Elsevier Editorial System(tm) for Journal of the Mechanical Behavior of Biomedical Materials
Manuscript Draft

Manuscript Number: JMBBM-D-17-00539R1

Title: A novel strategy to enhance interfacial adhesion in fiber-reinforced calcium phosphate cement

Article Type: Research Paper

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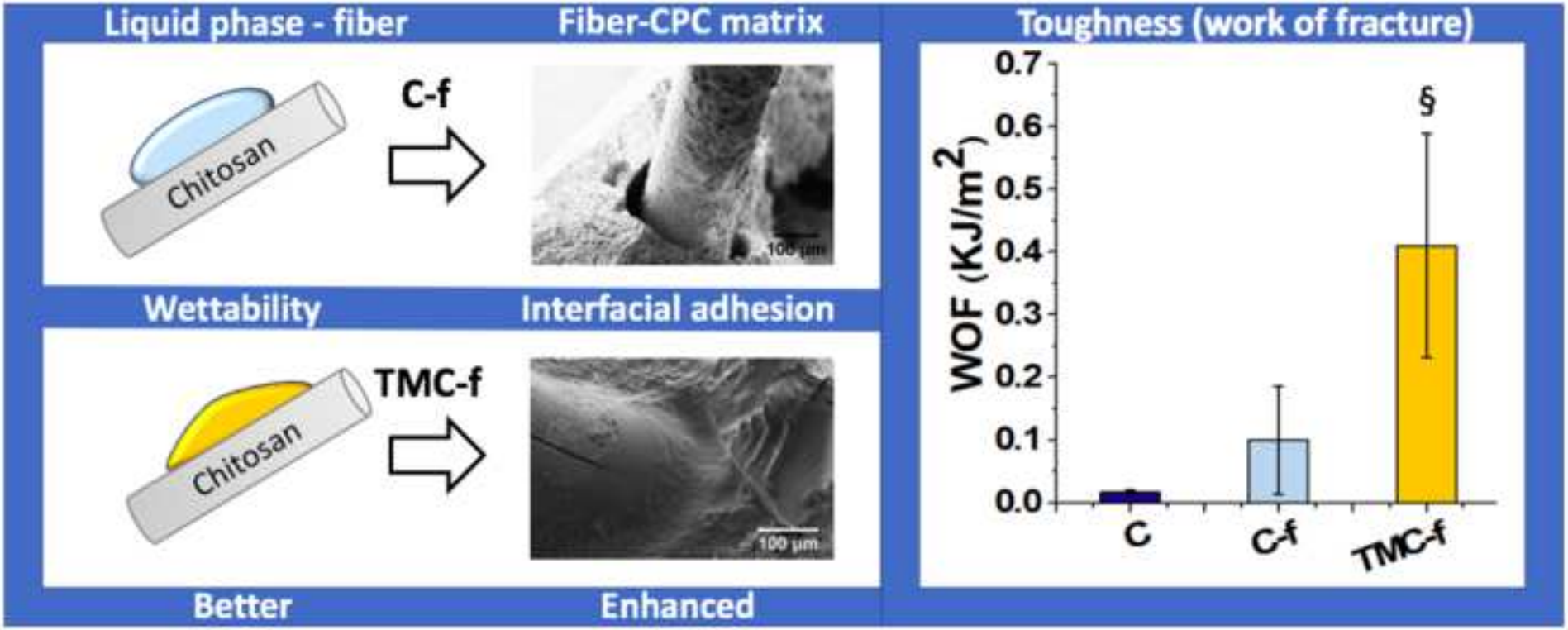
Abstract: Calcium phosphate cements (CPCs) are extensively used as synthetic bone grafts, but their poor toughness limits their use to non-load-bearing applications. Reinforcement through introduction of fibers and yarns has been evaluated in various studies but always resulted in a decrease in elastic modulus or bending strength when compared to the CPC matrix. The aim of the present work was to improve the interfacial adhesion between fibers and matrix to obtain tougher biocompatible fiber-reinforced calcium phosphate cements (FRCPCs). This was done by adding a polymer solution to the matrix, with chemical affinity to the reinforcing chitosan fibers, namely trimethyl chitosan (TMC). The improved wettability and chemical affinity of the chitosan fibers with the TMC in the liquid phase led to an enhancement of the interfacial adhesion. This resulted in an increase of the work of fracture (several hundred-fold increase), while the elastic modulus and bending strength were maintained similar to the materials without additives. Additionally the TMC-modified CPCs showed suitable biocompatibility with an osteoblastic cell line.

**Graphical Abstract (for review)**




Highlights

**HIGHLIGHTS**

• Calcium phosphate cements were reinforced with chitosan fibers and soluble chitosan

• The chemical affinity of fibers and matrix improved interfacial adhesion

• Mechanical reinforcement was achieved thanks to a good fiber-matrix adhesion

• Fiber-reinforced cements were significantly tougher than non-reinforced analogues

• The modified cement matrix supported osteoblast proliferation

# A novel strategy to enhance interfacial adhesion in fiber-reinforced calcium phosphate cement

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**Abstract**

Calcium phosphate cements (CPCs) are extensively used as synthetic bone grafts, but their poor toughness limits their use to non-load-bearing applications. Reinforcement through introduction of fibers and yarns has been evaluated in various studies but always resulted in a decrease in elastic modulus or bending strength when compared to the CPC matrix. The aim of the present work was to improve the interfacial adhesion between fibers and matrix to obtain tougher biocompatible fiber-reinforced calcium phosphate cements (FRCPCs). This was done by adding a polymer solution to the matrix, with chemical affinity to the reinforcing chitosan fibers, namely trimethyl chitosan (TMC). The improved wettability and chemical affinity of the chitosan fibers with the TMC in the liquid phase led to an enhancement of the interfacial adhesion. This resulted in an increase of the work of fracture (several hundred-fold increase), while the elastic modulus and bending strength were maintained similar to the materials without additives. Additionally the TMC-modified CPCs showed suitable biocompatibility with an osteoblastic cell line.

**Keywords:** calcium phosphate cement, chitosan, fiber reinforced, interfacial adhesion, toughness, work of fracture.

## Introduction

Calcium phosphate cements (CPCs) are ceramic materials, brittle by definition, with a porosity that can vary between 10 and 50 % depending mainly on the liquid to powder ratio used in their preparation (Espanol et al. 2009). The intrinsic brittleness derived from the microstructure and composition of these materials is one of the major limitations of their mechanical performance and has restricted their indication of use to non-load-bearing applications.

The toughness of CPCs ranges from 0.010-0.050 $kJ/m^2$ in their work of fracture (WOF) (Canal & Ginebra 2011), which is far below the work of fracture of bone, reported to be between 1.5 and 15 $kJ/m^2$ (Currey & Butler 1975). Although the bending strength values reported for CPCs are typically in the range of 5-15 MPa (Martin & Brown 1995; Ginebra et al. 2001), close to that of trabecular bone (estimated between 10 and 20 MPa) (Barinov 2010), their strain to failure is much lower (Xu et al. 2002). The brittleness of CPCs has recently been highlighted in a report on their strain-to-crack-initiation, which amounted to a mere 0.2% in compression (Ajaxon et al. 2017). An improvement of the mechanical performance of these materials, and particularly a mitigation of their brittle behavior, would significantly extend the applicability of CPCs.

For the last 15 years, several strategies have been evaluated to reinforce CPCs with fibers (Canal & Ginebra 2011; Krüger & Groll 2012). Fibers have been incorporated to the CPC matrix using different lengths (Xu et al. 2000; Pan et al. 2007), aspect ratios (diameter/length) (Xu et al. 2000; Zhang & Xu 2005; Zuo et al. 2010), orientations and textile constructs (mono/multifilaments, yarns, nonwovens, etc.) as reviewed by Canal *et al.* (Canal & Ginebra 2011). These approaches have allowed either an increase of the mechanical properties (Zhang & Xu 2005) or to couple good mechanical properties and macroporosity, increasing the degradation rate and allowing cell infiltration in the material (Xu et al. 2006; Xu et al. 2007). Nonetheless, up to now, little attention has been paid to the fiber-matrix adhesion**,** which is crucial for a successful load transfer, a prerequisite for an effective reinforcement (Nelson et al. 2002). The potential of the strategies based on enhancing the fiber-matrix interface has been highlighted recently by the improvement of

the reinforcement of PLA fibres through surface modification of the fibers by cold plasmas (Canal et al. 2014; Maenz et al. 2014).

Polymeric additives have earlier been used in CPCs with the purpose of improving their mechanical properties, injectability, resorption rate and biocompatibility (Dorozhkin 2009; Neumann & Epple 2006; Low et al. 2010; Perez et al. 2012; Engstrand et al. 2013). The polymers, which are often biodegradable, can be added to the matrix either solubilized in the liquid phase or as a second phase, as particles or fibers. Among the different polymers, chitosan is of interest mainly because it is biodegradable, biocompatible, and it can be processed into several products including flakes, fine powders, beads, membranes, fibers, and gels (Badawy & Rabea 2011).

In this work we used a strategy inspired by the acrylic bone cements, which consist of polymethyl methacrylate (PMMA) spheres embedded in a matrix of the same polymer (Ginebra 2009). The excellent adhesion between the PMMA particles and the PMMA matrix is due to the chemical affinity between the liquid and the solid phase. The methyl methacrylate monomer wets completely the PMMA powder, dissolving and repolymerizing the surface of the particles, creating a perfect continuity between the matrix and the filler. In our case, although it is not possible to induce a partial dissolution of the polymeric fibers due to the hydraulic nature of calcium phosphate cements, an attempt was made to enhance the continuity between fibers and matrix by adding to the cement matrix the same polymer used for the fibers.

Thus, the aim of this work was to develop a biocompatible fiber-reinforced CPC (FRCPC) with improved mechanical properties using chitosan as common polymer in the matrix and in the fibers, with the hypothesis that having an additive of similar nature would increase the chemical interactions between matrix and fibers, which would in turn result in a higher toughness. As chitosan is poorly soluble in water, trimethyl chitosan (TMC), which is a more soluble chitosan derivative (Domard et al. 1986), was added to the cement liquid phase, and chitosan fibers were used as reinforcing agents.

## 2. Materials and methods

### *2.1 Fiber reinforced calcium phosphate cements*

Fiber-reinforced calcium phosphate cements (FRCPCs) were prepared by mixing a solid phase containing α-tricalcium phosphate (α-TCP) and chitosan fibers with a liquid phase. The solid phase consisted of in-house made α-TCP obtained by solid-state reaction of a 2:1 molar mixture of calcium hydrogen phosphate ($CaHPO_4$, Sigma–Aldrich C7263) and calcium carbonate ($CaCO_3$, Sigma–Aldrich C4830) at 1400 °C for 15 h followed by quenching in air. The powder was first milled with 10 balls (d = 30 mm) for 15 min at 450 rpm. 2 wt% of precipitated hydroxyapatite (Merck, ref. n. 1.02143, Darmstadt, Germany) was added as a seed in the powder. Detailed powder characteristics are described elsewhere (Espanol et al. 2009).

To prepare FRCPCs, chitosan fibers were mixed with the CPCs powder. Chitosan monofilament with a 2% of acetylation degree, cylindrical cross section (diameter of 200 ± 10 μm), smooth surface and stress at break of 471 ± 13 MPa according to the manufacturer, were acquired from Medovent GmbH (ref. n. F1G-1206/01, Meinz, Germany). The fibers were cut to 8 mm length and blended with the powder phase of the cement to have a fiber content of 4, 8 and 12 wt%. The liquid phase used to enhance the interaction with the fibers in FRCPCs was 1 weight/volume % (w/v %) trimethyl chitosan (TMC) (Kytozyme, Belgium) in MilliQ water. CPCs and FRCPCs were prepared by mixing the solid phase with the liquid phase at a liquid to powder ratio (L/P) of 0.35 ml/g. Control materials for both the pristine cement and the FRCPCs with 8 wt% chitosan fibers were prepared using MilliQ water as liquid phase. The corresponding volume percentage (v/v %) of fibers (Table 1) was calculated using the density of the fibers (1.56 $g/cm^3$; provided by the manufacturer) and of α-TCP (2.866 $g/cm^3$), taking into account that cements were prepared using a L/P = 0.35 ml/g and estimating that the density of 1 w/v % TMC is the same as water (1 $g/cm^3$). As the solid phase was the same for all samples, the samples were named according to their liquid phase and the presence or absence of fibers in different amounts as described in **Table 1**.

The specimens to characterize specific surface area, crystalline phases, microstructure and compressive strength were prepared by filling Teflon cylindrical molds (6 mm diameter x 12 mm

height) with cement paste; specimens devoted to flexural tests were cast into rectangular molds of 3 x 4 x 50 $mm^3$; the specimens for biological characterization were shaped into discs (15 mm diameter x 1.5 mm thickness). In all cases the specimens were set in Ringer's solution (0.15 M sodium chloride solution) for 7 days at 37ºC.

*2.2 Physico-chemical characterization*

*a) Starting materials*

The static contact angle of chitosan films with water or 1 w/v % TMC solution as wetting liquids was evaluated. Chitosan films (Medovent GmbH, Meinz, Germany) with thickness of 0.05 mm were used as a model surface for the fibers. It was not possible to measure the contact angle on CPCs –and their composites with chitosan fibers– due to their inherent microporosity and hydrophilicity. This led to immediate water absorption after deposition of the water droplet on the surface, therefore impairing measurement of the contact angle. An Oca15+ contact angle meter (Dataphysics) was connected to a coupled camera device and the software SCA20 was used for data acquisition. A minimum of 5 replicates was performed for each group.

The pH evolution of the mixture of α-TCP powders with MilliQ water or 1% w/v TMC solution was measured by means of a multimeter (Multimeter Crison MM41, software ComLab EASY v 1.0) for 72 hours, at 37°C and at a L/P ratio of 200 ml/g.

*b) Cements*

Initial and final setting times of C and TMC pastes were measured with the standard Gillmore needles method (ASTM 1993). The assay consists in determining the time needed for the Gillmore needle to fail to make a perceptible circular indentation on the cement surface, counting as time zero the start of mixing. The initial and final setting times refer to the use of low and high pressure tips, respectively. Five specimens from each group were evaluated.

The specific surface area (SSA) of set cements was measured by nitrogen adsorption according to the BET method (ASAP 2020 Micromeritics). Around 3 g of material shaped in cylindrical specimens were used for the analysis.

The phase composition of set cements after 7 days immersion in Ringer's solution at 37ºC was measured by X-ray powder diffraction (XRD) using a Bruker D8 Advance device and scanning in Bragg-Brentano geometry using CuKα1 radiation (λ = 1.5406 Å) with detector PSD Lynx-eye. The scan step was 0.017° in the range between 5° and 75°, using 40 kV voltage and 40 mA intensity. The cement's phase composition was calculated with a semi-quantitative analysis (Chung 1974), which consisted in integrating the area of the three peaks with highest intensity and taking into account the reference intensity constant of their corresponding components. The peaks were identified by matching with patterns from the in Joint Committee on Powder Diffraction Standards for α-TCP (JCPDS No. 9–348) and HA (JCPDS No. 9–432).

The compressive strength of the unreinforced samples (C and TMC) was evaluated in cylindrical specimens (12 mm height x 6 mm diameter) at a cross-head speed of 1 mm/min using a Servohydraulic Testing Machine (Bionix 858, MTS Systems) equipped with a 2.5 kN load cell. At least six replicates were tested for each formulation. The materials were tested in wet conditions and on previously polished specimens to ensure parallel surfaces and full contact of the specimen surface with the piston.

The bending strength (BS), elastic modulus (E) and work of fracture (WOF) were measured by three-point bending in 3 x 4 x 50 $mm^3$ bars, using an outer span of 40 mm at a cross-head speed of 1 mm/min. The specimens were tested in wet conditions, right after extraction from the setting liquid (ASTM 2008). The following equations were applied to calculate the BS, E and WOF respectively:

$$BS = \frac{3PL}{2wt^2} \qquad \text{Equation 1}$$

$$E = \frac{PL^3}{4wt^3y} \qquad \text{Equation 2}$$

$$WOF = \frac{Area\ curve\ load\ vs\ displacement}{2wt} \qquad \text{Equation 3}$$

where P = load, L = span, w = width of the specimen, t = thickness of the specimen and y = deflection at the load point. A minimum of 8 specimens per group were tested in flexion.

The microstructure of the fractured surface of set cements was imaged by Field Emission Scanning Electron Microscopy (FE-SEM, Hitachi H-4100FE or FIB, Zeiss Neon40). The specimens were previously coated with Au/Pd (Emitech K950X metal evaporator) and a thin layer of colloidal silver (Electron Microscopy Sciences, ref. n. 12630) was painted on the side of the material in contact with the holder, to improve the conductivity of the materials.

*2.3 Biological characterization*

Pre-osteoblastic MG-63 cells (purchased from ATCC) were used to evaluate the effect of trimethyl chitosan modification of the cement matrix on the proliferation of the cells in direct contact with the materials. Cells were maintained in T-75 flasks in an incubator with a humidified atmosphere of 5% $CO_2$ in air at 37°C. DME/F12 (Thermofisher Scientific HyClone, ref. n. SH300023.01, Logan, UT, USA) supplemented with 10% fetal bovine serum (Thermofisher Scientific HyClone, ref. n. SH30071.03) and 1% penicillin/streptomycin (Lonza, BioNordika, ref. n. DE17-602E, Basel, Switzerland) was used for feeding the cells every other day. Cells were used for experiments upon 80% confluence. Prior to plating, cells were detached with a minimum amount of trypsin 0.25% (Fisher Scientific, Hyclone, ref. n. 10693313) diluted 2-fold with PBS.

The samples C and TMC were sterilized in 70 v/v% ethanol with orbital shaking and the solution was replaced every 10 minutes for 6 times. Afterwards the samples were rinsed in sterile PBS and pre-incubated for 1 h with supplemented media at 37°C.

Cells were seeded on the specimens (discs of 15 mm diameter x 1.5 mm thickness) at a density of 50 000 cells/$cm^2$ and cultured for 1, 3, and 7 days. The medium (1 ml) was changed daily. The complete study was performed twice using quadruplicate specimens for the cell proliferation assay and one specimen for SEM imaging. Moreover, as the behavior of the cells can be strongly influenced by the pH of the medium, the pH of the medium in contact with the specimens was monitored every day following the same conditions used for the cell culture .

At each time point the specimens were washed 3 times with PBS. The cells were lysed by incubating the samples with 0.1 v/v% Triton (Triton X-100, Merck, ref. n. 9036-19-5, Darmstadt,

Germany) in 500 µl of MEM medium (Hyclone, ref. n. SH30024.01) for 50 min at 37°C. The number of cells was quantified by measuring lactate dehydrogenase (LDH; Sigma-Aldrich, TOx7-1KT 091M6098, St. Louis, MO, USA) following the manufacturer's protocol. The absorbance was measured at 490 nm with a background absorbance of 690 nm. The absorbance values were transformed to cell number by using a standard curve.

The number of viable cells was visualized after 1, 3 and 7 days using live/dead staining kit (Life Technologies, USA). Briefly, the specimens were rinsed in PBS twice followed by 15 min incubation in staining reagents according to the manufacturer's protocol. The cells were then visualised using an Eclipse TE2000-U Optical microscope coupled to a Super High Pressure Mercury Lamp Power Supply CSHG-1 (Nikon Corporation, Japan). Representative micrographs were acquired in which live cells fluoresced green and dead cells fluoresced red.

The morphology of the MG-63 cells cultured on the cement specimens as well as the cement microstructures were visualized by Field Emission Scanning Electron Microscopy (FE-SEM, device: FIB Zeiss Neon40). Prior to observation cells were fixed with 2.5 v/v% glutaraldehyde (SERVA Electrophoresis GmbH, ref. n. 23114, Heidelberg, Germany) in PBS solution for 60 min at 4°C. Samples were subsequently rinsed twice in PBS and dehydrated in several steps with graded ethanol followed by further dehydration in hexamethylsilazane (Sigma Aldrich, ref. n. 440191).

*2.4 Statistics*

Statistical analysis was performed using a one-way ANOVA with Tukey's post-hoc test using Minitab 16 software (*Minitab, Inc*., State College, PA). A significance level of 0.05 was used and the sample groups compared were C, TMC, C-8f and TMC-4f, TMC-8f and TMC-12f.

## 3. Results

*3.1 Physico-chemical characterization*

The presence of 1 w/v% TMC in the liquid phase increased the setting time of the cement in comparison with that prepared only with water (**Table 2**). The pH of a cement slurry (200 ml/g) remained at 7.0-7.5 when 1 w/v % TMC was used as solution. In contrast, when only water was used, the pH increased from ca. 7 to 9.3 in less than 10 min, followed by a slow decrease to a neutral pH during 24 h (**Fig. 1**).

The crystalline phases of the end-products of the cementitious reaction were analyzed after 7 days (**Fig. 2**, **Table 3**). The highly pure (97%) α-TCP (**Fig. 2a** fully reacted into calcium deficient hydroxyapatite (CDHA, **Fig. 2b**) in the presence of water as liquid phase (C). The addition of 1 w/v % TMC in the liquid phase (TMC) slightly impaired the reaction and 6 % of α-TCP remained unreacted (**Fig. 2c)**. The end-product of TMC samples had a significantly lower SSA than that of C samples (**Table 3**).

The water contact angle of chitosan films was 71.2 ± 3.1° and the 1 w/v% TMC contact angle on the same surface was 10° lower (61.5 ± 3.6°), reflecting better wettability of chitosan with the polymer solution.

The presence of TMC in the liquid phase slightly increased the compressive strength with respect to the C sample of unreinforced cements **(Table 2**). The mechanical properties under bending of FRCPCs with different amounts of 8 mm long chitosan fibers (4, 8, 12 wt%) are shown in **Fig. 3**. The unreinforced cements (C and TMC) exhibited a catastrophic fracture (**Fig. 3a**) while the fiber-reinforced cements with water in the liquid phase (C-f8) resisted very low loads. On the contrary, when TMC was present in the matrix of fiber-reinforced cements (TMC-4f, TMC-8f, TMC-12f), the presence of the chitosan fibers prevented the catastrophic failure of the composites. The load-deflection curve for the FRCPCs with TMC in the matrix and different chitosan fiber contents displayed an initial peak followed by an increase in the load-bearing capacity of the material.

The presence of TMC in the liquid phase of the cements free of fibers caused an increase in the elastic modulus (**Fig. 3b**) and bending strength (**Fig. 3c**) in comparison with the samples whose liquid phase was only water, both for samples without fibers (TMC > C) and their analogues with fibers (TMC-8f > C-8f). This increase however was only significant ($p < 0.05$) among the fiber-

containing samples. The addition of fibers caused a significant decrease ($p < 0.05$) in the elastic modulus (**Fig. 3b**) and bending strength (**Fig. 3c**) for the cements prepared using water as liquid phase (C > C-8f), whereas these parameters were barely affected when TMC was present in the liquid phase when fibers were added, especially with 8 wt% of fibers (TMC ≈ TMC-8f) . Other amounts of fibers gave poorer outcomes (TMC-4f and TMC-12f). The addition of TMC in the liquid phase did not modify the toughness (measured as WOF) of the samples without fibers (C ≈ TMC). However, the addition of fibers caused an increase in the WOF, moderate when the liquid phase was pure water (C < C-8f), and more pronounced when TMC was added in the liquid phase (TMC < TMC-4f ≈ TMC-8f ≈ TMC-12f), the maximum value being reached for the TMC-8f sample **(Fig. 3d)**; this increase was however only statistically significant ($p < 0.05$) for the TMC samples. In any case, all FRCPC samples containing 1 w/v % TMC in the matrix showed at least 10-fold improvement in WOF with respect to pristine cement (C).

The SEM images of the fracture surfaces of different FRCPCs are shown in **Fig. 4**. Even if the fibers were aimed to be randomly distributed in the matrix, the molding process led to a partial alignment, as fibers of 8 mm were used and the 3-point bending molds were 4 mm wide. The images showed a good adhesion of fibers with the matrix of the TMC-8f cement (**Fig. 4d** and **4f**), where a continuous interface was observed, compared to the gap existing between the fibers and the matrix of C-8f (**Fig. 4c** and **4e**).

*3.2 In vitro* biological characterization

The number of cells attached to the material was quantified by LDH after lysing the cells. The cell number continuously increased on both C and TMC samples from 1 to 7 days, the values being consistently higher on C samples **(Fig. 5a)**. Regarding the potential modification of the cell culture medium by the presence of the materials, only small fluctuations in the pH were detected, from around 7.30-7.40 to 7.05-7.25 (**Fig. 5b**).

The images of the fluorescent staining of live and dead MG-63 cells on cement samples (**Fig. 6**) confirmed that the cells were alive and increasing in number with time, and with very small number of dead cells.

In agreement with the previous results, SEM images showed the spread morphology of the cells on the surface of both materials, a sign of good biocompatibility of the material **(Fig. 7)**.

## Discussion

Fiber Reinforced Calcium Phosphate Cements (FRCPCs) with improved fiber/matrix adhesion were obtained in this work by introducing a polymeric solution (trimethyl chitosan, TMC) in the cement matrix, with high affinity to the chitosan fibers that were randomly oriented in the matrix.

To the best of our knowledge, only two prior studies aimed to improve the adhesion of the fiber/matrix interface in CPC-fiber composites, but with a different strategy. These studies were based on the activation of the fiber surface by low temperature plasma treatments (Canal et al. 2014; Maenz et al. 2014). The first study was based on plasma activation of poly-L-lactic acid fibers to obtain new polar functional groups on the fiber surface, with the aim of improving their wettability and consequently obtain enhanced adhesion of the fibers to the apatitic cement (Canal et al. 2014). The results were quite promising since both flexural strength and WOF were improved in comparison to CPC reinforced with untreated fibers; but still not sufficient for the clinical needs. In the second study (Maenz et al. 2014), the same strategy was employed in brushitic cements to obtain more reactive functional groups on the surface of poly(lactic-co-glycolic) acid fibers, which were able to ameliorate the interfacial shear-stress. However, the WOF of the reinforced materials was not significantly improved.

In this study, the introduction of the polymeric additive in the liquid phase of CPCs caused some changes to the cement's final microstructure. As can be noticed through the pH evolution of α-TCP slurries (**Fig. 1)**, the addition of 1 w/v% TMC affected the setting reaction. When only water was used as liquid phase, a fast basification (in the range of minutes) occurred due to the α-TCP

dissolution and release of phosphate ions, followed by a slow decrease of pH (due to the precipitation of CDHA) until neutrality was reached. In contrast, when 1w/v % TMC was added to the liquid phase, the pH increase was gradual and slow (from pH 6.5 up to pH around 7 in 24 h). Similar behavior was obtained by Perez *et al.* (Perez et al. 2013) when collagen was used as additive in liquid phase. This "buffer-like" behavior was probably the cause of a slowing down of the dissolution-precipitation process of α-TCP in TMC samples, which led to slightly lower conversion to CDHA (6% of α-TCP remained unreacted after 7 days of setting, **Fig. 2** and **Table 3**) in TMC samples. This was in agreement with the slower setting of TMC cement (**Table 2**). In turn, the lower proportion of CDHA formed in TMC samples, which precipitates in the form of microcrystals, could contribute to the lower SSA of the TMC samples compared to the pristine cements (C) (**Table 3**).

Static contact angles were measured on chitosan films used as models for the surface of their fiber homologues. The water contact angle of chitosan was below 90° but still quite high (72°). This value was similar to others found in the literature for this material (Bumgardner et al. 2003) and corresponds to a slightly hydrophilic material. The wettability of chitosan with TMC solution was better than with water, which can be associated to the improved adhesion fiber/matrix as observed by SEM **(Fig. 4d** and **4e).** This improved interfacial adhesion may be essentially attributed to hydrogen bonds and van der Waals forces established between hydroxyl groups and amine groups of chitosan fibers and TMC present in the cement matrix, fostered by the enhanced wettability.

The addition of 1 w/v% TMC to the liquid phase of the cement without fibers tended to moderately improve compressive strength (**Table 2**). Xu *et al.* (Xu 2004) found a significant increase in the compressive strength of an apatitic CPC when another chitosan derivative was introduced in the liquid phase (15% chitosan lactate). The higher concentration they employed (15 % versus 1 % used in this study) together with the different structure of the molecule and the different pH of the liquid phase might have influenced the setting reaction and, thus, the strength of the final material. Interestingly, similarly to compressive strength, the addition of 1 w/v% TMC to the liquid phase resulted in materials with slightly higher elastic modulus and bending strength than the C samples,

although the differences were not statistically significant (**Fig. 3a** and **b**).

The addition of chitosan fibers had opposite effects depending on whether the cement matrix contained or not TMC. Blending chitosan fibers in the cements prepared with water (C-8f) caused a significant decrease in the elastic modulus (**Fig. 3b**) and bending strength (**Fig. 3c**) compared to the cements without fibers (C), probably due to poor fiber/matrix adhesion, as also confirmed by SEM images (**Fig. 4c** and **4e**). The poor fiber/matrix adhesion can be attributed to the relatively low wettability of chitosan with water, as well as to the fact that the chitosan fibers are smooth, so pull out may take place quite easily due to lack of physical interactions such as interlocking. This correlates well with other works (Xu et al. 2000; Xu & Quinn 2002; Xu 2004; Xu & Simon Jr. 2004; Gorst et al. 2006), where the addition of fibers or meshes caused a decrease in the elastic modulus with respect to the unreinforced sample. Interestingly, the elastic modulus (**Fig. 3b**) of the cement containing both chitosan matrix and 8 wt% chitosan fibers (TMC-8f) was similar to that of a cement containing only TMC solution in the matrix, which highlights the relevance of adding TMC in the matrix to increase the fiber-matrix adhesion. However, the elastic modulus decreased for lower (TMC-4f) and higher (TMC-12f) amounts of fibers. The decrease obtained with a too high amount of fibers agrees with previous work in the literature (Pan et al. 2007; Zuo et al. 2010) and confirms the need for adjusting the optimal fiber amount for each kind of fiber. Nevertheless, the elastic modulus of TMC-8f (10 ± 2 GPa) was quite close to that of cortical bone in bending (15-20 GPa) (Guo 2001). The same trend was observed for the bending strength **(Fig. 3c)**, since whereas the addition of 4 or 12% chitosan fibers (TMC-4f, TMC-12f) lead to a decrease of bending strength compared to the unreinforced cements, no statistically significant differences were observed between the specimens with 8% fibers (TMC-8f) and the TMC or C samples. Although overall the values registered for the bending strength were still far from the cortical bone values, which have been estimated between 50 and 200 MPa (Barinov 2010; Currey 1988), they are within the range estimated for trabecular bone (between 10 and 20 MPa) (Barinov 2010).

Regarding the fracture behavior, while CPCs (C and TMC) showed a catastrophic failure due to fast propagation from the initial crack (W.D. & Rethwisch 2009), FRCPC (C-8f, TMC-4f, TMC-8f

and TMC-12f) underwent multiple cracking, leading to a non-catastrophic failure **(Fig. 3a)**. However, the addition of fibers in the cement prepared with water as liquid phase (C-8f) result in a material with a very low load-bearing capacity in comparison with pristine cements (C). In contrast, when the fibres were blended with the TMC-containing matrix, the material showed a deflection-hardening behavior (Parra-Montesinos & Chompreda 2007), *i.e.*, it was able to sustain increasing amounts of load after first cracking, this leading to a remarkable and progressive increase of the WOF (**Fig. 3d**) from TMC-4f to TMC-8f. A further increase of the fiber content (TMC-12f) did not have a positive effect neither on the strength nor in the WOF. In the light of the SEM images (**Fig. 4d** and **4f**), this behavior can be associated to the enhanced matrix-fiber adhesion, which can increase the energy consumption during fracture through two mechanisms: i) the energy consumed during crack bridging due to the flexibility of the polymer; and ii) the higher consumption of energy in the frictional sliding during fiber pull-out. Previous strategies to improve the adhesion at the interface fibers-matrix employing $O_2$ plasma were able to achieve around 60% improvement in WOF (Canal et al. 2014), while our current approach reaches a 300% improvement in WOF. Notwithstanding, the value obtained for TMC-8f (0.4 kJ/m$^2$) is still lower than the WOF described for cortical bone, which has been reported to range between 1.5 and 15 kJ/m$^2$ (Currey & Butler 1975). In this respect, two combined effects acting as limiting factors could be pointed out for the present study: a) the poor tensile strength of chitosan fibers, especially in the wet state (Notin et al. 2006), which led to the breakage of some fibers during the bending test (**Fig. 4h**). The adhesion with the matrix exceeded the load-bearing capacity of the fibers, leading to fiber breakage, which hindered the toughening mechanism by fiber pull-out. The use of higher-strength chitosan fibers is expected to improve significantly the performance of this type of composites; and b) the smoothness of the fibers' surface. Modification of the surface topography to obtain rougher fibers, or employing yarns which are made of twisted fibers, would be interesting options expected to further improve the performance of this type of composites.

Regarding the biological characterization of these materials, a previous study by Wu *et al.* (Wu et al. 2013) evaluated the effect of the addition of chitosan fibers in a CPC on osteoblast-like cells. No

differences were observed between the pristine CPC and the FRCPC on cell adhesion, proliferation or viability (up to 14 days of cell culture). With this in mind, our focus in the biological studies was on the effect of the biocompatibility caused by the addition of 1 w/v % TMC (rather than fibers) in the matrix of the cements.

The biocompatibility (proliferation, cell function and cell morphology) of a human pre-osteoblast cell line (MG-63) was evaluated growing the cells directly on the surface of the materials. The number of cells gradually increased (**Fig. 5a**) in all samples during the 7 days of the study. Live/dead staining confirmed that cells were alive (**Fig. 6**) and images of SEM (**Fig. 7**) showed their spread morphology, which was a sign of good biocompatibility of the materials. The pH of the media in contact with TMC samples was hardly modified, especially after 2 days and on, being between 7 and 7.4 for all the samples (**Fig. 5b**), so this is not expected to influence cell adhesion or proliferation. Therefore, the higher number of cells on C samples compared to TMC samples (difference between 18% at day 1 and 26% at day 7) could be ascribed either to the differences in morphology and SSA of the samples **(Fig. 4 a** and **b, Table 2)** or to the presence of the polymeric TMC. However, TMC has been described as a biocompatible and non-toxic polymer (Follmann et al. 2012).

Overall, the approach taken in this study, consisting in adding a polymer of similar chemical nature both as a liquid phase and as fibers in the matrix of the cement, resulted in the production of a biocompatible fiber reinforced CPC, with a significantly higher toughness than the homologous CPC without fibers, and which under continuous load kept its integrity, with multiple cracking. Despite the higher toughness obtained by the enhanced adhesion at the interface, further investigation will be required in the future, as these materials are still far from the mechanical features of bone; the smoothness of the fibers may be a reason impairing a stronger adhesion between fibers and cement matrix, in addition to the limited tensile strength of the fibers leading to fiber breakage. Therefore, these parameters might be of interest for future work in this line.

## Conclusion

Fiber-reinforced calcium phosphate cements (FRCPCs) have been successfully prepared using trimethyl chitosan as additive in the liquid phase and chitosan fibers as reinforcing agent. The improved wettability of the fibers and its chemical similarity with the liquid phase of the cement enhanced their interfacial adhesion. The FRCPCs had a significantly improved toughness (measured as work of fracture) and at the same time the elastic modulus and bending strength were maintained in comparison to samples containing chitosan only as fibers or only as additive. The FRCPCs kept their integrity during fracture through multiple cracking and tension softening behavior. The biological characterization showed that cells adhered and proliferated on the cements containing TMC in the matrix as a sign of its good biocompatibility.

**Acknowledgements**: Authors acknowledge the "Generalitat de Catalunya" funding through a FI Scholarship of SG, the MICINN for the Ramon y Cajal fellowship of CC and the financial support in the MAT2015-65601-R project (MINECO/FEDER, EU). The research leading to these results received funding from the European Commission Seventh Framework Programme (FP7/2007-2013) under the Grant agreement n° 241879, through the "Reborne" project and through the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), project IG2011-2047. GM acknowledges Marie Curie Actions FP7-PEOPLE-2011-COFUND (GROWTH 291795) via the VINNOVA programme Mobility for Growth (project No. 2013-01260) and Lars Hiertas Minne Stiftelse (project No. FO2014-0334). Support for the research of MPG was received through the ICREA Academia award for excellence in research, funded by the Generalitat de Catalunya. Authors acknowledge Dr. Marta Pegueroles for contact angle measurement support. Kytozyme is acknowledged for providing the TMC.

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Figures

## FIGURES

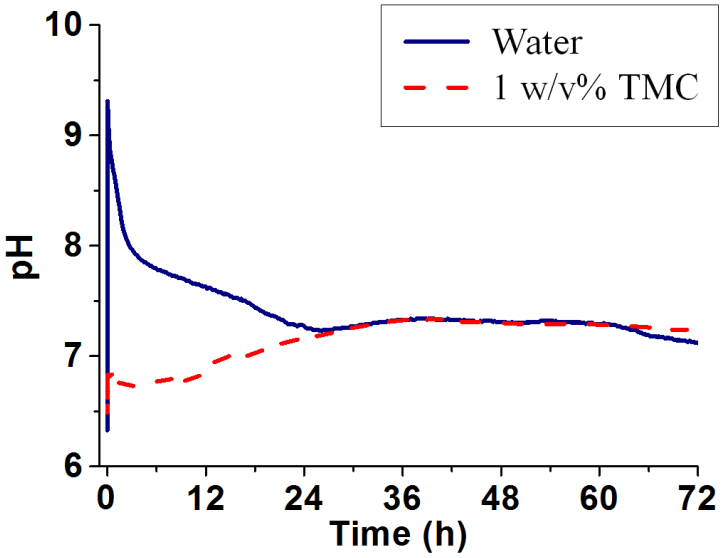


**Figure 1.** pH of α-TCP powder slurries (200 g/ml) in MilliQ water or in 1 w/v% TMC solution measured continuously at 37°C.

Figures

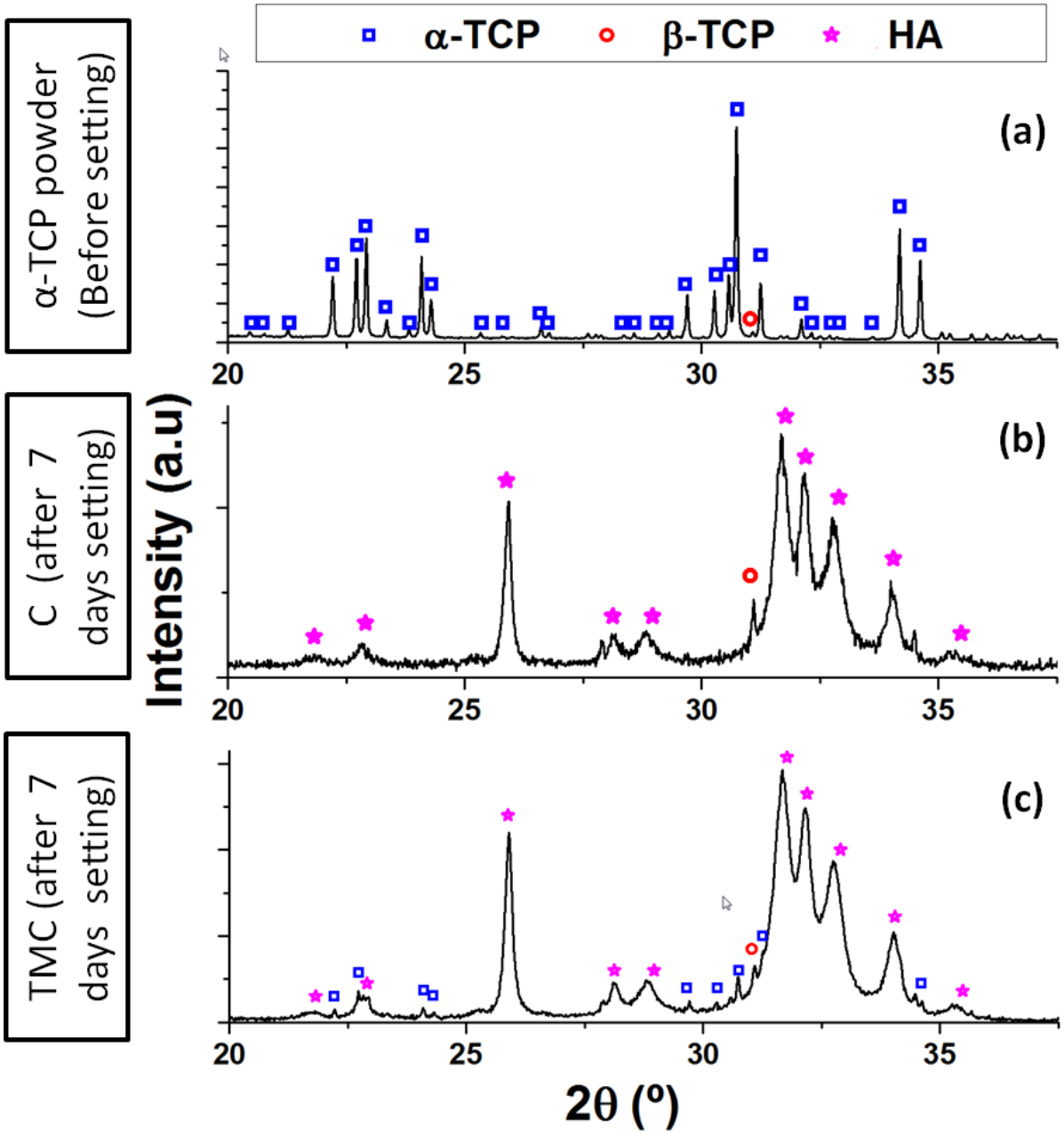


**Figure 2.** Crystalline phases determined by X-ray diffraction of a) initial powder α –TCP, b) sample C 7 days after setting, and c) sample TMC 7 days after setting.

Figures

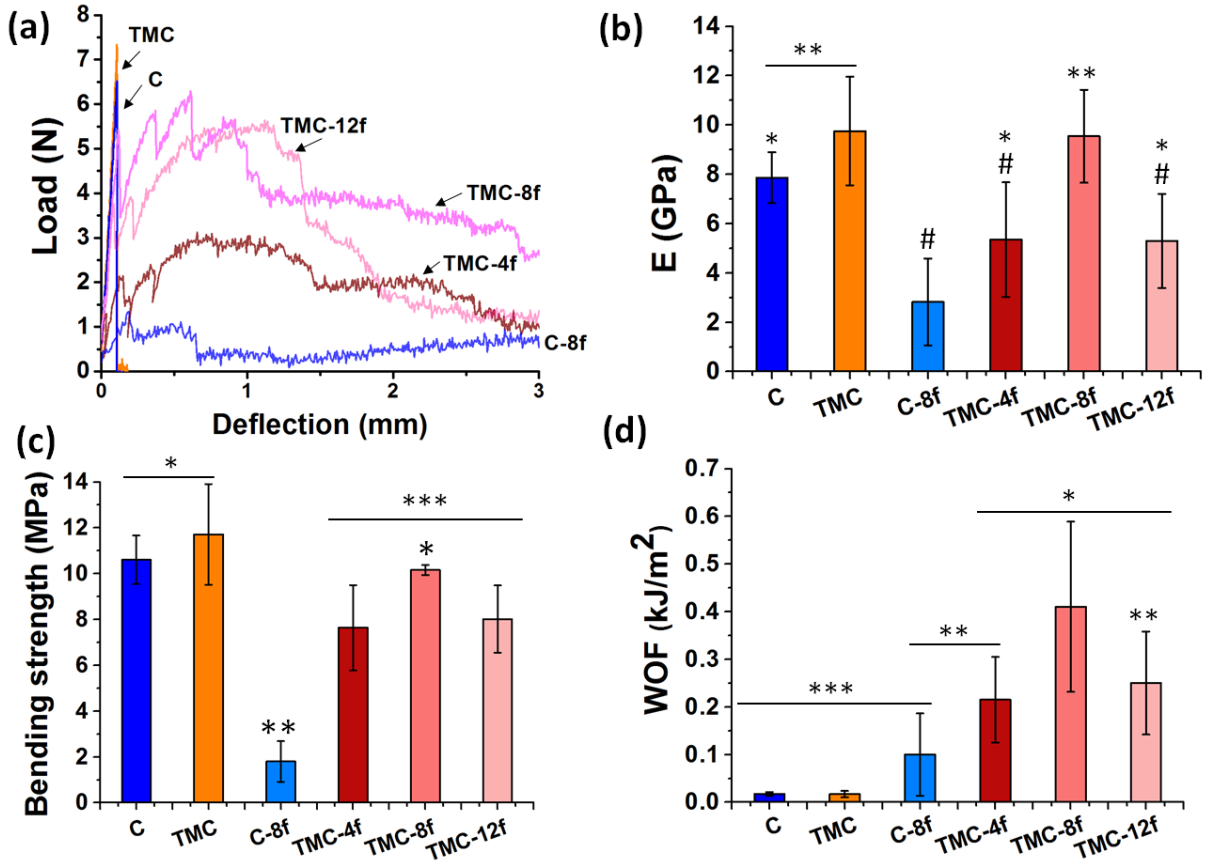


**Figure 3.** Mechanical properties under bending of unreinforced CPCs (C and TMC) and fiber-reinforced CPCs (C-8f, TMC-4f, TMC-8f, TMC-12f): a) Typical load/deflection curves, b) Young's modulus (E), c) bending strength, and d) work of fracture (WOF). Groups indicated with same symbol do not have statistically significant differences ($p > 0.05$).

Figures

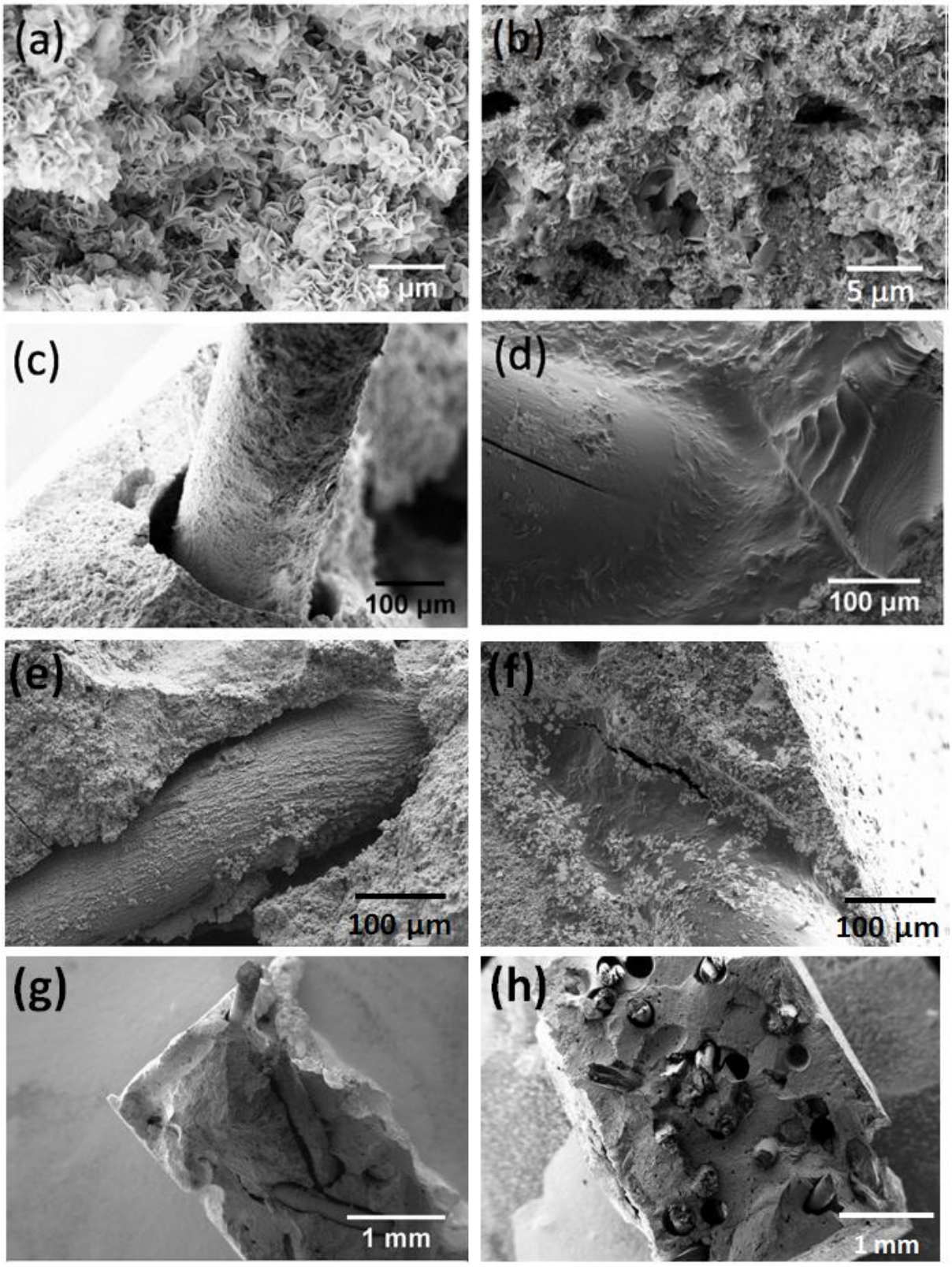


**Figure 4.** Microstructure taken by FE-SEM of the fracture surface of unreinforced cements and of FRCPCs set for 7 days: C (a), TMC (b), C-8f (c, e, g) and TMC-8f (d, f, h).

Figures

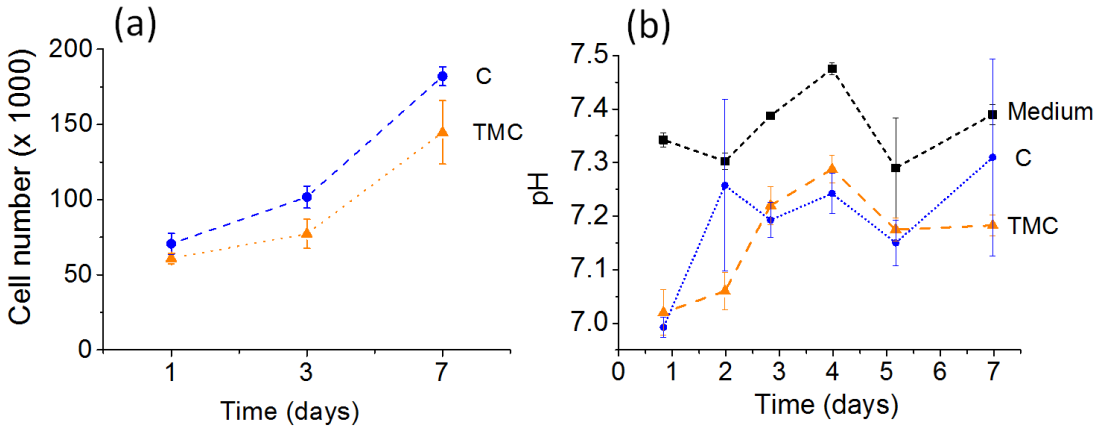


**Figure 5**. a) Number of cells adhered on the samples surface at different time points, and b) evolution with time of the pH of the cell culture medium (1 ml) in contact with a cement disc (15 mm diameter x 1.5 mm thickness).

Figures

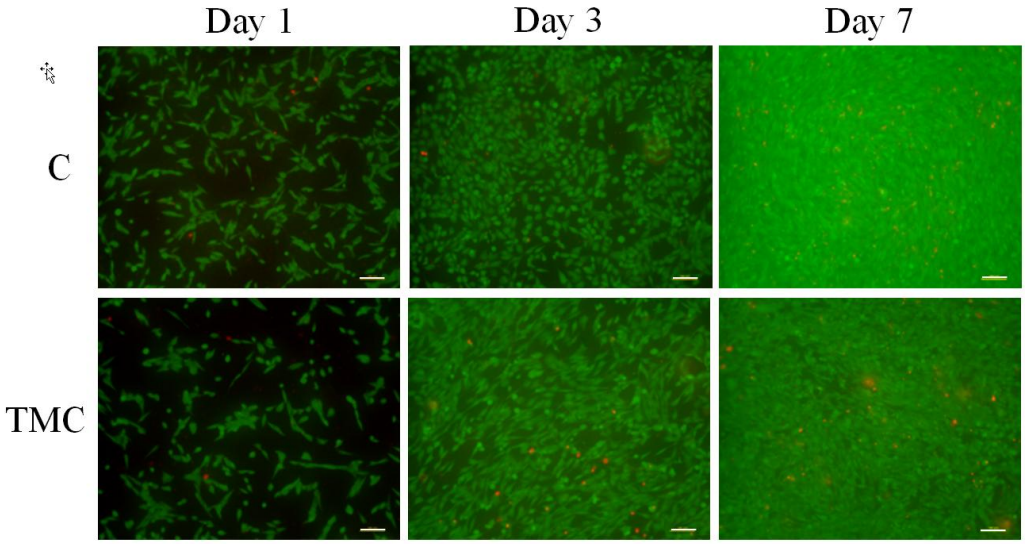


**Figure 6.** Live/dead images showing the alive cells (green) and the dead cells (red) on the C and TMC samples surface at different time points (bar = 100 μm)

Figures

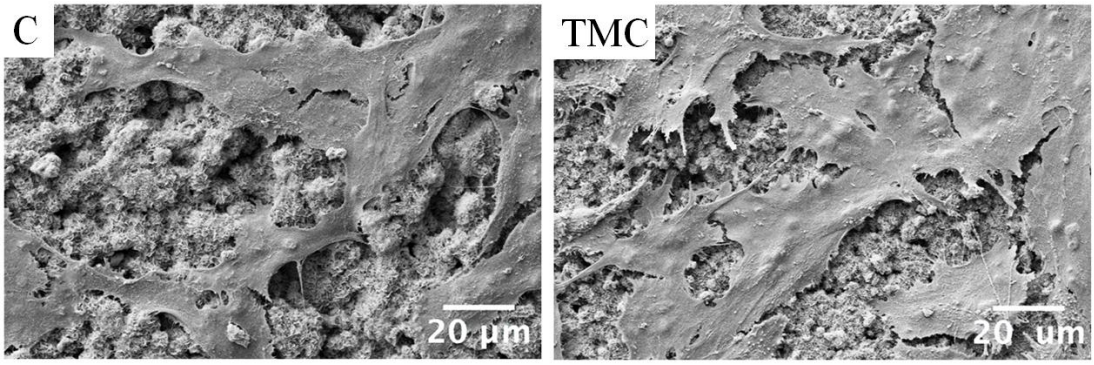


**Figure 7.** SEM images showing the morphology of cells cultured on C and TMC samples for 7 days.

# Tables

**Table 1**. Nomenclature used for the samples according to the liquid phase used and the presence of reinforcing fibers.

| Sample name | Liquid phase | Amount of reinforcing fibers (wt %) | Amount of reinforcing fibers (v/v %) |
|---|---|---|---|
| C | Water | 0 | 0 |
| TMC | 1 w/v % TMC | 0 | 0 |
| C-8f | Water | 8 | 10 |
| TMC-4f | 1 w/v % TMC | 4 | 5 |
| TMC-8f | 1 w/v % TMC | 8 | 10 |
| TMC-12f | 1 w/v % TMC | 12 | 14.5 |

**Table 2**. Setting time and compressive strength of CPCs using as liquid phase pure water (C) or 1% w/v TMC in water (TMC). Different letters indicate statistically significant differences ($p < 0.05$) between C and TMC for each group (initial setting time, final setting time and compressive strength).

| **Sample** | **Setting time (min)** | | **Compressive strength (MPa)** |
|---|---|---|---|
| | **Initial** | **Final** | |
| C | 15 ± 1 (a) | 49 ± 5 (a) | 31.3 ± 9.0 (a) |
| TMC | 20 ± 2 (b) | 68 ±19 (b) | 37.1 ± 4.9 (b) |

**Table 3.** Crystalline phases as determined by X-ray diffraction, and specific surface area measured by $N_2$ adsorption of the initial powder and set cements.

| | | **Phase (%)** | | | |
|---|---|---|---|---|---|
| | Material | **% α-TCP** | **% β-TCP** | **% HA** | **SSA ($m^2$/g)** |
| **Initial powder** | α-TCP | 97.0 | 3.0 | 0.0 | 0.975 |
| **Set cements** | C | 0.0 | 3.7 | 96.3 | 13.6 |
| | TMC | 6.2 | 1.8 | 92.0 | 10.5 |