# Sweet and Salted: Sugars Meet Hydroxyapatite

Monica Sandri,[a] Antonino Natalello,[b] Davide Bini,[b] Luca Gabrielli,[b] Laura Cipolla,*[b] Francesco Nicotra*[b]

[a] Institute of Science and Technology for Ceramics - National Research Council, via Granarolo 64, 48018 Faenza, Italy

[b] Dept. of Biotechnology and Biosciences, University of Milano-Bicocca, P.za della scienza 2, 20126 Milano, Italy
Fax +39(2)64483565; E-mail: laura.cipolla@unimib.it; E-mail: francesco.nicotra@unimib.it

*Received 12 May 2011*

Dedicated to Prof. Alfredo Ricci

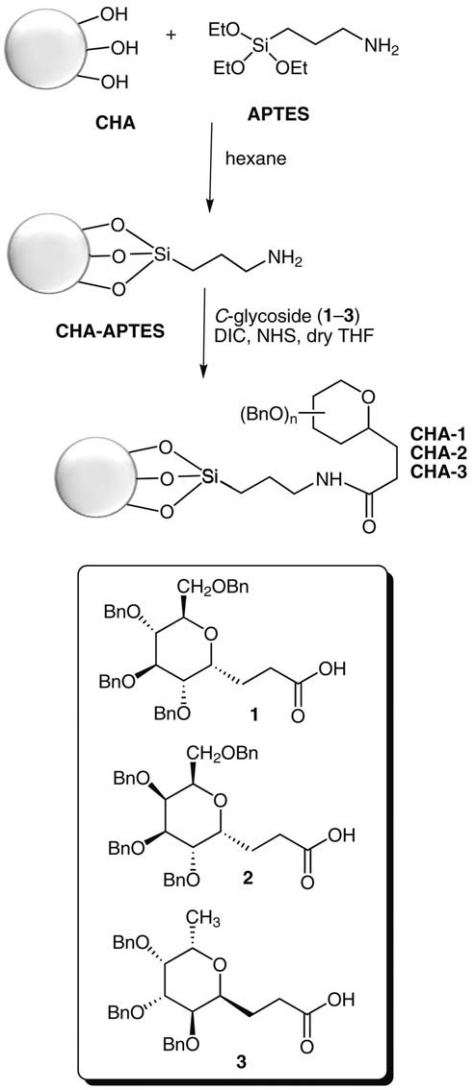
**Abstract:** Carbonated hydroxyapatite (CHA) has been successfully biodecorated with carbohydrate derivatives, via silylation of the hydroxy groups of the apatite and subsequent covalent bonding with a suitably functionalized carbohydrate moiety. The presented procedure opens the way toward covalent biofunctionalisation of CHA with carbohydrates.

**Key words:** carbohydrates, *C*-glycosides, biomaterials, hydroxyapatite, biofunctionalisation

Recent promising trends in biotechnology and tissue engineering are based on the development of advanced materials with biomimetic features created by designing and tailoring of specific surface properties, which improves biological responses and tissue compatibility.[1] Among various biocompatible materials, synthetic hydroxyapatite (HAp) and carbonate hydroxyapatite (CHA) are widely used in many biomedical applications.[2] HAp is a natural mineral ingredient of bones, teeth and calcified tissues in vertebrates. Synthetic HAp and CHA are used for human implant coatings possessing beneficial biocompatibility and osteoconductivity.

The combined use of inorganic materials, such as CHA, and organic signaling molecules is very a promising approach to tissue engineering.[3,4] To date, main efforts towards the covalent functionalisation of apatite has focused on the biodecoration of this inorganic material with whole proteins[5] or short peptide epitopes;[6] the most commonly used peptide for surface modification is RGD,[6a,b] which is the signaling domain derived from fibronectin and laminin.[7] Less exploited as cellular ligands for material functionalisation are carbohydrate epitopes; these are well-known to bind to cell surface receptors and allow cell–cell interactions.[8] In this context, the possibility of decorating hydroxyapatite with carbohydrates can be relevant.[9] To the best of our knowledge, no examples have been reported on hydroxyapatite biofunctionalisation with carbohydrates.

In this report, we present our preliminary results on the functionalisation of CHA with monosaccharide derivatives, to explore the possibility of covalently linking carbohydrate epitopes to hydroxyapatite, as a novel method of 'bioactivation' of this promising material. As model carbohydrates, perbenzylated *C*-glycoside derivatives of biologically relevant monosaccharides such as D-glucose, D-galactose and L-fucose, were used.




**Scheme 1** Methodology for CHA functionalisation with carbohydrate *C*-glycosides

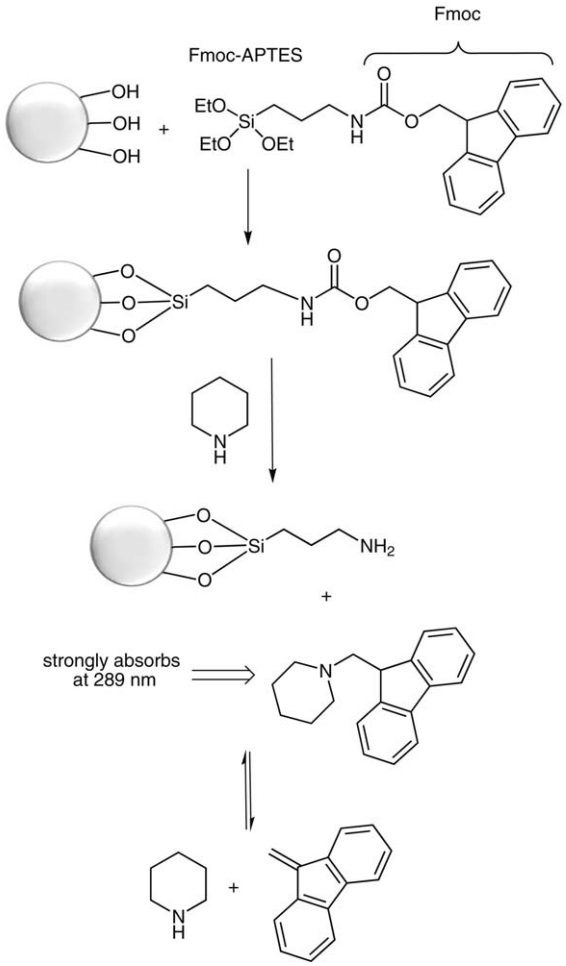
*C*-Glycosides present several advantages over their *O*-glycosidic counterparts:[10] their structure closely resembles that of the parent sugar, thus maintaining the biological information, but they lack the anomeric glycosidic bond that is usually subjected to degradation in vivo. In

*SYNLETT* 2011, No. 13, pp 1845–1848
Advanced online publication: 14.07.2011
DOI: 10.1055/s-0030-1260953; Art ID: S03811ST

addition, the benzyl protecting groups were used as probes to analyze the surface-decorated CHA.

CHA granules with dimensions in the 400–600 μm range, which were obtained from a nanostructured biomimetic CHA powder with a specific surface area of 36 $m^2/g$, were used for biodecoration with monosaccharide derivatives.[11] Because CHA possesses hydroxy groups, we envisaged the possibility of covalently linking suitably functionalized *C*-glycosides via an aminopropylsilane linker (Scheme 1). In more detail, the strategy for *C*-glycoside immobilization (Scheme 1) involved: (i) grafting of aminopropyltriethoxy silane (APTES) onto the surface of CHA granules in anhydrous hexane, and (ii) reaction of the amino groups on the CHA surface with the activated hydroxysuccinyl esters from *C*-glycosides in anhydrous THF as solvent.[6a]

To assess the degree of functionalization with the amino functionality, spectrophotometric analysis was performed after silanization of the hydroxyapatite with (3-triethoxysilylpropyl)carbamic acid 9*H*-fluorenylmethyl ester (Fmoc-APTES),[12] as shown in Scheme 2. Removal of the Fmoc group is usually achieved by treatment with piperidine in *N*,*N*-dimethylformamide (DMF). The mechanism of the Fmoc-deprotection reaction involves the formation of aromatic cyclopentadiene intermediates, which are rapidly eliminated to form dibenzofulvene, which is further scavenged by piperidine to form the Fmoc-adduct 1-[(9*H*-fluoren-9-yl)methyl]piperidine. This product strongly absorbs UV radiation at 289 nm, offering the potential to monitor the deprotection reaction by UV/Vis spectroscopy and quantifying the extent of functional-group loading on CHA. In this way, the degree of amino functionalization of hydroxyapatite was found to be 0.15 mequiv/g CHA.




**Scheme 2** Method used for the quantification of amino groups on the CHA surface

*C*-Glycosidic derivatives possessing a carboxylic group for the coupling reaction to the aminopropylsilane-functionalized CHA (CHA-APTES) were synthesized in a few steps from the suitably protected natural monosaccharide (for experimental details see the Supporting Information). Briefly, the α-allyl-*C*-glycoside was prepared by Sakurai reaction from the suitably protected parent monosaccharide;[13] the allyl *C*-glycosidic appendage was then functionalized with a carboxyl terminus,[14] which is a suitable functional group for the condensation reaction with the CHA-APTES, by hydroboration (9-BBN-H), followed by oxidation to the corresponding carboxylic acid (TEMPO).[15] The carboxylic group was finally activated as the succinyl ester (diisopropylcarbodiimide, *N*-hydroxysuccinimide) and then coupled to the amino group of CHA-APTES in anhydrous THF.

The CHA functionalized with carbohydrate derivatives was then characterized to determine (i) the chemical stability of the material upon chemical derivatization, and (ii) the effective biodecoration with carbohydrate derivatives.

To assess the stability of the material after chemical modifications, XRD analysis was performed; no phase change of the granules after functionalization could be detected — the material remained monophasic apatite. In addition, ICP analysis showed that the Ca/P molar ratio remained substantially unaffected after the chemical reactions. RX mapping of the elements by SEM-EDS analysis provided evidence for the homogeneous presence of Si, and for Ca and P, in the functionalized granules. TG analysis of the unfunctionalized CHA granules in air revealed a weight loss occurring up to 500 °C, which was attributed to dehydration of the absorbed and occluded water and to the removal of absorbed species.[16]

At higher temperatures, carbonate ions decompose, causing a weight loss due to $CO_2$ elimination; this allowed the extent of initial carbonation of the CHA granules to be estimated to be about 5.5wt%, which is comparable to the carbonate content found in samples of biological apatite (2–8wt%).

The STA analysis confirmed the presence of additional compounds besides CHA in the functionalized material, because the TGA and the DTA profiles changed moving from unfunctionalized CHA, to the functionalized derivative (Figure 1). In particular, DTA analysis showed, in both curves 2 and 3, the presence of significant peaks in

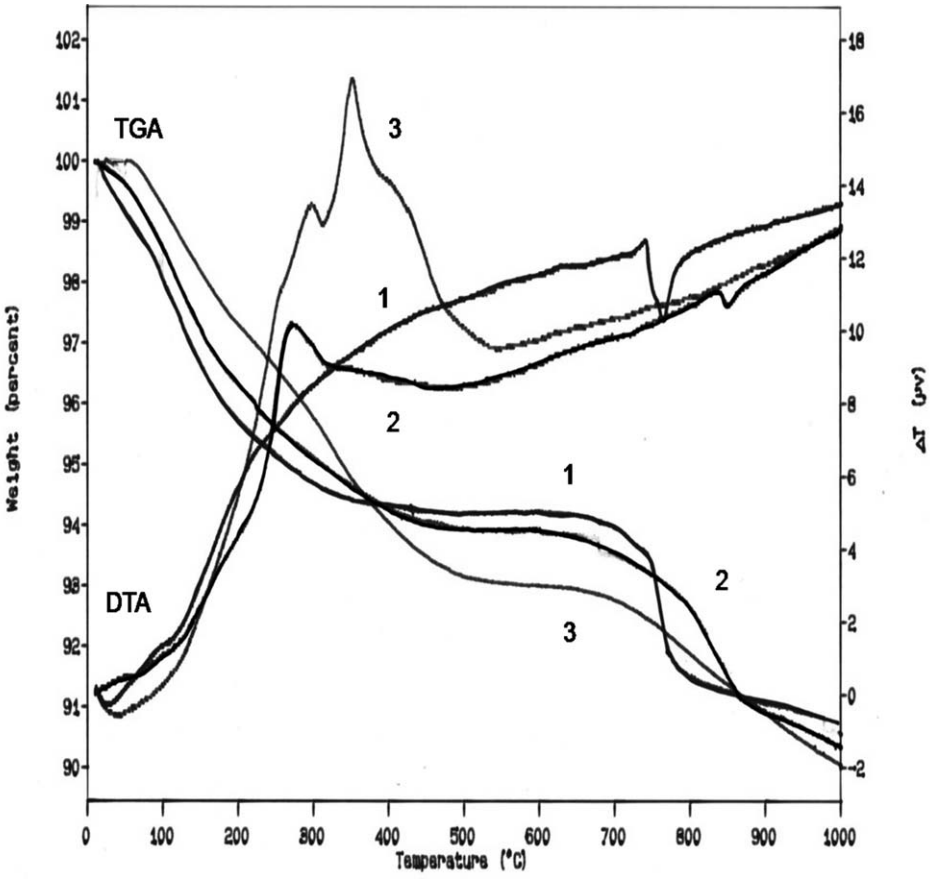



**Figure 1** TGA analysis of the HA samples: unfunctionalized CHA (curve 1), CHA-APTES (curve 2), and biodecorated CHA (curve 3)

the 250–450 °C temperature range that were attributed to the degradation of organic phases. In particular, the peak centered at 270 °C in curve 2 was correlated to the degradation of the silane group (APTES), and the multiple peaks in the 250–450 °C range of curve 3 were linked to the presence of both APTES and the connected carbohydrate moiety.

To further characterize the biofunctionalization of CHA and, in particular, to assess the effective biodecoration with carbohydrates, FTIR analyses were performed. In Figure 2A, we report the IR spectra of unfunctionalized CHA and those of CHA functionalized with 3-aminopropyltriethoxysilane (CHA-APTES) and with C-glycosides **1**–**3** conjugated to CHA via 3-aminopropyltriethoxysilane (spectra from CHA-1 CHA-3). All the spectra are dominated by the 1017 $cm^{-1}$ absorption peak due to the phosphate ($PO_4^{3-}$) vibration of CHA. Other absorption bands of CHA can be observed in the spectra, such as those due to the structural OH (3570 $cm^{-1}$), to phosphate (962 $cm^{-1}$), and to the B-type carbonate (couple at 1415 $cm^{-1}$ and at ~1455 $cm^{-1}$, and the peak at 872 $cm^{-1}$).[16,17] The presence of the typical peaks of the B-type carbonate confirmed that the carbonate ions are situated on the phosphate site of hydroxyapatite.[16]

To characterize the biodecorated materials, the spectral region 3100–2800 $cm^{-1}$ at which CH bond absorptions are typically found, is of valuable interest. Indeed (Figure 2 A), functionalization with 3-aminopropyltriethoxysilane leads to the appearance of several new bands, including the 2933 $cm^{-1}$ and 2863 $cm^{-1}$ peaks that are due, respectively, to asymmetric and symmetric $CH_2$ stretching. These bands are also present in the spectra of CHA biodecorated with derivatives **1**–**3** via 3-aminopropyltriethoxysilane. In addition, these last materials display absorption peaks in the 3091–3033 $cm^{-1}$ region due to CH vibrations of the aromatic rings present in the carbohydrate structure.

A more detailed inspection of the spectra reveals several additional features that further confirm the biodecoration of CHA. For instance, the formation of an amide bond between the silane group and the different compounds leads to a new absorption around 1650 $cm^{-1}$ (Figure 2 B) due to the C=O amide stretching vibration.[18] To discard the possibility of carbohydrate absorbtion onto CHA, and fully confirm the bonding between the amine group of CHA-APTES and the *C*-glycosides carboxyl group, we performed a second derivative analysis of the measured spectra (a resolution enhancement mathematical procedure). Because the C=O amide band has a reduced width compared to the CHA absorption in the same spectral region, in this way it is possible to clearly detect the formation of the new amide bond,[18] as can be seen in Figure 2 C (only CHA-2 is reported as an example). The presence of an absorption component around 1575 $cm^{-1}$ further confirms

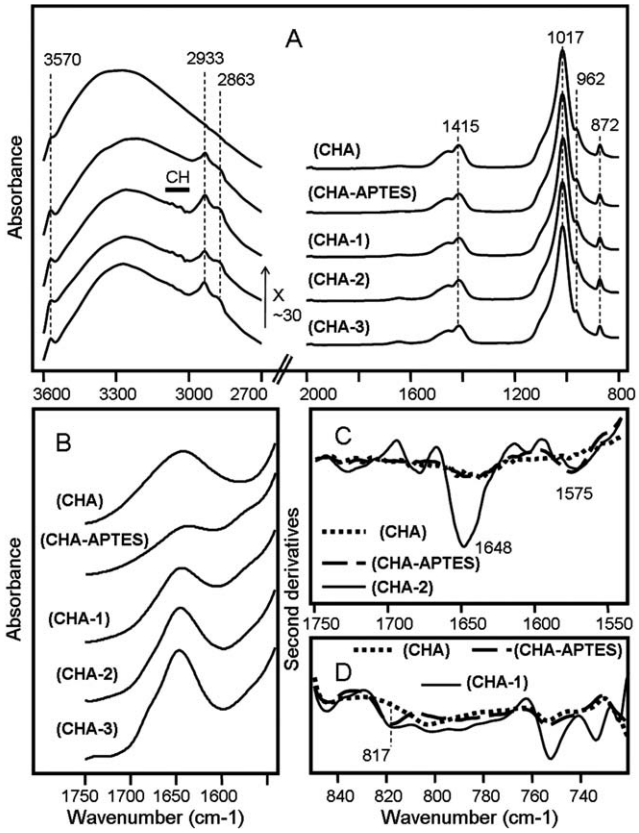



**Figure 2** FTIR analysis of CHA samples. Panels A and B: ATR/FTIR absorption spectra of hydroxyapatite (CHA) and of CHA functionalized with 3-aminopropyltriethoxysilane (CHA-APTES) and with compounds **1**–**3** (CHA-1, CHA-2, CHA-3). Panels C and D: Second derivatives of the absorption spectra reported in Panels A and B displayed in different spectral regions. Spectra are reported after normalization to phosphate absorption at 1017 $cm^{-1}$.

the formation of an amide bond. Indeed, this component, which is absent in the CHA spectrum, can be assigned to the $NH_2$ bending vibration in the case of CHA-APTES and to the NH bending (Amide II band) in that of CHA-2 (Figure 2 C). Similar results were obtained for the biodecoration with carbohydrates **1** and **3**.

Many additional bands were also observed after biodecoration that confirmed the success of our procedure. For instance, the absorption in the 720–760 $cm^{-1}$ region can be assigned to the aromatic ring vibrations (Figure 2 D). We should also report that materials CHA-APTES, CHA-1, CHA-2, and CHA-3 display a component around 817 $cm^{-1}$, which is absent in CHA, that can be assigned to a vibrational –O-Si- mode.[19]

With these data in hand, we can affirm that carbonated hydroxyapatite has been successfully biodecorated with carbohydrate derivatives. It should be noted that classical organic coupling reactions have been applied for the covalent functionalization of inorganic materials such as CHA. The presented procedure opens the way to the application of organic chemistry to the covalent biofunctionalization of CHA, which is an excellent biomimetic material, with suitable classes of biomolecules, such as carbohydrates; CHA functionalization with biologically active carbohydrate epitopes can upgrade this material from being biomimetic to being a bioactive material that is able to cross-talk with the surrounding cellular environment.

**Supporting Information** for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

## Acknowledgment

We gratefully acknowledge FONDAZIONE CARIPLO, project 2008/3175 and MIUR, project FIRB RBPO68JL9 for financial support.

## References and Notes

(1) (a) Lutolf, M. P.; Hubbell, J. A. *Nature Biotechnol.* **2005**, *23*, 47. (b) Ratner, B. D.; Bryant, S. J. *Annu. Rev. Biomed. Eng.* **2004**, *6*, 415P.
(2) (a) Yoshikawa, H.; Myoui, A.; Tamai, N.; Murase, T. *J. Artif. Organs* **2005**, *8*, 131. (b) Langer, R.; Vacanti, J. P. *Tissue Eng. Sci.* **1993**, *260*, 920.
(3) Schulz-Ekloff, G.; Wöhrle, D.; van Duffel, B.; Schoonheydt, R. A. *Micropor. Mesopor. Mat.* **2002**, *51*, 91.
(4) (a) Hench, L. L.; Polak, J. M. *Science* **2002**, *295*, 1014. (b) *Chem. Eng. J.* **2007**, *137*, Issue 1.
(5) Zurlinden, K.; Laub, M.; Jennissen, H. P. *Materialwiss. Werkst.* **2005**, *36*, 820.
(6) See for example: (a) Balasundaram, G.; Sato, M.; Webster, T. J. *Biomaterials* **2006**, *27*, 2798. (b) Durrieu, M.; Pallu, S.; Guillemot, F.; Bareille, R.; Amédée, J.; Baquey, C. H. *J. Mater. Sci.: Mater. Med.* **2004**, *15*, 779. (c) Nelson, M.; Balasundaram, M.; Webster, T. J. *Int. J. Nanomed.* **2006**, *1*, 339.
(7) Hersel, U.; Dahmen, C.; Kessler, H. *Biomaterials* **2003**, *24*, 4385.
(8) Kazuaki, O.; Jamey, D. *Cell* **2006**, *126*, 855.
(9) (a) Toole, B. P. *J. Clin. Invest.* **2000**, *106*, 335. (b) Acharya, C.; Hinz, B.; Kundu, S. C. *Biomaterials* **2008**, *29*, 4665. (c) Pourcelle, V.; Freichels, H.; Stoffelbach, F.; Velty, R. A.; Jerome, C.; Brynaert, J. M. *Biomacromolecules* **2009**, *10*, 966.
(10) Du, Y.; Linhardt, R. J. *Tetrahedron* **1998**, *54*, 9913.
(11) Landi, E.; Tampieri, A.; Celotti, G.; Langenati, R.; Sandri, M.; Sprio, S. *Biomaterials* **2005**, *26*, 2835.
(12) Cheng, K.; Landry, C. C. *J. Am. Chem. Soc.* **2007**, *129*, 9674.
(13) Hosomi, A.; Sakata, Y.; Sakurai, H. *Tetrahedron Lett.* **1984**, *22*, 2383.
(14) (a) Cook, B. N.; Bhakta, S.; Biegel, T.; Bowman, K. G.; Armstrong, J. I.; Hemmerich, S.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2000**, *122*, 8612. (b) Tang, P. C.; Levy, D. E.; Holme, K. M. Glycomed Inc. US5508387, **1996**.
(15) Huang, L.; Teumelsan, N.; Huang, X. *Chem. Eur. J.* **2006**, *12*, 5246.
(16) Landi, E.; Tampieri, A.; Celotti, G.; Vichi, L.; Sandri, M. *Biomaterials* **2004**, *25*, 1763.
(17) (a) Gibson, I. R.; Bonfied, W. *J. Biomed. Mater. Res.* **2002**, *59*, 697. (b) Rehman, I.; Bonfield, I. R. *J. Mater. Sci.: Mater. Med.* **1997**, *8*, 1.
(18) Susi, H.; Byler, D. M. *Methods Enzymol.* **1986**, *130*, 291.
(19) Petkova, V.; Yaneva, V. *J. Therm. Anal. Calorim.* **2010**, *99*, 179.