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Self-Hardening Hydrogel for Bone Tissue Engineering

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Summary: We have developed a self-reticulating polymer based on silanized hydroxypropylmethylcellulose (Si-HPMC). The aim of this study was to determine whether this Si-HPMC hydrogel with or without calcium phosphate granules could represent a potential scaffold for bone tissue engineering.
This study showed that Si-HPMC hydrogel didn't affect SaOS-2 and rat bone marrow cells viability. In addition, SaOS-2 cells are able to proliferate within Si-HPMC hydrogel containing or not calcium phosphate granules whereas Rat bone marrow cells proliferate only at the surface of calcium phosphate granules contained within Si-HPMC hydrogel. Finally, SaOS-2 cells seeded at the surface of reticulated Si-HPMC were not able to penetrate the hydrogel, while J774, a macrophage cells line, were able to move into the Si-HPMC hydrogel. These data indicate that Si-HPMC is a promissing scaffold for tissue engineering.

Keywords: biomaterials; cellulose; hydrogels; tissue engineering

Introduction

Tissue engineering is an emerging field of regenerative medicine which holds promise for the restoration of tissues. Over the past decade, tissue engineering has evolved from the use of nude biomaterials, which may just replace reduced area of damaged tissue, to the use of controlled three-dimensional scaffolds in which cells can be seeded before implantation. Bone and cartilage have taken advantage of the recent efforts in tissue engineering and have shown proof of concept in clinical situations.[1,2]

This hydrogel consist of hydroxypropyl methylcellulose (HPMC) chains grafted with silanol groups.[3,4] It has been demonstrated that this silanized HPMC (Si-HPMC) exhibits viscous and elastic properties allowing its injection.[5] These interesting rheological characteristics highlight the potential of using this Si-HPMC hydrogel in tissue engineering. In a previous work, we have demonstrated that Si-HPMC hydrogel represents a suitable scaffold for cartilage tissue engineering.[6–8]

The physiological process of bone remodelling confers to this tissue a strong-self healing capacity. With respect to this property, a great variety of natural and synthetic bone substitutes have been considered as means to repair bone loss. Suspension of calcium phosphate granules in a polymeric solution have been reported to be a convenient mean for the filling of bone cysts and alveolar sockets.[9–11] Contrarily to the acrylic bone cement, the injectable bone substitute (IBS) is bioactive, resorbable and osteoconductive. Despite a large body of evidence indicating that the above mentioned materials are suitable matrices for bone filling, neither CaP ceramics bone cements, nor IBS have osteogenic properties still too limited for large osseous reconstruction, particularly in territory with limited repair capacity (irradiated area). Therefore, the aim of this study is to investigate whether Si-HPMC

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hydrogel with or without calcium phosphate granules could represent a potential scaffold for bone tissue engineering.

Materials and Methods

Materials

The hydroxypropylmethylcellulose used was Methocel® E4M from the Colorcon Company. Reagent Dulbecco's modified Eagle medium (DMEM), antibiotics (penicillin, streptomycin, amphotericin B, gentamycin), trypsin/ethylenediaminetetraacetic acid (EDTA), fetal calf serum (FCS), and cell culture disposables (6- and 24-wells plates, T75 flasks, and 10-mm diameter dishes) were purchased from Life Technologies-GIBCO (Cergy Pontoise, France). Type IV collagenase, L-ascorbic acid, L-glutamine, dexamethasone and 1,25 $(OH)_2$ vitamin D_3 (calcitriol) were obtained from Sigma (Saint Quentin Fallavier, France). Glutaraldehyde was obtained from Merck (Fontenay sous Bois, France) and hexamethyldizilasane from Acros Organics (Noisy le Grand, France). For alkaline phosphaptase (ALP) histochemical detection, naphthol AS-BI, fast blue BB salt, paranitrophenyl phosphate (PNPP), and ALP buffer were purchased from Sigma.

Hydrogel Preparation

The synthesis of Si-HPMC was performed by grafting 14.24% of 3-GPTMS on E4M® in heterogeneous medium {Bourges, 2002 #7601}. Si-HPMC powder (3%, w/v) was solubilized in 0.2 M NaOH under constant stirring for 48 h. The viscous solution was dialyzed in 0.09 M NaOH water solution during 48 hours. After this treatment, the measured amount of silicon was 0.5% in weight. The solution was then sterilized by steam (121 °C, 30 min). To allow the formation of a reticulated hydrogel, two volumes of solution were mixed with 1 volume of a 0.13 M HEPES buffer. Thereafter, this hydrogel can be associated with calcium phosphate granules of MBCP™ (Biomatlante, Vigneux de Bretagne, France) at 10% (w/w). The MBCP™ granules sieved between 40 and 80 μm were mixed, under sterile condition, with the hydrogel before reticulation.

Cell Culture

Two cell lines were used in this study, the Saos-2 cells (ATCC HTB 85, human osteoblast-like cell derived from an osteosarcoma) and the J774 (ATCC TIB 67, murine macrophage cell line), as well as primary rat bone marrow cells (RBMC).

Cells were cultured in DMEM supplemented with 0.4 mM, L-glutamine and 10% FCS. Cells were seeded in 75-cm^2 tissue flasks. Cultures were maintained at 37 °C in a humidified atmosphere in the presence of 5% CO_2.

Isolation of Rat Marrow Stromal Cells

Rat bone marrow (RBMC) were prepared and cultured as previously described.[12] Bone marrow cells were seeded in 75-cm^2 tissue flasks at a final density of $1{\cdot}10^4$ cells/cm^2. Cells were cultured in medium consisting of DMEM containing 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mM, L-ascorbic acid, 15% FCS and dexamethasone (10^{-8} M). Non-adherent cells were removed 48 h after seeding by two vigorous washing with phosphate buffer saline (PBS), pH 7.4. Thereafter, the medium was changed twice a week.

Cell Proliferation Assay

Cell proliferation was evaluated by trypan blue exclusion dye experiments. Briefly, SaOS-2 and RBMC were allowed to attach in a 6 well plate at a final density of 10^6 cells/cm^2 and 10^5 cells/cm^2 respectively. After 24 h, culture medium was removed and 2 ml of Si-HPMC were added in each well. Samples were incubated at 37 °C for 1 h before adding 2 ml of culture medium. As a control, cells were also cultured in the absence of Si-HPMC. After 24 h and 48 h, hydrogel and culture medium were removed and the cells were detached by adding trypsine/EDTA for 2 min. the suspended cells were transferred into a

fresh culture medium and counted with Trypan blue staining that colors dead cells in blue. Results were expressed as the total number of cells per well.

Three-Dimensional Culture

SaOS-2 cells were mixed with Si-HPMC hydrogel alone or the composite Si-HPMC/MBCP™ at $1{\cdot}10^6$ cells/ml. 2 ml of cells/Si-HPMC or cells/SI-HPMC/MBCP™ were seeded in 6-well plates and incubated at 37 °C and 5% CO_2. After 1 h 2 ml of culture medium was added in each well and plates were incubated for 5, 8, 11, 26 days

Histochemical Staining

ALP activity was histochemically detected in six-well culture plates. Cell cultures were rinsed three times with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at 37 °C. After extensive washing, cells were incubated for 30 min at 37 °C with 0.5 mg/ml naphthol AS-BI phosphate in tris-buffer (pH 8.5) in the presence of 1 mg/ml fast blue BB salt. The identification of ALP-positive (ALP+) cells was determined under light microscope (magnification 400×).

Statistical Analyses

Results are expressed as mean ± the standard error of the mean (SEM) of triplicate determinations. Comparative studies of means were performed by using one-way ANOVA followed by post-hoc test (Fisher's projected least significant difference) with a statistical significance at $p < 0.05$.

Results

Cell Viability and Proliferation

The effect of Si-HPMC hydrogel on cellular viability and proliferation was assessed by scoring the cells after trypan blue staining (Figure 1).

Results showed that SaOS-2 cells proliferate significantly between 24 h and 48 h in both conditions (control and Si-HPMC). Interstingly, no significant difference was observed between the cells cultured in the presence of Si-HPMC and in control conditions.

The rat bone marrow cells cultured in control condition showed an increase in cell number between 24 h and 48 h. RBMC cultured in the presence of Si-HPMC exhibited a weak increase in cell number between 24 h and 48 h compared to the control conditions.

Cell Behaviour in Three-Dimensional Culture

When SaOS2 cells were seeded at the surface of the reticulated Si-HPMC hydrogel,

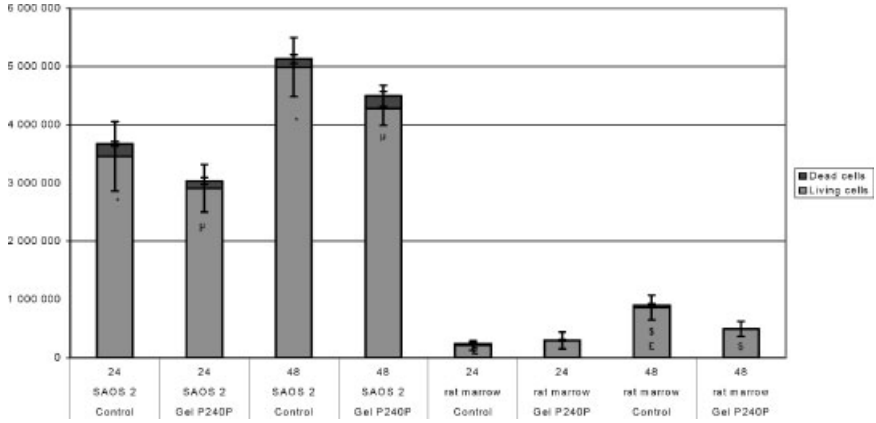

Figure 1.
Number of living or dead SaOS-2 or RBMC after 24 and 48 hours of culture in 2 dimensional conditions, with or without hydrogel on the cells. Results are expressed as the total number of cells per well.
* µ £ $ indicates that $p < 0.05$.

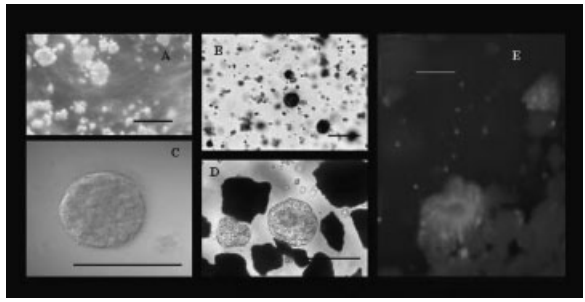

Figure 2.
(A) Microscopic aspect of osteosarcoma cells cultured at the surface of Si-HPMC hydrogel, 11 days, or (B) after 26 days in 3D culture into Si-HPMC hydrogel. (C) Microscopic aspect of SaOS2 cells after 26 days of 3D-cultured into Si-HPMC hydrogel or (D) into Hydrogel Si-HPMC/MBCP^{TM} confocal (nomarsky). (E) ALP staining of rat bone marrow cells after 11 days of 3D culture into Si-HPMC/MBCP^{TM} granules. ALP positive cells appear red. (Bars: 100 μm).

SaOS-2 cells were not able to move into the hydrogel. SaOS-2 cells grew and formed colony at the surface of the hydrogel (Figure 2A).

When SaOS-2 cells were cultured three-dimensionally within Si-HPMC Hydrogel, the SaOS-2 appeared isolated at the beginning of the 3D culture. After 24 h, cells are two by two (data not shown). The number of cells in these colonies increased with time to form nodules with size close to 100 μm in length after 26 days of culture (Figure 2B and C).

During three-dimensional culture within the Si-HPMC/MBCP^{TM} SAOS2 cell behavior was dependent on their location. SAOS2 cells in the Si-HPMC hydrogel, but not in contact to the calcium phosphate granules, grew in nodule as described for the cells within the Si-HPMC hydrogel alone (Figure 2D). On the contrary, SAOS2 cells in contact with the calcium phosphate granules adhered to the surface of granules adopted a fibroblastic shape and proliferated to form a thin layer of cells as it was observed by scanning electron microscopy (Figure 3).

Contrarily to SaOS-2 cells, RBMC three-dimensionally cultured within Si-HPMC/MBCP^{TM} didn't show any nodules formations inside the hydrogel. However, ALP staining revealed the presence of alkaline phosphatase positive cells spreading on the surface of BCP granules (Figure 2E).

In order to determine if cells could penetrate inside the hydrogel, J774 cells (macrophage-like) were seeded at the surface of reticulated Si-HPMC/MBCP^{TM}. Observation of transversal slide of Si-HPMC/MBCP^{TM} (Figure 4) exhibited J774 inside the Si-HPMC hydrogel. These results suggest that macrophage cells are able to penetrate within the hydrogel.

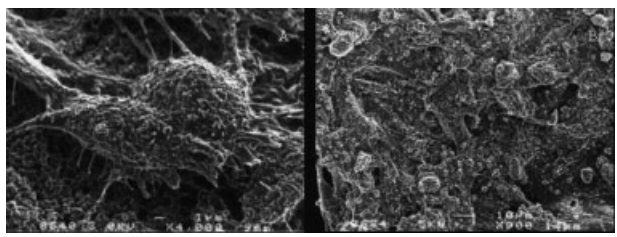

Figure 3.
Scanning Electron Microscopic aspect of SaOS-2 cells cultured in 3D within Si-HPMC/MBCP^{TM}. (A) 8 days, cells on BCP granule surface, (B): 26 days, cells on BCP granule surface.

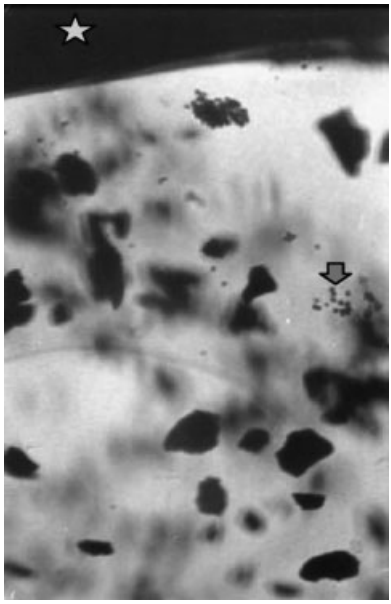

Figure 4.
Microscopic observation of transversal slide of Si-HPMC/MBCP^{TM} containing J774 cells (arrow) seeded on the surface (star).

Discussion

The field of tissue engineering is driving toward the development of strategies to restore damaged bone and cartilage. In this context, much attention has been paid to the development of three-dimensional matrices for the transfer and maintenance of cells in the recipient site. Therefore, we have developed a self-setting hydrogel named Si-HPMC. The aim of this study is to investigate whether Si-HPMC hydrogel could represent a potential three-dimensional matrix for bone tissue engineering. One of the first steps in the development of a novel scaffold is the evaluation of its cytotoxicity. In this work, we were first interested in examining the in vitro cytotoxicity of Si-HPMC. According to standards (ISO 10993-5: biological evaluation of medical devices-part 5: tests for in vitro cytotoxicity) we focused on cytotoxicity test by direct contact with SaOS-2 cells cultured in two dimension. SaOS-2 cells are a widely used cells line since it is a human osteoblast-like cell. However, SaOS-2 cells are derived from osteosarcoma and may exhibit low sensitivity to cytotoxic signals. Therefore, we aimed at confirming the cytocompatibility of Si-HPMC by using primary cells derived from rat bone marrow. In the present work, Si-HPMC didn't affect cell viability and proliferation of SaOS-2 and total rat bone marrow cells indicated by cell counting experiments. This lack of cytotoxicity of Si-HPMC has also been demonstrated with articular chondrocytes in a previous work.[6]

After demonstrating that Si-HPMC was not cytoxic with respect to osteoblastic cells, we evaluated whether three-dimensional culture within Si-HPMC containing or not calcium phosphate granules allowed osteoblast viability, proliferation and activity. Results indicate that SaOS-2 cells proliferate depending on their location within the Si-HPMC hydrogel containing calcium phosphate granules. On the contrary, rat bone marrow cells proliferate only at the surface of calcium phosphate granules in the Si-HPMC hydrogel. Moreover, this bone marrow cells grow on the surface of calcium phosphate granules exhibite a positive alkaline phosphatase activity. These results strongly suggest that calcium phosphate granules are needed for the osteoblastic differentiation of mesenchymal stem cells isolated from bone marrow.

Previous *in vivo* results[13] showed that the hydrogel was biocompatible without signs of inflammation nor osteolysis. In this preliminary *in vivo* study Si-HPMC with BCP granules was not an obstacle to bone colonization and bone ingrowth was similar to previous studies with non hardening a BCP suspension.[14] However, in this present study osteoblastic cells and bone marrow cells were not able to penetrate within the reticulated hydrogel. To investigate this discrepancy, we have used the J774 macrophagic cell line. Results show that macrophages were able to penetrate the hydrogel. This result suggests that some cells could move into and/or degrade the hydrogel before bone colonization and

substitution. Specific studies of degradation will be performed to elucidate this question.

Conclusion

These results highlight the potential of this Si-HPMC hydrogel as a scaffold for tissue engineering. This synthetic extra cellular matrix concept can be adapted to the cell behavior and the tissue which has to be regenerated. Diffusion and degradation process have to be controlled to adapt the chemistry to the application. The biomechanical and visco-elastic properties without and with cells are in progress to understand the influence of the three components, cells, their production and hydrogel.

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[1] R. Quarto, M. Mastrogiacomo, R. Cancedda, S. M. Kutepov, V. Mukhachev, A. Lavroukov, E. Kon, M. Marcacci, *N Engl J Med* **2001**, *344*, 385.
[2] M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, L. Peterson, *N Engl J Med* **1994**, *331*, 889.
[3] X. Bourges, P. Weiss, G. Daculsi, G. Legeay, *Adv Colloid Interface Sci* **2002**, *99*, 215.
[4] X. Bourges, P. Weiss, A. Coudreuse, G. Daculsi, G. Legeay, *Biopolymers* **2002**, *63*, 232.
[5] A. Fatimi, J. Francois Tassin, S. Quillard, M. A. V. Axelos, P. Weiss, *Biomaterials*, *In Press, Corrected Proof*.
[6] C. Vinatier, D. Magne, P. Weiss, C. Trojani, N. Rochet, G. Carle, C. Vignes-Colombeix, C. Chadjichristos, P. Galera, G. Daculsi, J. Guicheux, *Biomaterials* **2005**, *26*, 6643.
[7] C. Vinatier, D. Magne, A. Moreau, O. Gauthier, O. Malard, C. Vignes-Colombeix, G. Daculsi, P. Weiss, J. Guicheux, *J Biomed Mater Res A* **2007**, *80*, 66.
[8] C. Vinatier, J. Guicheux, G. Daculsi, P. Layrolle, P. Weiss, *Biomed mater eng* **2006**, *16*, 107.
[9] O. Gauthier, D. Boix, G. Grimandi, E. Aguado, J. M. Bouler, P. Weiss, G. Daculsi, *J Periodontol* **1999**, *70*, 375.
[10] O. Gauthier, R. Muller, D. von Stechow, B. Lamy, P. Weiss, J.-M. Bouler, E. Aguado, G. Daculsi, *Biomaterials* **2005**, *26*, 5444.
[11] P. Weiss, L. Obadia, D. Magne, X. Bourges, C. Rau, T. Weitkamp, I. Khairoun, J. M. Bouler, D. Chappard, O. Gauthier, G. Daculsi, *Biomaterials* **2003**, *24*, 4591.
[12] C. Maniatopoulos, J. Sodek, A. Melcher, *Cell Tissue Res* **1988**, *254*, 317.
[13] B. H. Fellah, P. Weiss, O. Gauthier, T. Rouillon, P. Pilet, G. Daculsi, P. Layrolle, *J Orthop Res* **2006**, *24*, 628.
[14] O. Gauthier, J. M. Bouler, P. Weiss, J. Bosco, G. Daculsi, E. Aguado, *J Biomed Mater Res* **1999**, *47*, 28.

