionalization of surfaces of different materials (silica gel, MCM-41, etc.) [30,33–35] with amino groups, which allows further immobilization of a large variety of organic molecules. Theoretically, surface functionalization with APTES should afford a homogeneous coupling between surface OH groups and silanols from hydrolysed APTES (figure 1a). However, the experimental result is a mixture of Si–O bond formation between the surface OH and hydrolysed APTES accompanied by physisorption that can also involve molecular inversion of APTES molecules (figure 1b and c, respectively) [36]. Hydrogen bonding between surface OH and silanols/amino groups, though weak interactions, are competing with the condensation reaction; these physisorption processes hamper/slow down the condensation reaction, which in some cases can be ensured by drying procedures [37]. The extent of the physisorption process deeply influences the efficacy of the covalent grafting of the silane on the material surface; here we show on CHA that the ratio between the Si–O bond formation and the physisorption, hence the efficacy of the functionalization, may be improved using TEOS–APTES mixtures.

On the basis of these considerations, three different experimental conditions were used and compared. Nanostructured biomimetic carbonate-substituted apatite (CHA), synthesized by a wet chemical route, was used to prepare granules of materials, with size in the range of 400–600 \( \mu \text{m} \) [38]. The powder particles were agglomerated using a wet-based process avoiding any additions of chemicals; then after drying at 80°C the granules were sieved and the specified fraction was collected. The granules are crystallographically pure: no secondary crystalline phases are detected by XRD besides nanocrystalline apatite (data not shown). The carbonate ions substitute in the phosphate site of hydroxyapatite (B-type CHA) as determined by FTIR spectroscopy (data not shown), amounting to 6% in weight (determined by TGA). The carbonation is thus in the range of the contents of the biological apatite.

CHA functionalization has been performed with three different procedures (scheme 1):

(A) direct APTES reaction with CHA (procedure A),

(B) one-step sol–gel procedure TEOS–APTES (procedure B) and

(C) two-step procedure TEOS–APTES (procedure C).

In more detail, the first strategy (scheme 1a) involved the direct grafting of APTES onto the surface of CHA granules in anhydrous hexane. Direct grafting of APTES has been widely
used for the modification of many kinds of materials (metals, glass, oxides, graphene oxide, etc.) and is reported as an efficient method for the coupling between inorganic and organic materials (such as polymers and proteins) [39–43].

In procedure B (scheme 1b), functionalization of CHA surface was performed via a sol–gel strategy [44], in the presence of a 1 : 1 TEOS–APTES mixture at pH 2. The sol–gel reaction of oxides precursors, for instance TEOS, proceeds at room temperature. The sol–gel reaction involves hydrolysis of the Si–O bonds and condensation of the resulting Si–OH groups, leading to the formation of a three-dimensional network of siloxane bonds. This network provides a stable backbone for the attachment of functional groups, such as the dansyl group in this case.

Scheme 2. (a–d) Dansyl functionalization of CHA samples.
temperature through (i) ‘activation’ of the oxides precursors by acid- or base-catalysed hydrolysis, (ii) polycondensation of the activated precursors or ‘monomers’ to oligomers leading to formation of transparent and stable nanometric dispersions of silica particles, and (iii) formation of a three-dimensional silica network by further polymerization and cross-linking of inorganic particles accompanied with solidification.

A two-step procedure was also used (procedure C, scheme 1):

- CHA was first reacted with TEOS in acidic conditions (CHA pre-activation step), thus introducing additional hydroxy groups on the material surface; after filtration and drying, the pre-activated CHA was reacted with APTES.

FTIR spectra in ATR mode have been recorded for each sample (figure 2) in order to verify the efficacy of CHA functionalization. The spectrum of bare CHA has been used as the reference. The spectrum of CHA is dominated by the 960 and 1030–1100 cm\(^{-1}\) bands due to the bending of PO\(_4^{3-}\). In addition, peaks around 1455 and 1415 cm\(^{-1}\) are indicative of the carbonate (CO\(_3^{2-}\)) presence in CHA. The formation of Si–O covalent linkages was verified by the presence of the absorption bands at approximately 3450, approximately 1100 and approximately 950 cm\(^{-1}\) corresponding to the stretching, asymmetric stretching and bending of silanol groups (Si–OH), respectively. Intensive bands in the region between approximately 1200 and 1100 cm\(^{-1}\) (figure 2 peaks 4, 5 and 6) represent the asymmetric stretching of siloxane groups (Si–O–Si). These bands correspond to a combination of Si–O–Si vibrational modes including those of bonds formed between the silane and surface, cross-linking between silane molecules at the surface, polymerization of silane and unreacted Si–O–C (ethoxy) groups [37]. In addition, two bands at approximately 3445 and 1640 cm\(^{-1}\) corresponding to N–H stretching vibration and NH\(_2\) bending mode of free NH\(_2\) groups, respectively, are particularly significant for the determination of the presence of amine functionalization of CHA [45]. From IR spectra, it can be highlighted that the use of TEOS (both in procedures B and C) allows a correct exposure of the amino groups of the propylamino chain of APTES: in fact, the presence of the sharp peak centred at approximately 1640 cm\(^{-1}\) (peak number 3) and the two peaks at higher wavelength (approx. 3500 cm\(^{-1}\), peaks 1 and 2) represent the primary amines of APTES that are actually absent in the spectrum of CHA functionalized by direct reaction of APTES (procedure A). Moreover, in both samples the vibrational modes of the CHA are almost completely covered by the silane chain, indicating a homogeneous functionalization of the samples. This observation is in complete agreement with reports on APTES reaction on glass surfaces [46]: it can be deduced that in procedure A an acid/base reaction between the surface OH and APTES amino groups is the main event occurring in CHA direct functionalization. After washing of the samples, the APTES is removed from the surface, thus justifying the absence of the primary amine vibrational modes in the corresponding FTIR spectra (figure 2).

In order to further confirm this hypothesis, ICP quantitative analysis was performed. The silicon content of the obtained samples was analysed using ICP–atomic emission spectrometry and results are reported in table 1 as wt% of Si ions with respect to CHA granules. The higher silicon contents are displayed by CHA functionalized by procedures B and C, both including the use of TEOS, thus confirming that TEOS may allow a higher degree of amine functionalization of CHA.

In order to assess also the reactivity of amino groups of CHA-modified surfaces, reaction with dansyl chloride was performed suspending CHA granules in dry THF (2 ml) containing 0.25 M dansyl chloride and 5% triethylamine, and kept under stirring for 4 h (scheme 2).

The dansyl functionalization of CHA was confirmed by NMR solid spectroscopy (figure 3). In fact, solid-state NMR methods were employed to monitor the degree of dansyl functionalization by the different protocols. Although NMR

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**Figure 2.** FTIR of CHA functionalized with the three different procedures.

**Table 1.** ICP analysis expressed as wt% of Si ions with respect to CHA granules.

<table>
<thead>
<tr>
<th>samples</th>
<th>Si%</th>
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<tbody>
<tr>
<td>(a) APTES</td>
<td>1.03</td>
</tr>
<tr>
<td>(b) TEOS–APTES procedure B</td>
<td>11.75</td>
</tr>
<tr>
<td>(c) TEOS–APTES two-step procedure C</td>
<td>8.45</td>
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is not indeed a sensitive method in terms of signal-to-noise ratio, and therefore signals should only be expected for a high level of attachment, the obtained spectra provided indications of the efficacy of the different protocols. The spectra obtained for the sample obtained after application of procedure A showed low signals for the dansyl moiety, although the APTES signals could not be properly detected. These data further highlight that the amount of APTES bonded to CHA exposing reactive amino groups is low, if compared to CHA functionalized by procedure B or C. In fact, a clear indication of a homogeneous attachment of the dansyl moiety, including aliphatic moieties from the amino-propyl chain, was obtained after analysis of the sample obtained by employing method B. Method C also provided relatively good spectra, although the spectrum showed a clear degree of heterogeneity in the dansyl incorporation. NMR was also performed on CHA samples directly treated with dansyl chloride without reactions with APTES or TEOS/APTES (figure 3d) in order to exclude chemi- or physisorption of the dansyl moiety to untreated CHA.

4. Conclusion

This work proposes a comparative study between different methods for the ‘decoration’ of CHA-based materials with free amino groups in an attempt to improve their potential for tethering of (bioactive) molecules. The methods including the use of TEOS treatments of CHA in addition to APTES proved to be more efficient for functionalization. In more detail, method B proved to be the most efficient one, considering the silicon content of the final material: this strategy allows surface functionalization in water conditions, avoiding organic solvents, that is in condition that are more ‘biocompatible’.

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