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MINERALIZATION OF REGENERATED CELLULOSE HYDROGELS INDUCED BY HUMAN BONE MARROW STROMAL CELLS

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Abstract

The proliferation of cultured human bone marrow stromal cells (HBMSC) on regenerated cellulose hydrogels was assessed. Regenerated cellulose hydrogels showed good rates of HBMSC proliferation, the cells exhibiting a flattened morphology, and after 22 days in culture, the cells had homogeneously colonized the surface of the materials. Moreover, since the early days in culture, between the surface of the materials and attached cells a continuous granulated hydroxyapatite layer was formed. It has been previously demonstrated *in vitro,* but without cells, that these materials did not mineralize. Hence, it seems that HBMSC promoted the mineralization of the surface.

Key Words: Regenerated cellulose hydrogels, human bone marrow stromal cells, cell proliferation, mineralization, calcium phosphates, hydroxyapatite.

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Introduction

The understanding of the interaction between bone cells and polymer surfaces is of paramount importance in the development of bone substitute biomaterials. A close apposition between bone and biomaterial, or osseointegration, is considered to be one of the major factors leading to a successful skeletal implantation (Hosseini *et al.*, 2000; Albrektsson, 1998; Friedman *et al.*, 1993). It is widely accepted that the attachment of osteoblast-like cells to material surfaces may lead to improved bone integration and hence longer-term stability of load-bearing implants (Hosseini *et al.*, 2000; Albrektsson, 1998; Friedman *et al.*, 1993; Puleo and Nanci, 1999). Recent advances from tissue-engineered orthopaedic biomaterials confirm that adequate substrates for bone cell growth and differentiation constitute adequate scaffolds to prepare functional hybrid biomaterial/new bone 3D structures (Yaszemski *et al.*, 1996; von Recum *et al.*, 1999; Patrick *et al.*, 1998; Burgess and Hollinger, 1998).

Osteoblasts are anchorage-dependent cells and their attachment to synthetic surfaces is a complex process involving cell attachment and spreading, focal adhesion formation, and extracellular matrix formation and reorganization (Lamba *et al.*, 1998). Materials surface properties influence the composition of the adsorbed protein compounds, which in turn regulates how cells respond to the material (Lamba *et al.*, 1998; Horbett, 2004; McFarland *et al.*, 1999; Healy *et al.*, 1996). Understanding the relationship between material surface properties, adsorbed molecules and cellular responses is essential to designing optimal material surfaces for implantation and tissue engineering.

Cellulose is the world's most abundant natural, renewable and biodegradable polymer. Similarly to other polysaccharides, cellulose has a long background in medical applications, essentially due to its lack of toxicity (monomer residues are part of metabolites found in the human body), water solubility or high swelling ability, and stability to temperature and pH variations (Hon, 1996; Franz, 1986). The biocompatibility of unmodified and derivatised cellulose is well documented (Hon, 1996; Franz, 1986; Martson *et al.*, 1998; Chauveaux *et al.*, 1990; Miyamoto *et al.*, 1989; Ikada, 1989). Oxidized cellulose has been used for decades as a wound dressing and for osseous regeneration (Dias *et al.*, 2003; Uysal *et al.*, 2003; Galgut, 1990; Skoog, 1967; Degenshein *et al.*, 1963), and regenerated cellulose sponges have shown to promote

bone regeneration (Martson *et al.*, 1998, 1999). Regenerated cellulose hydrogels (cellulose regenerated by the viscose process, CRV®) have been investigated as implantable materials in orthopaedic surgery as an attachment material for the femoral component in hip prostheses, in place of the acrylic cement (Gross *et al.*, 1992; Chauveaux *et al.*, 1990; Poustis *et al.*, 1994; Pommier *et al.*, 1990). It was envisaged to take advantage not only of its good matching with mechanical properties of cortical bone, but also of its hydroexpansivity, therefore allowing a satisfactory primary fixation to hard tissue. The biocompatibility and osteoconductive properties of these regenerated cellulose hydrogels have been demonstrated *in vivo* (Gross *et al.*, 1992; Chauveaux *et al.*, 1990; Pommier *et al.*, 1990).

In the context of a program aimed at investigating the biological behaviour of modified cellulose, the *in vitro* biocompatibility of regenerated cellulose hydrogels was evaluated with cultured HBMSC, in terms of cytotoxicity and cell attachment. It was demonstrated that these materials are cytocompatible and promote attachment of HBMSC to a good extent (Granja *et al.*, submitted).

In the present work, the mineralization accompanying the proliferation of HBMSC on regenerated cellulose hydrogels was investigated.

Materials and Methods

Regenerated cellulose hydrogels (cellulose regenerated by the viscose process, CRV®) were a generous gift from Hexabio (Bordeaux, France), and were obtained by a previously described method (Pommier *et al.*, 1990). CRV discs (10 x 2 mm) used in cell cultures were machined from blocks dried in air at room temperature. The discs were dialyzed against distilled water for 24 hours before use, in order to remove contamination resulting from machining. All chemicals were of research grade purity, and used without further purification.

Sterilization. After packing, samples were γ-irradiated for 8 h and 30 min at room temperature in the absence of oxygen, in a CIS Bio-Industries (Gif sur Yvette, France) equipment, model IBL 337, loaded with 13000 curies of ^{137}Cs, in order to absorb a total dose equal to 25 kGy.

Cell cultures. Osteoblast-like cells were isolated from human bone marrow stromal cells (HBMSC), according to Vilamitjana-Amédée *et al.* (1993), with some modifications. Briefly, human bone marrow was obtained by aspiration from the iliac crest of healthy donors (aged 20-50 years) undergoing hip prosthesis surgery. Cells were pooled and separated into a single suspension by sequentially passing the suspension through syringes fitted with 16, 18 or 21 gauge needles. After centrifugation for 15 min at 800g, the pellet was resuspended in Iscove Modified Dulbecco's Medium (IMDM, from Gibco, InVitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) fetal calf serum (FCS, from Gibco) and 10^{-8} M dexamethasone (Sigma, St. Louis, MO, U.S.A.). Cells were then plated into 75 cm^2 cell culture flasks (Nalge Nunc, Rochester, NY, U.S.A.), at a density of $5x10^5$ cells/cm^2, and incubated in a humidified atmosphere of 95% air 5% CO_2 at 37ºC. Three days later, the medium was removed, replaced 2 times with the complete medium supplemented with 10^{-8} M dexamethasone, and then every 3 days with IMDM containing 10% FCS (v/v). Subculturing was performed using 0.2% (w/v) trypsin and 5 mM ethylenediaminetetraacetic acid (EDTA). Cell differentiation was followed by the measurement of alkaline phosphatase activity and osteocalcin synthesis, as described previously (Vilamitjana-Amédée *et al.*, 1993). All cell assays described were performed using at least 6 replicates for every condition tested.

Cell proliferation. Cell proliferation assays were performed as described by Mosmann (1983). Cell attachment and proliferation on tissue culture polystyrene (TCPS) of plastic culture dishes was used as the positive control. In order to prevent cell attachment to plastic dishes, an agarose layer (2% (v/v) in 0.1 M phosphate buffered saline (PBS at pH 7.4) was poured into 24-well plates, and constituted the negative control (Amédée *et al.*, 1994). Cellulose discs were then placed onto this agarose layer and incubated overnight, at 37ºC, in IMDM. Thereafter, discs were seeded with a density of 5000 cells per cm^2 in 10% (v/v) FCS in IMDM. Cultures were incubated at 37ºC in a humidified atmosphere. The culture medium was replenished every 3 days. Cell growth was quantified at 1, 3, 8, 15 and 22 days, by measuring the cell metabolic activity by the MTT assay (based on the observation that a mitochondrial enzyme of viable cells has the ability to metabolize a water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into an insoluble formazan salt). Briefly, 3 h after incubation of MTT at 37ºC, the MTT solution was removed, the insoluble formazan crystals formed were dissolved in dimethylsulfoxide and 100 µL were aspirated and then poured into another 96-well plate, for absorbance measurement at 540 nm. The intensity of the staining obtained is directly proportional to cell proliferation (AFNOR, 1994; ISO, 1992). Agarose was used as the negative control. Results are expressed as percentage of cell growth on TCPS, which is defined as 100%.

Light microscopy. Observations of unfixed cells were carried out on cell culture plates, using an Olympus IM inverted microscope.

Cell morphology and surface characterization. Scanning electron microscopy (SEM) was carried out in order to study cell morphology on the culture discs. Samples were fixed by 15 min immersion in a 2% (v/v) glutaraldehyde in 0.15 M cacodylate buffer (pH 7.3). Samples were washed with 0.15M cacodylate buffer for 10 min, then dehydrated by successive immersions in ethanol solutions (from 25 to 100%), then finally dried by the CO_2 critical point technique, and Au-sputtered before observation. SEM analyses were carried out at 15 and 20 keV for 60 s, using a Hitachi (Tokyo, Japan) S-2500 scanning electron microscope. Mineralized areas were analysed using an energy dispersive x-ray analysis system (Noran Voyager, Thermo Electron Corp., Philadelphia. PA, USA).

The mineralized surfaces of the samples were further characterized by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy with a

Perkin Elmer (Wellesley, MA, U.S.A.) 2000 FT-IR Spectrometer, using the Split Pea accessory (Harrick Scientific Corporation, Pleasantville, NY, U.S.A), equipped with a silicon hemispherical crystal. All samples were run at a spectral resolution of 4 cm^{-1}.

Statistical verification of results. The results were statistically analyzed by one-way ANOVA using SPSS (Chicago, IL, U.S.A.) 10.0.01. The level of significance was set at $p < 0.05$.

Results

As can be observed in Fig. 1, HBMSC proliferate at a good rate over regenerated cellulose hydrogel discs, achieving proliferation rates of 72.9% ± 13.7 after 15 days in culture and 76.7% ± 24.7, after 22 days, as expressed as percentage of the proliferation observed in the TCPS controls. Differences found to cell proliferation over agarose at these time points were statistically significant.

SEM observations (Fig. 2) clearly demonstrate these findings. Cells adapted very closely to the surface and

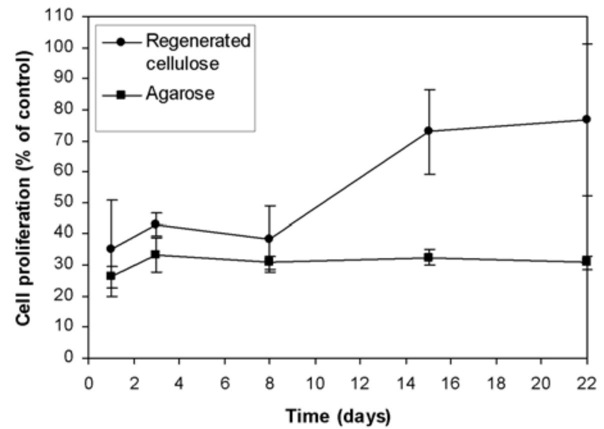

Figure 1. Proliferation rates of human bone marrow stromal cells on regenerated cellulose hydrogels, compared to the negative controls over agarose. Data are expressed as % of proliferation of the positive controls on tissue culture polystyrene.

Figure 2. SEM micrographs of the proliferation and attachment of human bone marrow stromal cells on regenerated cellulose hydrogels after: a) and d) 3 days; b) and e) 15 days; and c) and f) 22 days. At lower magnification, in Figs. 2a, 2b and 2c, the cells constitute the darker areas. In Figs. 2c and 2f it can be seen that the surface is almost totally covered by the cells.

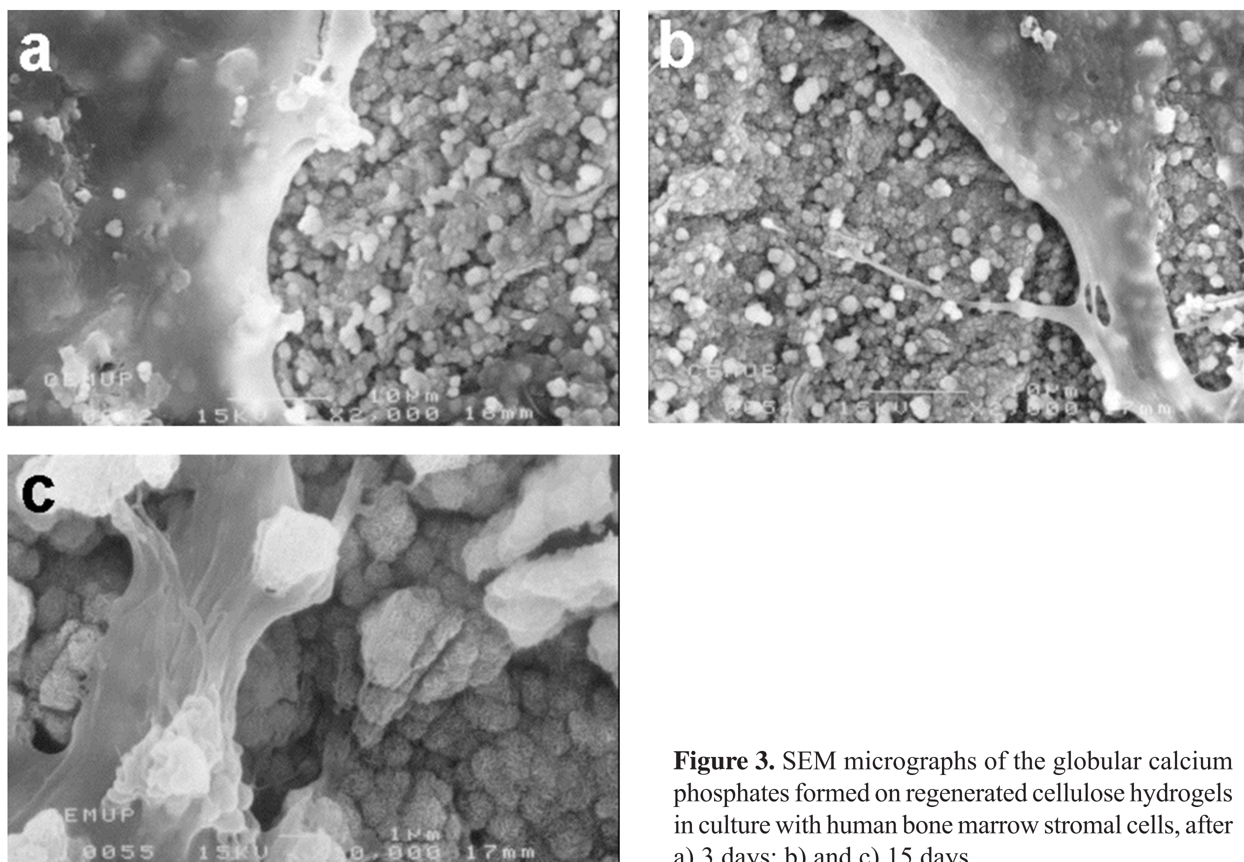

Figure 3. SEM micrographs of the globular calcium phosphates formed on regenerated cellulose hydrogels in culture with human bone marrow stromal cells, after a) 3 days; b) and c) 15 days.

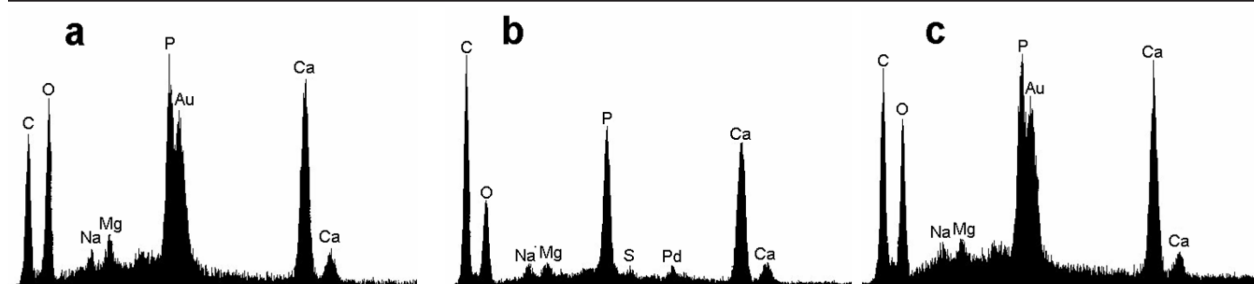

Figure 4. EDS microanalysis spectra of the calcium phosphates formed on regenerated cellulose hydrogels in culture with human bone marrow stromal cells, after a) 3 days, b) 15 days and c) 22 days.

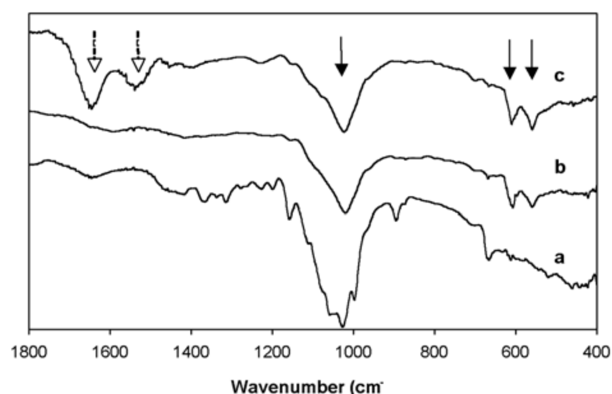

Figure 5. ATR-FTIR spectra of samples analysed, in the 400-1800 cm^{-1} window, showing the formation of an hydroxyapatite layer with its characteristic ν_3 (at about 1016 cm^{-1}) and ν_4 PO_4 (at 557 and 605 cm^{-1}) vibrations (solid arrows): a) regenerated cellulose hydrogel; regenerated cellulose in culture with human bone marrow stromal cells after: b) 3 days, and c) 22 days.

exhibited a flattened morphology. Contact between adjacent cells was also observed in every case. The amount of cells on the surfaces increased with time (Figs. 2a to 2f), until the substrate could hardly be observed, after 22 days, since it was almost completely covered by cells (Figs. 2c and 2f).

Figs. 2d through 2f seem to indicate that the surfaces contacted by cells are changing in the course of time from the original structure of regenerated cellulose. At further magnifications (Fig. 3), a homogeneous layer featuring globular structures, which was later identified as calcium phosphates and hydroxyapatite, could be distinguished between the cell layer and the cellulose surface. Cells are observed to attach very closely to those globular structures, following their topography.

The Ca/P ratio of the calcium phosphate formed, as analysed by EDS microanalysis (Fig. 4), was found to decrease from about 1.88, after 3 days in culture, to about 1.59, after 22 days.

ATR-FTIR analyses of the calcium phosphates formed (Fig. 5) showed that they are apatitic since, in addition to the OH bands, the characteristic ν_3 (at ca. 1016 cm^{-1}) and ν_4 PO_4 (at 557 and 605 cm^{-1}) vibrations were observed, which are typical of apatitic minerals. Furthermore, after 22 days in culture (Fig. 5c), where cells covered the material surface, two additional peaks, centered at 1539 cm^{-1} and 1643 cm^{-1} (dashed arrows) were observed.

Discussion

In previous studies, contact angle measurements confirmed the moderate hydrophilicity of regenerated cellulose hydrogels (Granja *et al.*, 2001a). It was demonstrated that HBMSC attached at a good extent on regenerated cellulose hydrogels and that these materials are cytocompatible (Granja *et al.*, submitted). Their ability to promote HBMSC proliferation was also quantified and results obtained showed that they are adequate substrates for HBMSC proliferation and growth. However, HBMSC morphology on these substrates has not been investigated.

In the present investigation, proliferation of HBMSC on regenerated cellulose hydrogels was observed to a relatively high extent. Changes in proliferation were low in the first 8 days and then increased gradually towards the 15th day. HBMSC cultured on regenerated cellulose hydrogels attached very closely, exhibited a flattened morphology, and contact between adjacent cells was observed, which is typical of well-attached and grown cells (Puleo and Bizios, 1992; Shelton *et al.*, 1988). These findings indicate that regenerated cellulose hydrogels not only allow a good attachment of HBMSC with spreading and acceptable proliferation of the cells. After 22 days in culture, the materials surface was almost completely colonized by well-attached HBMSC.

The good HBMSC attachment and proliferation found can be attributed to the peculiar properties of regenerated cellulose hydrogels for biomedical applications, namely their high purity and high water uptake or swelling, causing less physical damage to cells (Dumitriu and Dumitriu, 1996; Smetana, 1993), associated with their moderate hydrophilicity. In the literature, the role of hydrophilicity in cell adhesion has been widely discussed and, despite the difficulty in isolating this property from the other influencing ones, the general conclusion seems to be that moderate hydrophilicity favours cell adhesion, and extreme values inhibit this phenomenon (Smetana, 1993; Lee *et al.*, 1997; Choee *et al.*, 2004; Webb *et al.*, 1998). A certain amount of free hydroxyl groups on these hydrogels is also expected to favour cell adhesion. It was previously reported by Curtis *et al.* (1986) that moderate hydroxylation enhances cell adhesion and extreme values prevent it.

Analysis of the ATR-FTIR spectra showed peaks centered at 1539 cm^{-1} and 1643 cm^{-1}, on surfaces after 22 days in cell culture, which can be attributed to amino acids from cellular structures, since those surfaces were nearly totally covered by cells.

After contact with cells, the surface of regenerated cellulose hydrogels was significantly changed, as depicted in Figs. 2 and 3. SEM observations at high magnifications, along with EDS microanalysis and ATR-FTIR spectroscopy, showed that a hydroxyapatite layer was formed between the materials surface and attached HBMSC. In addition to the OH bands, the ν_3 and ν_4 PO_4 vibrations were assigned, which are typical of apatitic minerals (Bohic *et al.*, 1998; Dasarathy *et al.*, 1993). In previous studies, the mineralization of regenerated cellulose hydrogels was investigated and it was shown that these materials did not mineralise in Simulated Body Fluid (SBF), except when they were previously treated with calcium chloride (Granja *et al.*, 2001b; Granja *et al.*, 2001c). In the present study, untreated cellulose materials mineralised in HBMSC culture, which can only be attributed to the action of cells themselves, and/or to the culture medium used. In fact, it can be deduced that HBMSC proliferated to a higher extent only on the apatitic mineral layer formed and not on the cellulose surface. The mechanism governing mineralization on regenerated cellulose hydrogels in HBMSC culture must be further investigated in order to better understand and to promote this same behaviour on skeletal implant biomaterials.

At present, only a few available polymeric biomaterials have shown the capability of constituting adequate substrates for bone cell attachment, growth and differentiation in such a way that they allow cells to produce mineralised tissue. Some cues can be summarized from the present work, namely: an hydrogel structure, availability of free OH groups and moderate hydrophilicity. The emerging field of tissue engineering, and more specifically for orthopaedic applications, requires materials that promote the proliferation and growth of viable bone cells expressing their phenotype, in order to promote the formation of new bone on the surface of the materials, or in a 3D porous structure (von Recum *et al.*, 1999; Burgess and Hollinger, 1998). At present, among the most promising substrates for tissue engineered bone tissue are porous biodegradable scaffolds made from polymers belonging to the family of the poly(α-hydroxy) acids, namely poly(lactide), poly(glycolide), and their copolymers, mainly due to their good biocompatibility, good interaction with bone cells, adjustable biodegradation in the human body into metabolic residues, commercial availability, among other adequate properties (von Recum *et al.*, 1999; Ishaug *et al.*, 1997). However, on these materials no direct bone contacts have been observed, and some complications were reported *in vivo*, namely inflammatory reactions at the site of implantation, or even tissue necrosis, which were attributed to the release of lactic acid and consequent local tissue acidification (von Recum *et al.*, 1999; Bergsma *et al.*, 1995; Vert *et al.*, 1994). Cellulose cannot be considered as a biodegradable alternative since it is not digestible in the human organism (Miyamoto *et al.*, 1989; Hayashi, 1994). Nevertheless, the interesting properties revealed in the present work, associated with its adequate mechanical properties, lack of toxicity and long experience in medical applications, make it an interesting alternative, as a porous material, for many applications where, at present, no adequate degradation rates seem to match specific requirements, as

is the case of orthopaedic applications, where mechanical properties constitute a major requirement. Furthermore, it has been reported that several chemical modifications can make it biodegradable, if considerable changes in the higher order structure of cellulose are performed (Miyamoto *et al.*, 1989; Hayashi, 1994). In addition, several other biodegradable polysaccharides have promising properties as biomaterials, although relatively little attention has been paid to their application e.g., in the orthopaedic field.

Conclusions

Regenerated cellulose hydrogels are biocompatible and showed good rates of HBMSC proliferation, with cells exhibiting a flattened morphology on materials surface. After 22 days in culture, cells homogeneously colonized the materials surface. Moreover, between the materials surface and attached cells, a granulated hydroxyapatite layer was formed homogeneously. In previous studies, using simulated body fluid without cells, mineralization did not occur. Hence, it seems that either HBMSC or the culture medium alone were responsible for promoting the mineralization of the surface which also seemed the prerequisite for proliferation.

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Discussion with Reviewers

Reviewer I: Does the culture medium contain beta-glycerophosphate or ascorbic acid, and if not, have these investigators performed other experiments with the medium containing beta-glycerophosphate and ascorbic acid, and can compare the results?
Authors: The culture medium in the present experiment was only supplemented with dexamethasone. No, we have not performed such experiments, thus this comparison was out of the scope of the present experiment.

Reviewer I: Are regenerated cellulose hydrogels biologically safe?
Authors: Regenerated cellulose is biologically safe, similarly to several cellulose derivatives that have found biomedical applications. Cellulose membranes for haemodialysis are among the most widely used polymers in therapy. In particular, several other studies have

confirmed the biological safety of regenerated cellulose, as indicated in the introduction.

Reviewer I: Is regenerated cellulose hydrogel truly biodegradable in human body?
Authors: Cellulose is usually not degraded in the body, since humans do not possess adequate enzymes to do it. However, functionalized cellulose can be rendered biodegradable through changes in its higher order structure. Furthermore, as indicated in the introduction, oxidized regenerated cellulose has been claimed to be slowly degradable in the body. The product Surgicel® (trademark from Johnson & Johnson) is widely used as a resorbable wound dressing and as haemostatic agent.

Reviewer I: Is regenerated cellulose hydrogel non-immunogenic in the human body?
Authors: The present authors have not studied immunogenicity of this particular type of cellulose, although several related investigations are indicated in the introduction. Depending on their composition, cellulose and its derivatives can elicit only a mild immunologic response after implantation. Some works related to blood contact applications have described cellulose as a polymer eliciting a low immune response (low phagocytosis by macrophages and low interleukin-1 release), and inducing a comparatively higher activation of the complement system.

J. de Bruijn: Can the authors speculate on the mechanism of mineralised deposit formation after already 3 days of cell culture? This can obviously not be *de novo* bone formation. Have the authors evaluated whether conditioned medium from control HBMSC cultures can also result in the apatitic layer formation when added to the regenerated cellulose hydrogels (in the absence of cells)?
Authors: Concerning the mechanism of mineralized deposit formation, we do not want to speculate at this point, but surely the whole phenomenon cannot be regarded as *de novo* bone formation. As stated in this manuscript, mineralization was not expected, and hence this control with culture medium without cells was not included in the experimental design. The referee is right in the sense that it is not possible to be sure that this mineralization is a result of the cultured HBMSC, or if only the medium contributed to this phenomenon. This aspect was added to the discussion and should be adequately clarified in future studies.

R. Oreffo: Given the lack of biodegradability of cellulose and requirement of functionalisation, could the authors indicate an orthopaedic application envisaged in the first instance?
Authors: In the first instance, it was envisaged to take advantage of its good mechanical properties and hydroexpansivity, coupled with biocompatibility, as a sealing material for the femoral component in hip prostheses, in place of the acrylic cement, as well as a dyaphyseal obturator. It could also be used as a bone filler in non load-bearing applications.

H. Plenk: Could the authors discuss SBF (simulated body fluid) experiments which showed that a mineralized layer is deposited on a variety of so-called “bioactive” but also “bioinert”materials without the presence of cells?
Authors: Mineralization in SBF has been widely investigated using several materials, as a means to ascertain their bioactivity. Studies in SBF were also carried out using these same cellulose materials and showed that no mineralization occurred in SBF, as indicated in the discussion. The composition of SBF and of the cell culture medium are clearly distinct, and thus no comparisons were attempted. The presence of cells further makes the present study undoubtedly divergent from studies in SBF.

H. Plenk: Were the authors aware of the studies by Kristen *et al*. (1977, 1978), and later by the people in Leiden (Li *et al*., 1997a,b) on bone reactions to wood, and that mineralization occurs naturally in several species of wood?
Authors: We are well aware of those studies and they are thoroughly discussed in our previous papers concerning mineralization studies of cellulose in SBF (references are provided in the introduction). However, as discussed in the previous item, such studies seem clearly different from *in vitro* studies where cells are present.

H. Plenk: While in the studies of Kristen *et al*. (1977, 1979) direct bone contacts to wood surfaces could be shown, no direct bone contact, but only a foreign body response was observed at the cellulose interfaces in your referenced study of Gross *et al*. (1992). Thus, the Gross *et al*. (1992) cellulose material, as well as the PLA-PGA materials, are not “osteoconductive” in my understanding. Could you comment on that?
Authors: You are right that the study from Gross *et al*. (1992) is incomplete, but further evidence of osteoconduction on cellulose is provided in the paper by Chauveaux *et al*. (1990) (text reference).

H. Plenk: When you observed this mineral deposition underneath the HBMS cells, why did they then not produce the bone nodules as in similar cell culture studies? Consequently, can you speculate from your results, if direct bone apposition would occur *in vitro* and *in vivo*?
Authors: The present study is focused on the analysis of the mineral layer formed between proliferating cells and the material surface. No differentiation studies were performed, although we are currently carrying out such studies using oxidized regenerated cellulose. However, from the present studies, it can speculated that the used materials, pre-colonized with cells, could promote direct bone apposition.

Additional References

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