A new Phosphorylated Polysaccharide for Biomedical Applications: Generation of 3D Scaffolds with Osteogenic Activity and Coating of Titanium Oxide Surfaces

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Summary: A new phosphorylated derivative of carboxymethylcellulose and amidic carboxymethylcellulose containing one phosphate group for each disaccharide unit was synthesized using sodium trimetaphospahte (STMP) as the phosphating agent. The new polysaccharide was characterized by infrared spectroscopy (FT-IR) and the amount of phosphate groups was determined by elemental analysis. These modified polysaccharides were used both to prepare 3D scaffolds and functionalize titanium oxide surfaces with the aim to improve the osseointegration with the host tissue. The presence of phosphate groups modify the physical-chemical properties of the hydrogels with respect to the native ones. The evaluation of the bioactivity of the phosphorylated carboxymethylcellulose hydrogels towards osteoblast-like cells showed a significant increase in the osteocalcin production. The modified surfaces were chemically characterized by means of X-ray photoelectron spectroscopy (XPS) and FT-IR, whereas the surface topography was analysed by Atomic Force Measurements (AFM) measurements before and after the polysaccharide coating. In vitro biological tests using osteoblast-like cells demonstrated that phosphorylated carboxymethylcellulose functionalized TiO_2 surfaces promoted better cell adhesion and significantly enhanced their proliferation. These findings suggest that the phosphate polysaccharide both as a 3D scaffold and as a surface coating promotes osteoblast growth potentially improving the biomaterial osseointegration rate.

Keywords: 3D scaffolds; biomedical applications; osteogenic activity

Introduction

Bone formation is the result of a sequence of events that starts from the recruitment and proliferation of osteoprogenitor cells from surrounding tissues followed by osteoblastic differentiation and mineralization.[1] Many efforts have been made to find methods for improving the integration of a biomaterial with the bone tissue.[2] On one hand it has been searching a biomimetic scaffold with a good biocompability, bioactivity (favouring of bone apposition), and adequate mechanical properties able to assure skeletal functions. On the other hand, the target is to optimize the properties of surface materials with good performances by manipulating the surface chemical composition and the surface topography.[3] However, it is widely accepted that surface chemical and topographical cues play a key role in the osseointegration process which is a major determinant of the implant

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success.[4] Among the different strategies adopted to turn polymeric biomaterials with low or no tendency to induce osseointegration into mineralising ones, the introduction of phosphate groups has been reported as a successful approach because they act as nucleating centres since anions attract and sequester Ca^{2+} ions. Titanium and titanium-based alloys have been used as excellent materials for dental implants, artificial hip joints and bone screws thanks to their high biocompatibility and their good mechanical properties being similar to bone. Despite all these excellent properties, a true adhesion between the bone and the metal surface has not been observed, yet.[5] For these reasons, many efforts have been made in the past to optimize the properties of titanium oxide surfaces by the introduction of specific functionalities. For example, recently, self assembled monolayers of alkane phosphates or phosphonates have been prepared on titanium oxide (TiO_2) surfaces to tailor physical-chemical properties of the surface such as wettability, surface charge, bio-fouling properties.[6]

In this paper, we introduced phosphate groups along the chain of carboxymethylcellulose (CMC) and amidic carboxymethylcellulose (CMCA) which are negatively charged polysaccharides containing two and one carboxylate groups for each disaccharidic unit respectively. The modified polysaccharides was physico-chemically characterised and used for:

a) the generation of a three-dimensional matrix by the cross-linking of the polymer chains
b) the coating of titanium oxide surfaces

The native and phosphorylated CMC and CMCA were cross-linked; their swelling and rheological properties as well as their biocompability and bioactivity towards osteoblast-like cells were evaluated. The chemistry of the modified TiO_2 surfaces was characterised by XPS and grazing angle equipped FT-IR; information about surface topography, roughness and stiffness was obtained by AFM measurements. The stability of phosphorylated CMC coating has been evaluated under physiological condition at 37 °C for 7 days. In vitro biological tests using osteoblast-like cells were performed in order to assess the cell behaviour compared to non-functionalised TiO_2 surfaces.

Materials

The sodium salt of CMC (MW 200.000, carboxymethylation degree 0.9 ± 0.1 per monosaccharidic unit) was supplied by Hercules S.p.A. Commercially titanium oxide disks (grade 2, diameter 12 mm, thickness 1 mm) were supplied by L.I.M.A-Lto S.p.A. (Italy). All the other chemicals were purchased from Fluka Sigma-Aldrich (Switzerland) and used without further purifications.

Methods

Synthesis of the Phosphorylated CMC and CMCA

A 1% aqueous solution (pH 12 NaOH 2 M) was added with trisodium trimetaphosphate (STMP) in a molar ratio of 1:1 of hydroxylic groups of the polymer and STMP. The mixture was maintained under stirring for 2 hours then dialysed against deionised water and lyophilised.

Crosslinking of the Polysaccharides

The phosphorylated CMC (CMCP) and CMCA (CMCAP) were crosslinked using a previously reported procedure[7] in order to obtain a cross-linking degree of 100%.

Coating of TiO_2 Surfaces

CMCP-Na containing one phosphate group for each disaccharidic unit was allowed to adhere onto freshly cleaned titanium oxide surfaces. Once cleaned and acid etched, the disks were dried and immediately dipped into a CMCP-Na aqueous 5 mM solution. The surfaces were maintained for 48 hours at R.T. to allow the polymer to bind to the surface, then they were rinsed with double distilled water (even under flow) and dried with filtered nitrogen flow.

Physico-Chemical Characterisation of the Polymers

ATR spectra of the phosphorylated polysaccharides were recorded on a Bio-rad FTS 6000 in the spectral region between 4000 and 750 cm^{-1} following the classical procedure. As previously experimented, 64 scans were averaged at a resolution of 1.0 cm^{-1}. The phosphorylated CMC and CMCA were analysed for their C, H, P and N content by elemental analysis.

Chemical and Morphological Characterisation of the Hydrogels

The water up-take (W.U) was determined for each sample and calculated using the following formula:

$$W.U. = [(W_s - W_d)/W_d] \times 100$$

where W_s and W_d are the weight of the swollen and dried hydrogels, respectively.

The rheological behaviour of the hydrogels was evaluated by a strain controlled AR2000 Rheometer (TA Instruments; UK) at the controlled temperature of 25 °C. The frequency ranges from 0.1 to 10 Hz. $G'(\omega)$ (shear storage modulus) and $G''(\omega)$ (shear loss modulus) were measured to get information about the elasticity ($G'(\omega)$) and the viscous character ($G''(\omega)$) of the material.

Surface Characterisation of CMCP Functionalized TiO_2 Disks

The surface chemical composition of native, treated with acid mixture and CMCP functionalised TiO_2 disks, was investigated by XPS analysis. XPS spectra were recorded in an UHV chamber equipped with a hemispherical electron energy analyser (VSW HA150) and a non-monochromatized Al Kα (1486.6 eV) X-ray source. The surface topography was viewed by Atomic Force Microscopy (Solver Pro, NT MDI Instruments, Russia). AFM images were acquired in air in non-contact mode with a sharpened gold coated silicon tip (spring constant of 2.5–10 N/m). Surface roughness parameters, (R_a) and (R_q) of five characteristic scan areas (scan size 5×5 μm^2) of the different substrates were determined using the standard formula.[8]

Cell Cultures on CMCP and CMCAP Hydrogels and Surfaces Functionalised with CMCP

A suspension of 100 μl of osteoblast cells at a concentration of 0.5×10^5 cells/ml was seeded for each hydrogel sample previously sterilised with ethylene oxide. At the end of the experiment, the supernatant was collected from the wells and the release of Bone Alkaline Phosphatase (CICP, Metra CICP EIA Kit, Quidel, USA) was determined. Finally, the cell proliferation reagent WST-1 was performed to assess cell proliferation and viability. The supernatants were quantified spectrophotometrically with a reference wavelength and the results have been reported as optical density. All the data were statistically analysed.

Cell behaviour was studied also on bare and functionalised TiO_2 disks using the same cell line. Cells were plated at the concentration of 1.5×10^4 cells/cm^2/100 μl on three samples for each type (native TiO_2, etched TiO_2 and CMCP TiO_2). The cell response in terms of adhesion, cell shape and proliferation was investigated by Scanning Electron Microscopy (SEM) and cell counts.

Results and Discussion

Phosphorylation of Polysaccharides and Physico-Chemical Characterisation of the CMCP and CMCAP

The mechanism of the phosphorylation reaction involves the opening of the STMP cycle by one of the hydroxyl group of the polymer. At basic pH and in dilute solutions the charged carboxylate groups cause a repulsion among the polymer chains promoting the attack on the hydroxyl groups of the same chain more than the attack on the hydroxyl groups belonging to different chains. On the contrary, at higher concentrations of the polymer the binding between different chains is favoured with the release of pyrophosphate affording the formation of a 3D scaffold.[9] IR analysis of

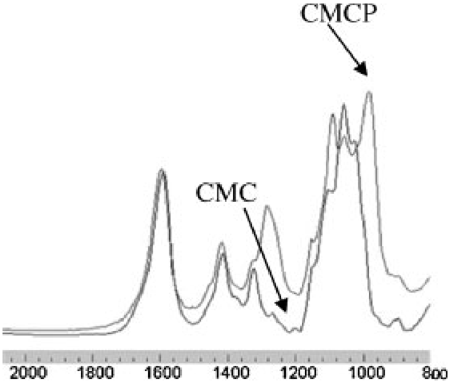

Figure 1.
IR spectra of native CMC polymer (blue one) and CMCP polymer (red one). Spectral region between 2000 and 800 cm^{-1}.

the phoshorylated CMC showed the presence of three new shoulders: one centred at 1286 cm^{-1} ascribed to free P=O stretching and two bands one at 1090 cm^{-1} and another at 990 cm^{-1} relative to the O–P–C stretching (Figure 1). A similar spectrum with the appearance of the same three bands was recorded for CMCAP. Elemental analysis showed that there was one phosphate group per disaccharidic unit for both native and amidated CMC.

Crosslinking of the Polysaccharides and Chemical Characterisation of the Hydrogels

The cross-linking reaction involves the formation of an amidic bond between the amine groups of the diamminopropane, which works as a cross-linker, and the carboxylate groups of the polysaccharides previously activated. The amount of the activating agent was determined to obtain a crosslinking degree of 100%. The IR analysis of the hydrogels confirmed the formation of the network and the absence of free carboxylate groups. The presence of phosphate groups along the polysaccharide chains drastically decreased the water uptake of the hydrogels with respect to the non phosphorylate ones. In fact, the W.U. of cross-linked CMCP was 1200 whereas the W.U of cross-linked CMC was about 4000. The same trend was found for CMCA based. The presence of phosphate groups also induced a significant increase in the mechanical properties of the hydrogel. In fact, G′ increased from 5.8 KPa to 1 MPa at 10 Hz in the presence of phosphate groups (Figure 2). A similar increase was noticed for the CMCA based hydrogel. The morphological analysis (data not shown) confirmed the mechanical properties of the hydrogels: the presence of phosphates give the structure more compact in comparison with that of the same non-phosphorylated hydrogels.

Surface Characterisation of CMCP Functionalized TiO_2 Disks

The XPS analysis of the native TiO_2 substrate showed the presence of titanium, oxygen, carbon and nitrogen (Table 1a). The C and N are due to adventitious contamination which can not be completely removed.[10] No presence of Ti metal was detected meaning that all the surface is covered by an oxide layer thicker than 10 nm (XPS penetration depth). The XPS of H_2SO_4/H_2O_2 treated TiO_2 showed that the acid treatment caused a decrease of the percentage of C(1s) and the disappearance of N(1s) signal due to the removal of any residual organic traces (Table 1b). Table 1c summarizes the experimental binding energies and relative assignment of O(1s), Ti(2p), C(1s), P(2p) and Na(1s) peaks after the CMCP coating. The increase of C(1s) signal together the appearance of P(2p) signal evidenced that the CMCP polymer adhered to the TiO_2 surface. The peak fitting of the O(1s) showed that there were at least two components of the O(1s) signal one at 530.1 eV, typical of metal oxide and another at 532.6 eV due the polymer binding to the surface. The peak fitting of C(1s) showed that beside the contribution of adventitious carbon, the main peak components located at 286.3 eV can be attributed to carbon singly bonded to oxygen, while the other components at 287.9 eV to carbon double bonded to oxygen (–C–O–C– and –C=O groups). Surface infrared analysis

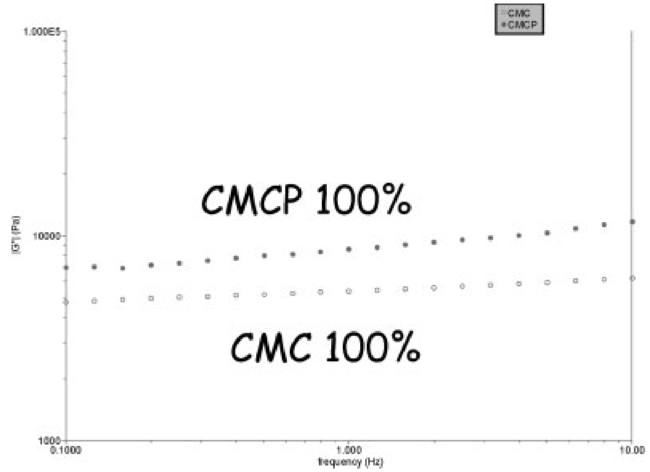

Figure 2.

Rheological behaviour of native and phosphorylated CMC hydrogels. Measurements were performed at 25 °C in the frequency range 0.1–10 Hz. The presence of phosphate groups increased the rheological properties of the hydrogel.

Table 1.

Surface elemental composition (atomic percentages) for native TiO_2, H_2SO_4/H_2O_2 treated TiO_2 surface and c) CMCP functionalised TiO_2 surface.

Table 1a

Element	Assignment	B.E. (eV)	Atomic composition (%)
O(1s)	TiO_2	530.1	51.5
Ti(2p 1/2)	TiO_2	458.6	38.5
Ti(2p 3/2)	TiO_2	464.4	
C(1s)	CH_2, CH_3	285.0	7.8
N(1s)	N–H, C–N	400.1	1.2

Table 1b

Element	Assignment	B.E. (eV)	Atomic composition (%)
O(1s)	TiO_2	530.1	52.0
Ti(2p 1/2)	TiO_2	458.6	41.8
Ti(2p 3/2)	TiO_2	464.3	
C(1s)	CH_2, CH_3	285.0	6.2

Table 1c

Element	Assignment	B.E (eV)	Atomic composition (%)
O(1s) (1)	TiO_2	530.1	50.0
O(1s) (2)	P–O–R P–O–H	532.6	
Ti(2p 1/2)	TiO_2	458.5	12.2
Ti(2p 3/2)	TiO_2	464.4	
C(1s)(1)	CH_2, CH_3	285.0	16.7
C(1s)(2)	C–O–C, C–O–P,	286.3	
C(1s)(3)	O–C–O, C=O	287.9	
P(2p)	$C{-}O{-}P{=}O(O)^{-}{}_2$	134.1	1.9
Na(1s)	PO_4^{3-} Na^+	1071.9	19.1

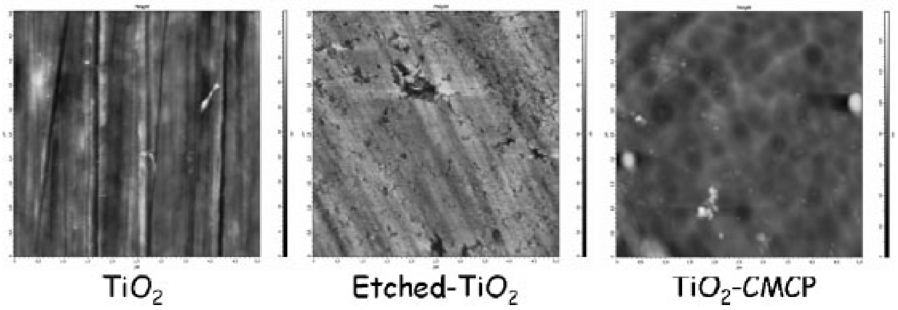

Figure 3.
AFM scans (scan size 5 × 5 μm^2) of a) TiO_2 native substrate, b) etched TiO_2, c) CMCP functionalized TiO_2 surface.

evinced the presence of a thin layer of CMCP onto TiO_2 surface.[11] The shift of the bands ascribable to the phosphate groups, was interpreted as an evidence of the fact that the phosphate groups of CMCP were involved in the bond with titanium oxide surfaces. The shift and in some cases the disappearance of the double peak in the region 1280–1320 cm^{-1} was also observed in previous works.[12] AFM analysis (topography mode and phase mode) of the CMCP coated surfaces showed the presence of a homogeneous coating all over the surface (Figure 3). Roughness analysis showed that TiO_2-CMCP surfaces is smoother than etched TiO_2 ($R_a = 8.0 \pm 0.4$ nm, $RMS = 11.2 \pm 0.7$ nm for CMCP/TiO_2, $R_a = 10.9 \pm 0.5$, $RMS = 14.0 \pm 0.9$ nm for etched TiO_2).

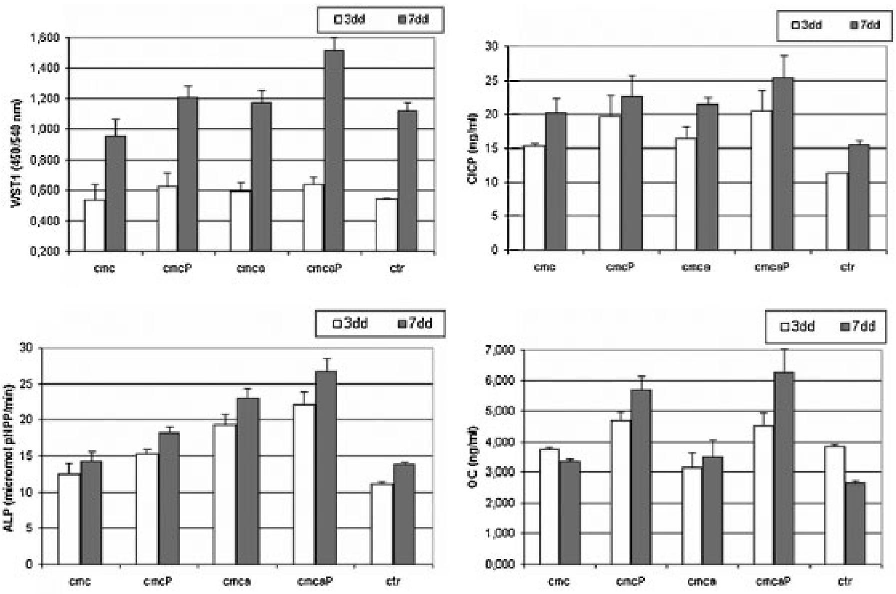

Figure 4.
Osteoblast proliferation, differentiation and synthetic activity after 3 days (white column) and 7 days (grey column) of culture with the different hydrogels and the tissue culture polystyrene (ctr). WST1 3 days ns; 7 days CMCP, CMCA vs CMC ($p < 0.05$), CMCAP vs CMCP CMCA ($p < 0.005$), CMCAP vs CMCP, CMCA $p < 0.005$. ALP 3 days CMCA vs CMC, ctr $p < 0.05$ CMCAP vs CMC, CMCP, ctr $p < 0.05$; 7 days CMCA vs CMC ctr $p < 0.05$, CMCAP vs CMC ctr $p < 0.005$ and CMCAP vs CMCP $p < 0.05$. CICP 7 days CMCAP vs CMC, ctr $p < 0.0001$; OC 7 days CMCP vs CMC, ctr $p < 0.05$, CMCAP vs CMC, CMCA, ctr $p < 0.05$.

Biological Tests

Biological tests demonstrated that both CMCP functionalized TiO_2 surfaces and CMCP and CMCAP based hydrogels were able to promote the adhesion and the proliferation of MG63 cells. In particular, CMCP and CMCAP hydrogels increased significantly the production of collagene type I, osteocalcine and alkaline phosphatase with respect to the non phosphorylated hydrogels. (see Figure 4). In particular CMCAP hydrogel always had higher levels of significance when compared with the CMCP and non phosphorylated ones.

Conclusions

A new phosphorylated CMC based derivative was successfully synthesized. The same polysaccharide was used for the generation of 3D scaffolds and the coating of titanium oxide surfaces. The phosphorylated hydrogels showed modified physico-chemical properties with respect to the native ones. In particular, the presence of phospahte groups induces a decrease of the water up-take and a correspondent increase of the mechanical properties. The functionalised TiO_2 surfaces was completely covered by a homogeneus layer of CMCP which is bound to the titanium oxide through the phosphate groups of the polymer.

Biological tests (i.e. production of collagen type I, osteocalcin and alkaline phosphatase) performed using osteoblast-like cells cultured on such materials emphasized the good performance of CMCP (both as a coating and as a 3D scaffold) towards bone-like cell.

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