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Advanced protocol to functionalize CaP bioceramic surface with peptide sequences and effect on murine pre-osteoblast cells proliferation.

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ABSTRACT

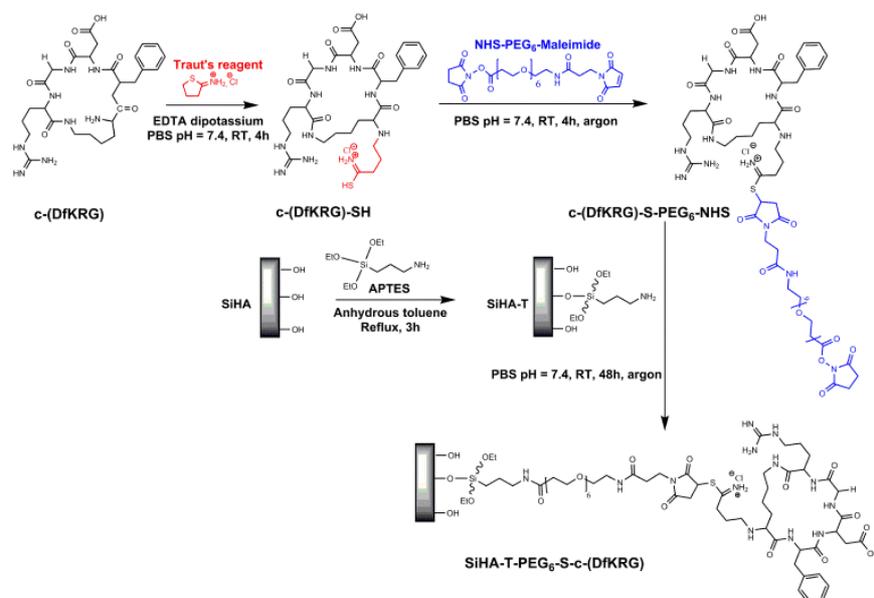
To bring osteoinductive properties to calcium phosphate (CaP) bioceramics, a silicon-substituted hydroxyapatite was functionalized by integrin-adhesive cyclic-pentapeptides (c-(DfKRG)). A new two-step protocol was set up to immobilize peptides at low and controlled density on the ceramic surface and limit contamination by adsorbed molecules. To this aim, a spacer bearing c-(DfKRG)-S-PEG₆-NHS molecule was synthesized and bonded to an organosilane previously covalently bonded to the ceramic surface. The functionalized ceramic was tested *in vitro* for MC3T3-E1 murine pre-osteoblasts. CaP ceramic surface retained good biological properties thanks to low density of bonded molecules preserving part of the bioactive CaP surface free of bioorganic molecules. The final SiHA-T-PEG₆-S-c-(DfKRG) was shown to increase cell density and to improve proliferation. Furthermore, the use of a strong and stable covalent bond between inorganic and organic parts prevented early burst release of the peptide and increased the persistence of its bioactivity over time. So, this CaP ceramic associating c-(DfKRG) by covalent grafting could be considered as promising new biomaterials for bone tissue engineering.

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Calcium phosphate hydroxyapatite (HA), is a bioceramic material commonly used as bone graft substitute. It is highly biocompatible, osteoconductive and bioactive¹. Silicon is known to be involved in early-stage bone formation² and for several years a new silicon-substituted hydroxyapatite (SiHA) has been investigated.³⁻⁶ However, HA or Si-HA ceramics do not exhibit intrinsic osteoinductive ability. This limitation could be overcome by the addition of biologically active molecules (*i.e.* peptides or proteins) involved in bone formation to the calcium phosphate (CaP) apatite. In this way, active biomolecules targeting cell adhesion and proliferation are of interest to improve the biological properties of the material. The interaction of bone cells with proteins belonging to the extracellular matrix (ECM) is a key point for the first steps of bone healing.⁷ Numerous works have shown that the RGD (Arg-Gly-Asp) sequence is the smallest active peptide able to induce a cellular response.^{8,9} Several research teams have highlighted the influence of linear or cyclic RGD-containing pentapeptide on biomaterials surface to improve cell attachment and they have shown that the presence of c-(DfKRG) induces a better and stronger adhesion in the early times (3 h) after cell seeding.^{10,11} Ceramic functionalization by peptides can be processed by two routes: adsorption of the peptides on the implant surface, or covalent binding. Because of its easy implementation, adsorption of RGD peptides on HA has been chosen by several groups.^{12,13} As reviewed by Carragee *et al.* in the case of bone morphogenetic protein BMP2, the major drawbacks of the adsorption methods are the poor control of both the adsorption process and the biomolecule delivery (kinetics and actually released amounts).¹⁴ In order to better control these processes, the covalent grafting of RGD sequences onto various surfaces (titanium,^{15,16} polyethylene terephthalate (PET),¹⁷ cellulose,¹⁸ glasses¹⁹ and apatites such as hydroxyapatite¹¹ or apatite-wollastonite²⁰) has been developed. Nevertheless, a series of three successive steps of

chemical reactions is classically used in order to functionalize the material surface: (i) silanization of the material surface, (ii) immobilization of an organic cross-linker agent and finally (iii) the biomolecule attachment. These successive chemical steps can be challenging with the potential absorption of chemical reactants or by-products on the material surface. Moreover, the initial silanization leads usually to the formation of a continuous polymer coating on the ceramic surface, thus decreasing the biological properties of the latter.

In this context, the present work is devoted to surface functionalization of SiHA ceramic by covalent grafting of cyclo-RGD at an optimized low-density distribution to improve the biological properties. The cyclo-RGD, known to primarily enhance cell adhesion as described above, was chosen to evaluate the efficiency of the grafting method. The low density of immobilized peptides will keep most of the substrate surface free from RGD to maintain the interactions between the CaP substrate and the cells (*i.e.* osteoconduction). To this end, low density SiHA surface silanization by 3-aminopropyltriethoxysilane (APTES, noted T) was first performed so as to prevent the formation of a polymer layer that would cover the whole ceramic surface. Second, a spacer bearing cyclo-(DfKRG) prepared separately was covalently grafted onto SiHA-T. This grafting strategy consists of only a two-step protocol, which allows for a better control of the purity of the reaction products and ruling out unwanted contamination of the surface by adsorption of organic reactants or reaction side-products. The functionalized biomaterial was characterized by means of mass spectrometry, UV-visible spectroscopy, X-ray photoelectron spectroscopy and confocal microscopy. Finally, it was evaluated *in vitro* in order to check the activity of bonded peptide on cell attachment and proliferation (1, 4 and 7 days).



Scheme 1 Synthesis of the spacer bearing c-(DfKRG)-S-PEG₆-NHS peptide and its grafting on silanised SiHA-T ceramic.

A commercial c-(DfKRG) peptide (American peptide) was chemically modified on its lysine by the 2-mercaptoethylamine hydrochloride (Traut's reagent) to produce a c-(DfKRG)-SH peptide providing a clickable thiol function (Scheme 1). After purification by Sephadex® size exclusion chromatography, only one peak at 11.36 min corresponding to pure c-(DfKRG)-SH was observed. Then, the c-(DfKRG)-SH peptide was functionalized on its thiol function by NHS-PEG₆-Maleimide according to the thiol-ene click reaction strategy.²¹ The reaction was realized in pH 7.4 PBS solution and was monitored by TLC. The pure product (white powder with 79.9% of reaction yield) was lyophilized. The purity of the product was confirmed by ESI-MS/MS analysis (see Fig S1 in supplementary materials).

SiHA (Ca₁₀(PO₄)_{5.6}(SiO₄)_{0.4}(OH)_{1.6}) powder was synthesized by an aqueous precipitation method.⁴ SiHA ceramic pellets (Ø = 10 mm) were sintered at 1200 °C for 30 min in a Super Khantal furnace under air atmosphere and then they were mirror polished.⁶ Silanization of pellets was realized by heating them for 3 h, under reflux, in a solution of APTES in anhydrous toluene under argon atmosphere (scheme 1, see also supplementary materials). After reaction, the pellets were washed successively with 10 mL of toluene, ethanol and diethylether under sonication (10 min). Finally, the pellets were dried in a ventilated oven at 100 °C for 1 h and stored in a desiccant.

Primary amine sites of SiHA-T pellets were quantified by UV-Vis photometric methods.²² To check the distribution of the organosilane on the ceramic pellet surface, a fluorescent dye (5-fluorescein isothiocyanate isomer I (FITC)) was reacted with the primary amine function of SiHA-T. In order to discriminate the fluorescence from FITC possibly adsorbed on the ceramic surface, a bare (un-silanised) SiHA pellet was used as a negative control. The remaining FITC in the labeling solution and in the washing solution was quantified by spectrofluorometry. For an initial FITC solution of 5×10⁻⁶ M, after immersion of the SiHA-T pellets, only a remaining concentration of 2.6×10⁻⁸ M was measured in the supernatant. After the first washing of the SiHA-T-FITC pellets with absolute ethanol and under mild ultrasonic treatment, the concentration decreased to 3.0×10⁻⁹ M and after the second one to 1.2×10⁻¹⁰ M.

Pictures obtained by confocal fluorescence microscopy of SiHA-T pellets and un-silanised SiHA pellet (used as control) after reaction with FITC are presented in Figure 1. The control surface seems homogeneous with no FITC (Fig 1B). Only a weakly uniform green color due to auto-fluorescence of the pellet is observed. SiHA-T-FITC surface shows good and uniform dispersion of the fluorescent

dye and consequently of the grafted organosilane (Fig 1A). This confirms the absence of a continuous polymer layer covering the ceramic surface, as expected from the silanization settings.

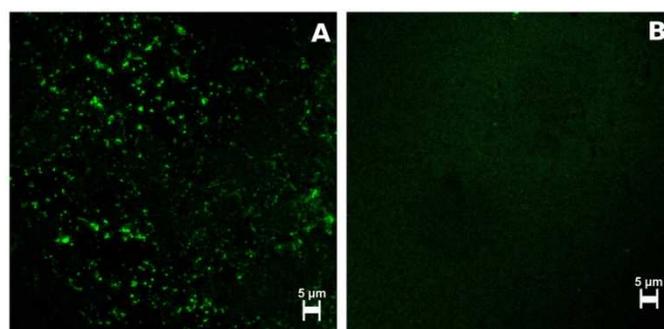


Figure 1 Confocal fluorescence microscopy: distribution of FITC on the surface of (A) silanised SiHA-T pellet and (B) un-silanised bare SiHA pellet. Images are squares of 90×90 µm (lens ×100).

XPS analyses of the ceramic surface are given in Table 1. As expected, Ca, P and Si elements were detected at the surface of the bare SiHA pellet. SiHA-T ceramic clearly showed the presence of the nitrogen coming from the APTES.

Table 1 XPS atomic composition (in mass percent) of the ceramics surface.

Sample	C	O	P	Ca	Si	N	S
SiHA	26.3	46.4	10.2	36.3	1.4	/	/
SiHA-T	58.3	30.8	4.7	6.7	2.6	1.4	/
SiHA-T-PEG ₆ -S-c-(DfKRG)	22.5	50.9	8.0	13.4	0.7	2.2	trace ²

¹SiHA-T-PEG₆-S-c-(DfKRG) pellet analysed after two rounds of 7 days of cell culture.

²Sulphur detected in amount below the significant quantification limit.

Immobilization of the c-(DfKRG) peptide at the surface of silanised ceramics was obtained by the addition of SiHA-T pellets and c-(DfKRG)-S-PEG₆-NHS into a PBS 10X pH 7.4 solution (5 mL) (Scheme 1). The mixture was vigorously planetary stirred (231 rpm) at RT for 48 h under argon. Then the ceramic pellets were washed with 3 mL of fresh PBS 10X pH 7.4 and sonicated for 5 min in order to eliminate potential organic molecules adsorbed onto the

ceramic surface. Finally, the peptide functionalized SiHA-T-PEG₆-S-c-(DfKRG) pellets were lyophilized and stored in desiccant at RT.

The potential enhancement of cell adhesion and proliferation on the surface of functionalized SiHA ceramic with the adhesive c-(DfKRG) peptide was investigated *in vitro* using murine pre-osteoblast cells MC3T3-E1. This cell line is commonly used to study biocompatibility, and osteoconductive properties of various biomaterials designed for bone engineering.²³⁻²⁵ The cell behavior was evaluated at three different time points: 1, 4 and 7 days. In a first instance, the cytocompatibility of the SiHA-T-PEG₆-S-c-(DfKRG) was assessed. As all the terminal amines of APTES may not have reacted during the step of c-(DfKRG) immobilization (scheme 1), the cell viability was also checked on the SiHA-T to ensure that the possible unreacted APTES remaining on the ceramic surface was not deleterious. Whatever the time course point, the cell morphology is not affected by any of the chemically modified biomaterials (Fig 2A), which is consistent with previous works.^{26,27}

The cell population density (*i.e.* the number of cells by unit surface) was determined after 1 day and 4 days (Fig 2B & 2C). After one day of growth on the different materials (Fig 2B), the cellular density was significantly lower on glass coverslip when compared to the ceramics (0.9×10^4 on glass and from 1.8×10^4 to 2.0×10^4 on ceramics, $p < 0.0001$). When the ceramic pellets were compared to each other, there was no significant difference. Thus, SiHA is very efficient as a support surface for cell attachment and adhesion. Moreover, the surface modifications due the functionalization do not decrease this ability. Four days after seeding (Fig 2C), the cell density on pellets increased to reach values between 1.1×10^5 and 2.1×10^5 , about ten times those measured after 1 day. In brief, cell population is growing on all ceramics. However, cells were significantly less dense on the c-(DfKRG) functionalized pellet in comparison with both the SiHA and SiHA-T pellets, $p < 0.0001$. This could be due to the inhibitory effect of RGD peptide associated to apatite on cell density that has been reported in the literature.^{28,29} This effect could relate to the peptide immobilization settings: the surface density of the c-(DfKRG), the nature the the peptide (amino-acids flanking RGD), the cross-linker chain and the material substrate itself.³⁰ The cell density on glass coverslips was close to those observed on SiHA or SiHA-T pellets showing that the presence of APTES had clearly no influence on cell tissue growth.

The proliferation rate of MC3T3-E1 cells seeded onto the bare SiHA, SiHA-T and SiHA-T-PEG₆-S-c-(DfKRG) pellets was assessed by studying the incorporation of EdU into their DNA during DNA replication (S-phase) just before cell division (Fig 2D). After four days, the cell proliferation rate on SiHA-T-PEG₆-S-c-(DfKRG) was nearly twice that found on SiHA samples (respectively 7.2% *vs.* 13.7%, $***p = 0.0161$) whereas the proliferation rate was much lower for cells growing on glass coverslips (1.9%). The number of replicating cells counted on SiHA-T was not significantly higher than the number of replicating cells found on SiHA (9.1% *vs.* 7.2%, $p = 0.7304$). This confirms the results of cell density. In conclusion, APTES, in low concentration at the surface of CaP ceramics, has no influence on cell behavior.

After 4 days, cells reach confluency whatever the conditions are (Fig 2A). Lower proliferation observed on SiHA and SiHA-T at day 4 can be due to the higher cell density when compared with SiHA-T-PEG₆-S-c-(DfKRG), which causes a proliferation arrest *in vitro*. As cell density was lower on the latter substrate surface (Fig 2C), cells keep on proliferating (Fig 2D). Thus, a delayed cell proliferation may be considered on SiHA-T-PEG₆-S-c-(DfKRG) due to the peptide.

In order to assess the robustness of the covalent bonding of the c-(DfKRG) peptide and APTES to the ceramic, the cells were removed from the different SiHA pellets, which were further cleaned, sterilised and used again for cell culture of MC3T3-E1 pre-osteoblasts. The same biological tests as the previous ones were done for 4 and 7 days (Fig 3).

No toxicity was recorded during any of the repeated experiments with a viability rate close to 99%. After 4 days, the cell density was significantly increased on SiHA-T and SiHA-T-PEG₆-S-c-(DfKRG)

in comparison with bare SiHA pellet ($p < 0.0001$ in each case) (Fig. 3A) and the proliferation rates were increased on SiHA-T-PEG₆-S-c-(DfKRG) when compared with the other substrates (Fig. 3C). After 7 days, the cell density was still significantly higher on SiHA-T-PEG₆-S-c-(DfKRG) (Fig. 3B). After 4 days, the proliferation rate was significantly higher on SiHA-T-PEG₆-S-c-(DfKRG) than on SiHA or SiHA-T (Fig. 3C). After 7 days, the proliferation rates were globally lower when compared to those obtained at day 4 (Fig. 3D).

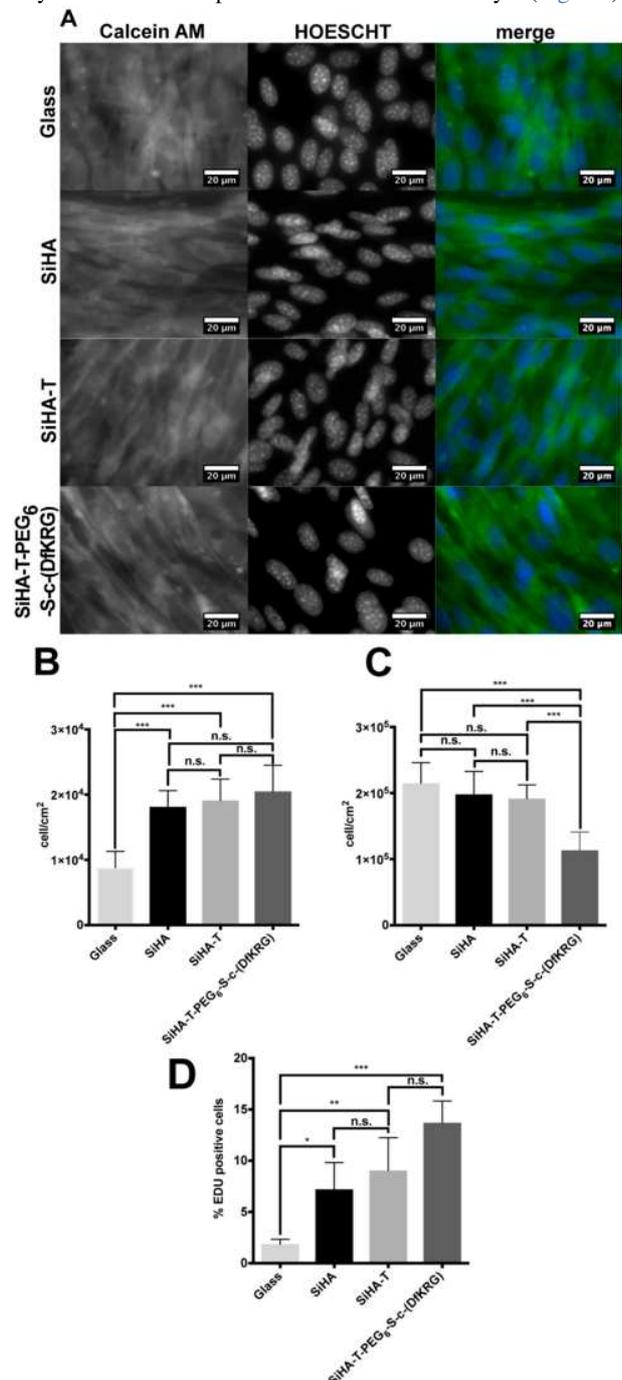


Figure 2 MC3T3-E1 murine pre-osteoblast cells behavior. (A) Living cell morphologies 4 days after seeding. Cells were stained with 10 μ M calcein AM and nuclei by 20 μ M Hoescht 33343 (scale bar length is 20 μ m). (B) Cell density after 1 day (B) and (C) 4 days of culture. (D) Cell proliferation rate after 4 days of culture. Percentage of cells incorporating EDU was calculated from microscopy images by taking into account the number of cells positive for EDU versus the total number of nuclei. $*p < 0.05$ was considered as significant (n.s. non-significant; $**p \leq 0.01$; $***p \leq 0.001$).

This is probably because of a phenomenon of contact inhibition due to cell confluence. As a result the proliferation rate was not significantly higher for SiHA-T-PEG₆-S-c-(DfKRG) when compared with the SiHA control ($p = 0.0605$) or SiHA-T. On the SiHA-T

substrate, the proliferation diminished more slowly between 4 and 7 days, resuming a rate comparable to that measured on SiHA-T-PEG6-S-c-(DfKRG) (Fig. 3C and Fig. 3D).

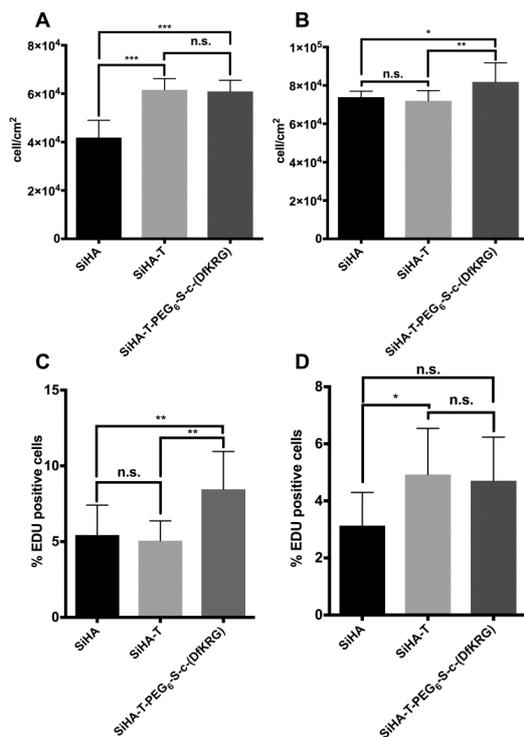


Figure 3 MC3T3-E1 murine pre-osteoblast cells behaviour after cultivation on SiHA, SiHA-T and SiHA-T-PEG₆-S-c-(DfKRG) pellets reused after a first cell seeding. Cell density at (A) 4 days and (B) 7 days after the cell seeding on the substrate. Proliferation rate evaluated after (C) 4 days and (D) 7 days of culture. $p < 0.05$ was considered as significant (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

A third round of cell culture on these SiHA pellets was done after a second cleaning cycle and gave the same variations in terms of cell density and proliferation rate at 4 and 7 days as those observed after the first reuse (data not shown). The XPS analysis of SiHA-T-PEG₆-S-c-(DfKRG) ceramic surface after two successive rounds of cleaning and cell culture of seven days still shows the presence of nitrogen, C-O and C=O contributions proving the sturdiness of the organic-peptide modified surface (Table 1 and Fig. S2 in supplementary materials).

In this work, an organic-inorganic materials associating silicon-substituted hydroxyapatite and c-(DfKRG) peptide has been developed, thanks to a new advanced protocol of functionalization allowing the covalent bonding in low amount of an organic molecule containing c-(DfKRG) on the SiHA ceramic surface. The c-(DfKRG)-S-PEG₆-NHS peptide was synthesized before being bonded to an APTES anchor previously bonded to the SiHA ceramic surface. The final SiHA-T-PEG₆-S-c-(DfKRG) organic-inorganic bone substitute was biologically tested and showed a superior biological behavior. Two successful consecutive reuses proved the preservation of both its structural integrity and its biological activity. This approach of the functionalization of bone substitutes allows better results in comparison to previous works reported in the literature. Indeed, using this new protocol, we demonstrated that it is possible to:

- (i) limit contamination by adsorbed products due to successive *in situ* synthesis steps of classically used protocols,
- (ii) enhance the biological behavior of CaP ceramic substrates with a low dose-efficient amount of peptide while preventing its burst release by strong covalent immobilization,
- (iii) use organic molecules in sufficiently low density to preserve the CaP surface partially free of organic molecules and thus the bioactive properties of this material.

Using the same strategy, the grafting of other peptides, proteins or growth factors of interest in bone tissue engineering will be performed.

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Supplementary data

Supplementary data and experimental procedure for the synthesis associated with this article can be found in the online version, at

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Graphical Abstract

31.

Advanced protocol to functionalize CaP bioceramic surface with peptide sequences and effect on murine pre-osteoblast cells proliferation.

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