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# Chemical–Physical Characterization and *in vitro* Preliminary Biological Assessment of Hyaluronic Acid Benzyl Ester–Hydroxyapatite Composite

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**ABSTRACT:** HYAFF®11 is a biocompatible, biodegradable benzyl ester of hyaluronic acid. However, in order to use it for orthopedic application, its mechanical performance needs to be improved. In this study, a novel composite based on HYAFF®11 polymer matrix reinforced with hydroxylapatite (HA) has been developed. Its advantage is having a similar component of the mineral phase of bone resulting in favorable osteoconductive properties. The present study has examined the compressive mechanical and surface chemical-physical properties of the novel HYAFF®11-HA composite. Preliminary biological investigations, including pH and cytotoxicity studies of the material extracts, have also been performed using an *in vitro* primary human osteoblast-like cell model. Moreover, protein, especially fibronectin adsorption has been investigated following incubation in culture medium and human plasma. The results show a grainy surface topography composed mainly of C, P, and Ca, with a Ca/P atomic ratio indicating HA on the composite surface. Mechanical analysis shows an improvement of the compressive properties of HYAFF11 matrix, both in the dry and swollen states, with values in the range of that of spongy bone. No cytotoxic effects and no inhibition of cell proliferation have been observed in the presence of the material extracts with pH values within acceptable ranges for cell vitality. Protein studies reveal a similar pattern, but a higher amount of fibronectin following incubation in human plasma when compared with culture medium. The results show that the novel HYAFF®11-HA composite shows a great potential for application in orthopedic fields, especially as vertebral trabecular bone substitute.

**KEY WORDS:** hydrogel, composite, surface analysis, stress analysis, cell response, protein adsorption, biocompatibility, osteoblast, *in vitro* tests, cell culture.

## INTRODUCTION

**M**usculoskeletal conditions are becoming an increasing health concern in aging population. Common treatments in fracture repair and total joint replacement still use traditional methods, such as autograft and allograft for bone repair, both of which have limitations.

The most commonly used biomaterials for orthopedic applications are natural and modified natural materials, as well as metals, ceramics, and synthetic polymers. In order to achieve specific properties not attainable by single-phase materials, it is now possible to design composites combining polymers, ceramics, and metals in order to obtain tailor-made devices for applications in various biomedical fields. Synthetic polymeric composites are very attractive biomaterials because of their similarities with most of the structural living tissue, composed of macromolecular composites.

Owing to their biodegradability, high biocompatibility, and nontoxicity, many natural polysaccharides have more recently been used for biomedical applications. Particular attention has been given to the

largest glycosaminoglycans (GAG), hyaluronic acid or hyaluronan, a linear anionic polysaccharide composed of repeating disaccharide units of glucuronic acid and *N*-acetyl-glucosamine with the following molecular structure:  $-\text{[D-glucuronic acid (1-}\beta\text{-3) } N\text{-acetyl-D-glucosamine (1-}\beta\text{-4)]}_n\text{- [1]}$ . Hyaluronic acid is heterogeneously distributed in various tissues, where it plays diverse roles with regard to structural, rheological, physiological, and biological functions in the body. Physical and biological characteristics of hyaluronan in its purified form, such as water solubility, rapid resorption rate, and short half-life in the tissues, limit its possible biomedical applications. As a result, several attempts have been made to modify the molecular structure of high molecular weight purified hyaluronan in order to obtain a more stable solid material, preserving its biocompatibility and noninflammatory properties [2].

HYAFF® is a class of hyaluronan derivative polymer obtained by esterifying the free carboxyl group of glucuronic acid, with different types of alcohol (aliphatic, arylphatic, cycloaliphatic, and others) in order to reduce the hydrophilic, negatively charged, carboxyl groups of the polyanionic polysaccharide, thereby increasing its hydrophobic components. This results in a decrease in solubility and a slight increase in structural rigidity of the macromolecule, caused by the interaction of hydrophobic group organized in hydrophobic patches. The benzyl ester of hyaluronan, termed HYAFF®11, is a well-characterized HYAFF® polymer from both the chemical-physical and biological point of view [3]. The structure of HYAFF®11 is characterized by the presence of numerous hydrophobic benzyl groups along the polymeric chain, probably organized in hydrophobic patches. This gives the molecule a more rigid and less mobile conformation, reducing its ability to interact freely with water.

In order to improve the mechanical performance of HYAFF®11, a novel composite adopting calcium hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , (HA) as reinforcement was designed. It is highly biocompatible and shows good bioaffinity by stimulating osteoconduction along its surface and being slowly replaced by host bone post implantation [4–6].

The aim of this study is to develop and characterize this novel composite based on HYAFF®11-HA 14/86 (w/w). Mechanical performance of the material with compressive tests in dry and wet conditions was evaluated. Surface chemical-physical properties, especially topography and stoichiometry composition, were also analyzed.

A preliminary biological examination was also performed to analyze the pH values and the cytotoxicity of the composite extracts after long-term incubation in culture medium. In addition, protein, especially

fibronectin adsorption were investigated following incubations in culture medium containing fetal calf serum (FCS) and in human plasma, in order to simulate an *in vivo* environment.

## MATERIALS AND METHODS

### Materials

The composites used in this study were obtained using the fully benzylated ester of hyaluronic acid (HYAFF<sup>®</sup>11, HATBA, batch 0.100.00, average Mw: 200,000 Da; Fidia Advanced Biopolymers (FAB), Albano Terme, Italy) reinforced with 86% (w/w) ratio of HA (batch N1904, particle diameter: 2–10  $\mu\text{m}$ ; P205 grade from IRC in Biomedical Material-QMW, London, UK).

A solution of 8% (w/w) HYAFF<sup>®</sup>11 in dimethylsulfoxide (DMSO, Sigma-Aldrich) was prepared under a slow magnetic stirrer at room temperature ( $25 \pm 2^\circ\text{C}$ ) in a glass container. The homogeneous mixture was mixed at room temperature with HA in order to obtain the final composition of HYAFF<sup>®</sup>11–HA 14/86 (w/w) composite. The composite paste was placed in the selected shapes (discs, 14 mm diameter and 3 mm high, except for mechanical tests) and then washed with ethyl alcohol (95%, Ashland Chemical) for 7 days, three changes a day. The samples were washed with bidistilled H<sub>2</sub>O (Carlo Erba) for 7 days, three changes a day, and then dried under air for 3 days.

HYAFF<sup>®</sup>11 alone was used as a control for compressive properties and protein analysis, and was prepared as described here.

### Mechanical Characterization

All the tests were performed at least in triplicate.

#### *Compressive Properties*

Cylindrical specimens (6 mm diameter and 12 mm length) of HYAFF<sup>®</sup>11 and HYAFF<sup>®</sup>11–HA composite were tested (INSTRON testing machine model 4204; crosshead speed of 1 mm/min) according to ASTM D 695 in dry and swollen conditions.

### Surface Chemical–Physical Characterization

#### *Surface Topography*

The samples were sputter coated (Emitech 500, 4 min at 20 mA,  $10^{-1}$  mBar) before examination under a Jeol JSM 5500 LV at an accelerating voltage of 15 keV.

### *Surface Chemistry*

The samples were sputter coated (Emitech K550) and then analyzed by energy dispersive atomic X-ray (Philips XL 30; LaB<sub>6</sub> filament; EDAX CDU leap detector) for elemental analysis.

### ***In vitro Biological Assays***

Thermanox (TMX) discs (Life Technology) as negative, nontoxic control and polyvinylchloride (PVC) discs (Life Technology) as positive, toxic control were the controls used.

All the tests were performed at least in triplicate.

### *Elution pH Measures*

The samples were placed into a 24-well tissue culture plate and incubated in 1 mL of culture medium at 37°C on a plate shaker. At the selected time points (1, 3, 7, 14, 21, 28, 35, and 42 days) the aqueous extracts were collected and replaced with 1 mL of fresh medium and the plate placed back at 37°C. The pH of the eluted medium was measured using a pH meter (Corning 240) in triplicates.

### *Elution Cytotoxicity Assays*

Primary human osteoblast-like cells (HOBs) were isolated from trabecular bone using a previously described method [7]. Briefly, the bone chips were cultured under sterile conditions until 'osteoid seams' were seen, then digested using collagenase (100 U/mL) (Sigma) and trypsin (300 U/mL) (Sigma) solution. Following isolation, expansion, and characterization, the obtained cells were used for the cytotoxicity tests.

The sterile samples were placed into a 24-well tissue culture plate incubated in 1 mL of culture medium at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. At the selected time points (1, 3, 7, 14, 21, 28 days) the aqueous extracts were collected in sterile condition, replaced with 1 mL of fresh medium and the plate was placed back in the incubator. One hundred microliters of  $1 \times 10^5$  cell/mL cell suspension were seeded into a sterile 96-well culture plate (Beckton Dickinson) and incubated until almost confluent, usually 24 h. The culture medium was then replaced with aqueous extract (100 µL/well) and the plates were further incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere for 72 and 48 h. Ten microliters of MTT (Sigma) solution (5 mg/mL in phenol- and ascorbic acid-free culture medium containing FCS) was added to each well, the plates were incubated for a further 4 h and then the medium was removed by inversion. One hundred microliters of DMSO (Merck)

was added to each well and mixed for 20 min until the crystals were completely dissolved. Absorbance was measured by a Dynatech MR 700 plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

### *Protein Adsorption Studies*

The sterile samples were placed in a 24-well plate, washed in PBS (Oxoid Ltd) and then equilibrated with 1 mL of FCS-free culture medium for 30 min. The samples were then incubated either in a culture medium containing 10% FCS or in human plasma obtained from healthy human donor blood in static conditions at 37°C for 1 h. The specimens were removed, washed carefully in 1 mL of PBS for 5 min at room temperature, placed in a new 24-well plate and proteins were eluted for 1 h at 37°C by 200  $\mu$ L of SDS-PAGE sample buffer: (2% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 10% (w/v) glycerol, 0.02% (w/v) bromophenol blue in 62.5 mM tris-HCl, pH 6.8). Supernatants were withdrawn and boiled for 3 min. After cooling, 20  $\mu$ L of each elutions were analyzed by a 10% SDS-PAGE electrophoresis using a Bio-Rad Miniprotean II Module. Electrophoresis was performed at 100 V, 46 mA for 90 min according to Laemmli's method [8]. As controls, 1 : 10 diluted complete DMEM and 1 : 10 diluted human plasma were also used. Protein molecular weight was evaluated by loading SDS-PAGE standard (Bio-Rad, Mw range 205,000–7600 Da: cat. 161-0318). Polyacrylamide gels were stained for 1 h using a Silver Stain Plus Kit (Bio-Rad). Following electrophoresis, proteins were blotted onto nitrocellulose Hybond membranes (Amersham) using a Mini Trans Blot Module (Bio-Rad). Membranes were blocked with 1% (w/v) gelatin in PBS and incubated with 50  $\mu$ g/mL primary antibody solution containing rabbit antibodies (Dako) against either human or bovine fibronectin (Fbn) overnight at room temperature. Goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Dako), diluted 1 : 2000 in the same buffer, were used as secondary antibody. Bands were detected using 0.03% (w/v) 3-amino-9-ethylcarbazole and 0.03% (w/v) H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) in 0.1 M sodium acetate (Sigma-Aldrich), pH 5.2, as substrates.

## RESULTS

### **Mechanical Characterization**

#### *Compressive Tests*

The compressive elastic modulus ( $E$ ), the maximum compressive strength ( $\sigma_{\max}$ ) and the maximum deformation ( $\varepsilon_{\max}$ ) were evaluated

for HYAFF<sup>®</sup>11–HA composite and HYAFF<sup>®</sup>11 samples in dry and swollen states (Table 1, Figure 1(a) and (b)).

The results showed an increase in the mechanical properties for the composites due to the reinforcing HA phase. For the HYAFF<sup>®</sup>11, the fracture was not detectable for the plastic behavior of the materials. The same behaviors were obtained in the swollen state. In the latter case, a decrease in the compressive properties of both HYAFF<sup>®</sup>11–HA and HYAFF11 HYAFF<sup>®</sup>11 samples were observed, mainly due to the plasticizing effect of the absorbed water. The water content at the equilibrium state of HYAFF<sup>®</sup>11 has been reported as  $32 \pm 2\%$  [9] while for the HYAFF<sup>®</sup>11–HA composite it was,  $5 \pm 1\%$  due to the presence of HA.

### Surface Topography

The samples showed a rough surface consisting of spherulites  $< 10 \mu\text{m}$  in diameter (Figure 2).

### Surface Chemistry

The EDAX analysis of the sample surfaces showed the following elements – Ca, P, C, Na, and Si. The Ca/P atomic ratio value of 1.75, obtained for the composite, was close to both the typical value of stoichiometric HA and the value obtained for the HA used for the test composite preparation. No traces of S were detected (Figure 3).

### pH Determination of the Eluted Media

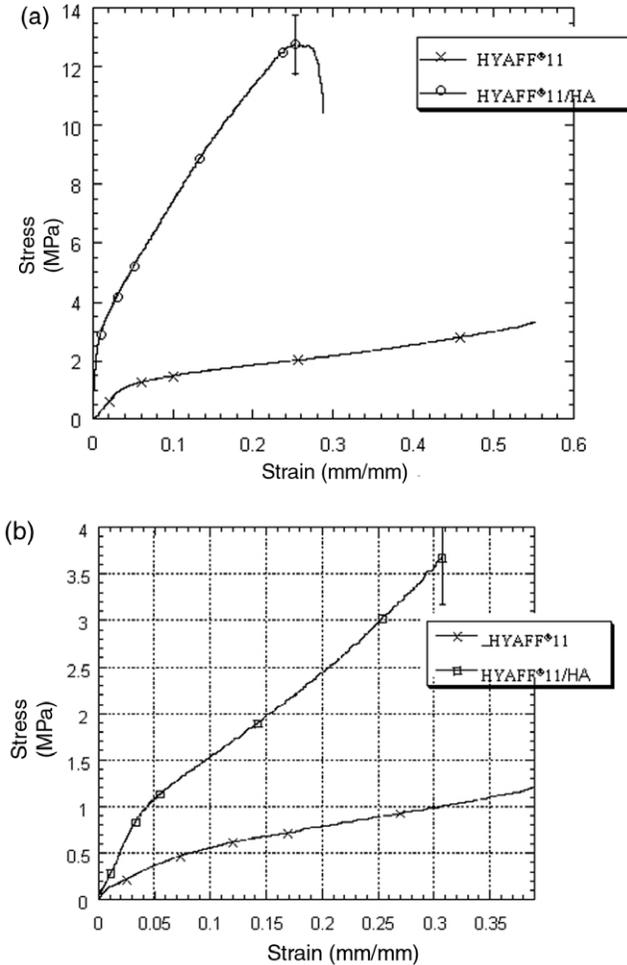
The results obtained showed no significant differences in the pH values of the elution collected, remaining approximately in the range of 7–8 from day 1 to day 42 of sample incubation in the culture medium.

### Elution Cytotoxicity Assay

No evidence of cytotoxicity was observed after 48 h (Figure 4(a)) and 72 h (Figure 4(b)) of incubation with HOB cells in the presence of

Table 1. Compressive properties of HYAFF<sup>®</sup>11–HA and HYAFF<sup>®</sup>11 in dry and swollen states.

	$E$ (MPa)	$\sigma_c$ (MPa)	$\epsilon$ (mm/mm)
Material (Dry)			
HYAFF <sup>®</sup> 11	$27 \pm 5$	–	–
HYAFF <sup>®</sup> 11–HA <sup>(14/86)</sup>	$340 \pm 40$	$12 \pm 1$	$0.24 \pm 0.01$
Material (Swollen)			
HYAFF <sup>®</sup> 11	$10 \pm 2$	–	–
HYAFF <sup>®</sup> 11–HA <sup>(14/86)</sup>	$70 \pm 4$	$4 \pm 1$	$0.31 \pm 0.01$



**Figure 1.** Compressive stress–strain curve of HYAFF®11–HA and HYAFF®11, in (a) dry and (b) swollen states.

sample extracts at the selected time points, as shown by the comparison with the TMX and PVC control extracts.

### *Protein Adhesion*

A similar protein adsorption profile was observed following incubation of the materials in culture medium containing FCS (Figure 5(a)) and human plasma (Figure 5(b)), respectively. The SDS-PAGE for the HYAFF®11–HA specimen and the HYAFF®11 samples, incubated in human plasma showed a band with molecular weight around

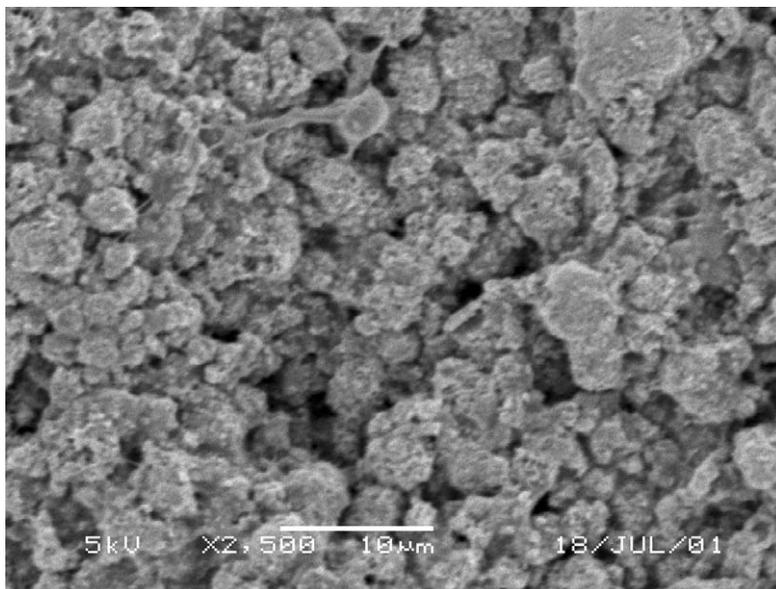


Figure 2. Scanning electron micrograph of HYAFF®11-HA composite sample surface.

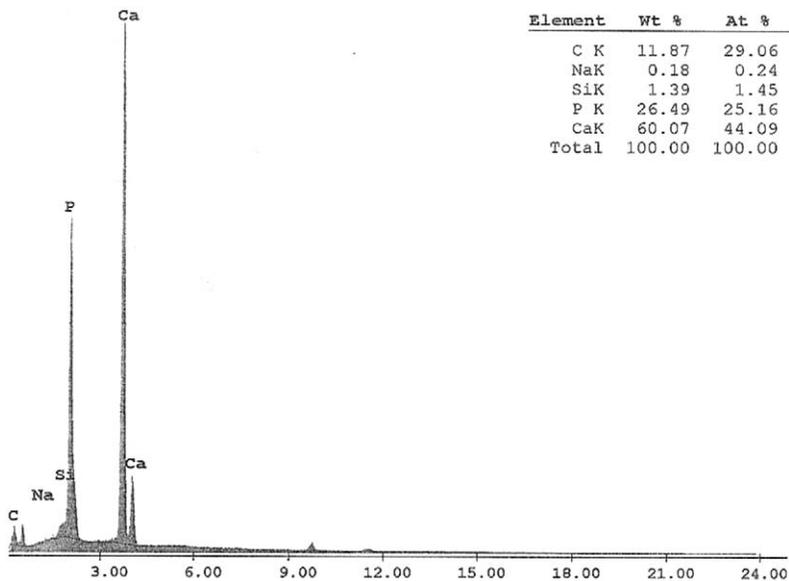
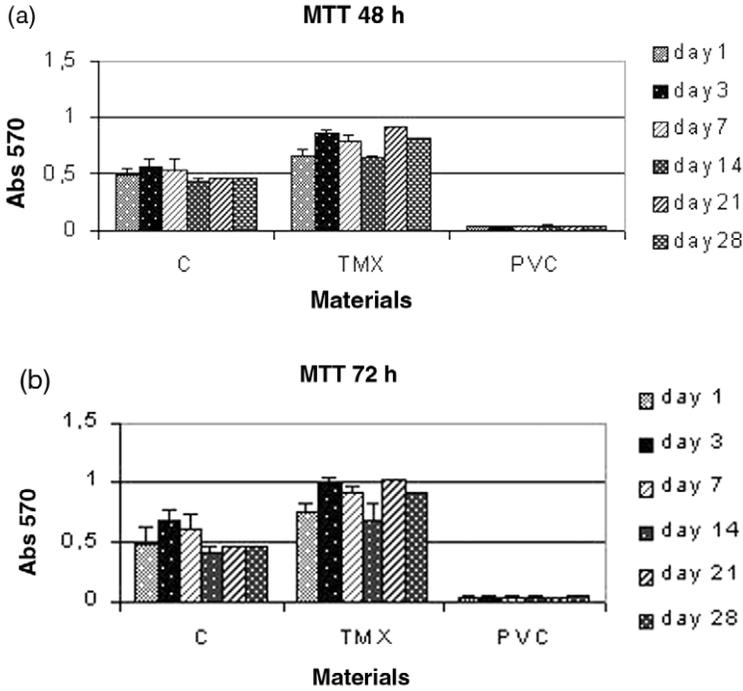


Figure 3. EDAX analysis of HYAFF®11-HA composite sample surface.



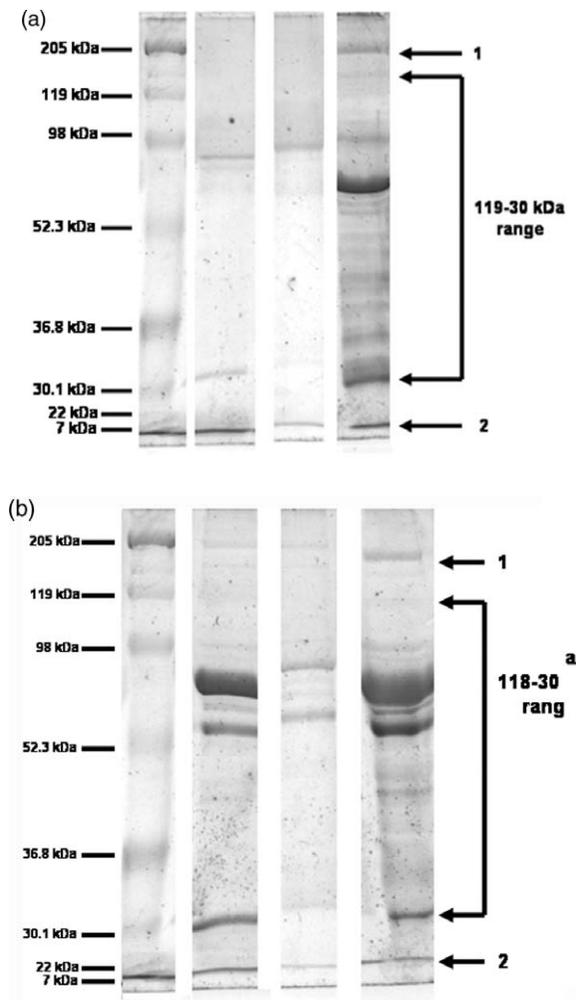
**Figure 4.** MTT test on HOB cells cultured (a) for 48 and (b) 72h in culture medium previously incubated for 1, 3, 7, 14, 21, and 28 days with HYAFF<sup>®</sup>11-HA composite samples (C).

205 kDa (arrow 1) as well as protein adsorption in the range of 119–30 kDa (indicated range) and around 7.6 kDa (arrow 2). With the exception of the 205 kDa band, protein adsorption was visible in the above-mentioned ranges also for materials incubated in culture medium containing FCS.

From the immunoblot analysis, fibronectin was undetectable for all the materials incubated in the culture medium containing FCS, but was clearly visible for samples incubated in human plasma (Figure 6).

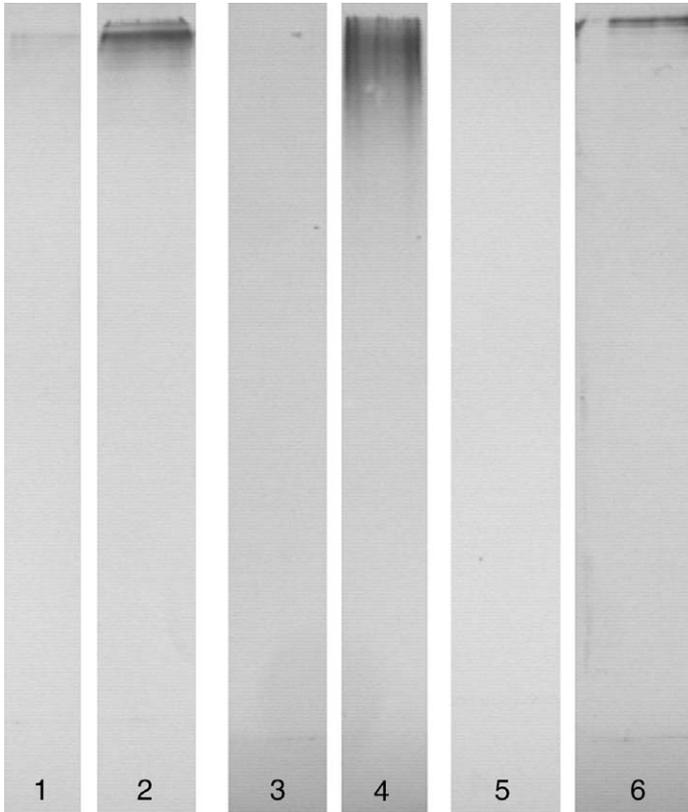
## DISCUSSION

In the present study, mechanical and surface chemical-physical characterization as well as a preliminary biological assessment were performed on a novel HA-reinforced hydrophilic polymer, based on HYAFF<sup>®</sup>11 polymer matrix, HYAFF<sup>®</sup>11-HA 14/86 (w/w).



**Figure 5.** SDS-PAGE of samples incubated (a) in culture medium and (b) human plasma: lane 1: molecular weight standard; lane 2: HYAFF<sup>®</sup>11-HA composite; lane 3: HYAFF<sup>®</sup>11; lane 4: relative incubation medium. Arrows and arrow range indicate protein with a different adsorption profile on the tested surfaces (for details see text).

Appropriate mechanical properties are crucial requirements in the design of bone-substitute materials. Both HYAFF<sup>®</sup>11 and HA are known to be bioactive, but when used alone their mechanical properties are not sufficient and hence, limit their use. Improvements in composite technology have resulted in the design of substitute materials



**Figure 6.** Immunoblot of samples incubated in culture medium (lane 1: HYAFF<sup>®</sup>11–HA composite; lane 3: HYAFF<sup>®</sup>11; lane 5: culture medium) and human plasma (lane 2: HYAFF<sup>®</sup>11–HA composite; lane 4: HYAFF<sup>®</sup>11; line 6: human plasma).

with enhanced properties. The compressive tests have shown that it is possible to have a composite structure with compressive properties in the range of that of spongy bone. As presented in Table 1, the composite shows compressive properties in the range of vertebral trabecular bone [10]. The higher reduction of the HYAFF<sup>®</sup>11–HA compressive properties in the swollen state is due to the poor adhesion between HA and HYAFF<sup>®</sup>11, which is enhanced by the presence of water, and the presence of voids (Figure 2) generated during the preparation.

Surface topography affects important parameters involved in the cell–material interaction. Topography influences cell anchorage, orientation, alignment, and migration by recognizing surface features and

reacting to them [11,12]. Rugophilia has also been described to play a role, whereby a roughened surface increases cell, especially, osteoblast response to materials [13,14].

The HYAFF®11-HA composite structure showed a grainy surface with exposed HA particles, thus indicating a potentially favorable surface topography for cellular adhesion and proliferation.

In addition to topography, surface stoichiometry is another important requirement for biomaterials to promote cellular adhesion. It has been reported that exposing HA increases the osteoconductive properties with material surfaces directly in contact with cells, creating a favorable surface for enhancing osteoblast adhesion [15-21].

Surface stoichiometry analysis on the test samples showed the presence of the elements C, P, and Ca, the expected elements in the material composition. The detected traces of Na and Si might be due to impurities related to solvents/disposables used during the specimen preparation process or to the sample holders used before/during the EDAX analysis. The absence of S in HYAFF®11-HA composite samples indicated that they were effectively purified from the toxic DMSO used during the composite sample preparation.

The results relating to the Ca/P atomic ratio of the composite sample surface indicated the presence of HA on the surface, suggesting a composite surface microenvironment suitable for HOB cell attachment. EDAX allows the analysis of a sample surface layer that can have a maximum thickness of a few nanometers, so the described results show that the preparation method adopted, phase inversion and casting, and also the high concentration of HA used allow HA particles to be present not only in the bulk but also on the composite surface. It should be noted, however, that the values obtained from the EDAX analysis were semiquantitative. This was a result of the analysis being performed directly on the 'as-cast' sample surface, which was irregular and during the analysis, each point was not equidistant from the EDAX microscope probe. The sample surfaces were not previously treated for smoothing, thus presenting a natural surface similar to that exposed to cells once implanted *in vivo*.

Cellular response is known to be affected by the release of leached products as a result of the degradation process stimulated upon incubation in an aggressive environment, such as that in the human body. It has been documented in the literature that pH changes of the surrounding environment affect cellular activity [22,23].

The pH measurements of the supernatant collected for the composite for all the selected time points showed pH values ranging from 7 to 8. It has been reported in literature that alkaline environment with

pH values above 9 can affect cell activity [23]. It can be concluded that the reactivity of the material with the surrounding fluids was able to create a favorable microenvironment for cell viability.

Furthermore, from the cytotoxicity tests performed, there was no indication of any considerable deterioration in cell viability as assessed with the long-term eluted extracts. The results showed that there was no release of toxic leachables from the composite eluted over the selected period of time.

In order to analyze the protein pattern on the sample surface more accurately, SDS-PAGE was performed on specimens incubated both in culture medium containing FCS and, to better simulate an *in vivo* environment, in human plasma. The presence of fibronectin, adhesion protein signalling for HOB cells, was analyzed. Fibronectin, once adsorbed onto a material surface, has been shown to mediate and increase cell adhesion and spreading [12].

The SDS-PAGE results showed a similar pattern of protein adsorption for the test HYAFF<sup>®</sup>11-HA composite and the HYAFF<sup>®</sup>11 when incubated in culture medium. These observations imply that the cellular response of the composite should be similar to that of the HYAFF<sup>®</sup>11 control. Although the adsorption of proteins from medium supplemented with FCS was, as expected, lower than the clinically reflective incubation in plasma, the pattern of adsorption was not significantly different, suggesting a similar behavior of the composite when placed in an *in vivo* environment. This hypothesis is correlated by the immunoblotting of fibronectin. Fibronectin was not detected for the samples incubated in the culture medium containing FCS and for the culture medium itself, probably as a result of the dilution of FCS concentration in the culture medium to a level undetectable for Western Blot. It was however, clearly visible in those specimens incubated in human plasma.

## CONCLUSIONS

Mechanical analysis showed that the presence of the HA improved the compressive properties of the HYAFF<sup>®</sup>11 matrix both in the dry and the wet states, with values in the range of that of spongy bone.

HYAFF<sup>®</sup>11-HA 14/86 (w/w) composite sample surface analysis showed a grainy topography, the presence of C, P, and Ca with small traces of Na and Si impurities, and a Ca/P atomic ratio close to the typical value of stoichiometric HA and to the value obtained for the HA used in sample preparation.

The *in vitro* cytotoxicity tests showed no evidence of toxic leachables from the composites and acceptable pH values for cell survival and proliferation.

Protein adhesion analysis showed a similar protein pattern in culture medium with FCS and human plasma; with a higher amount of fibronectin detected in the latter.

In conclusion, these results indicate that the novel HYAFF®11–HA composite has potentials for a wider range of application in the field of orthopedics, especially as vertebral trabecular bone substitute.

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