

Biocompatible porous ceramics for the cultivation of hematopoietic cells

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Abstract Porous ceramics made of alumina and hydroxyapatite were created using a protein foaming method. Porosity and pore size distribution were successfully varied by means of chemical modification of the foaming protein Bovine serum albumin (BSA). The effectiveness of the BSA and of its chemical modifications as well as the influence of the dispersing agent were investigated using synchrotron tomography. Resulting porous ceramic materials were used as three-dimensional substrates for the cultivation of human peripheral stem cells. The cells proliferated and differentiated in culture. Five cell lines consistent with human blood cell lines were observed.

Keywords Protein foaming · Alumina · Hydroxyapatite · Cell cultivation

Introduction

Hematopoiesis develops in a three-dimensional matrix located in the embryo and fetus, in extraskelatal blood vessels, in the liver and spleen, later, postpartum, increasingly in the bone marrow. The interaction of hematopoietic and mesenchymal stem cells through direct contact and mediators is supported by the mechanically protecting compartment of mineralised bone [1]. The process of such interaction is not yet fully known. An example was recently given for the role of transforming growth factor beta and bone matrix proteins 2, 4 and 7 for the creation of hematopoietic precursors [2]. Hematopoietic stem cells are usually grown in culture flasks that do not contain a specific three-dimensional structure. It was found that the proliferation of cells can be influenced by a given microenvironment. Attempts to use a three-dimensional matrix for the culture of hematopoietic progenitor cells were published [3–6]. There are also publications describing the benefit of three-dimensional culture vessels for the expansion and proliferation of cells [7, 8]. Significant differences exist when comparing normal and in vitro hematopoiesis on three-dimensional substrates. The mechanical protection of cell proliferation in ceramic compartments with interconnecting pores can be beneficial. The lack of mesenchymal stem cells and the lack of cell adhesion receptors in the largely absent extracellular matrix do not represent normal conditions. The lack of normal perfusion of the porous structure can also provide an additional negative influence.

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The aim of this study was to explore the differentiation of human peripheral stem cells seeded in three-dimensional constructs with ceramics. Pore sizes and pore size distribution of normal trabecular bone are in the range of 75 μm –500 μm [9–12]. According to this size range, two ceramic substrate materials were developed, consisting primarily of alumina (Al_2O_3) or hydroxyapatite (HA). Attempts were made to influence the pore size and the pore size distribution of the foamed ceramic. As a consequence of the undefined foaming character of the protein, the control of pore size and its distribution cannot be handled easily. It was shown that chemical modification of the protein, i.e. a glycation or acetylation yields a required functionality of protein. The influence of the inorganic dispersant on rheology was also investigated, as well as the generation of pores in the three different processing steps.

Materials and methods

Materials

For the development of ceramic foams, two different powders were used: Al_2O_3 -powder AKP 50 (Sumitomo Chemicals Co., Japan) with a purity of 99.99%, particle size between 0.1 and 0.3 μm , and a specific surface of 10 m^2/g (measured with BET); and Hydroxyapatite (HA) powder (CFB Budenheim, Germany) type B powder NF pretreated by heat for better workability, particle size between 0.5 and 4 μm , and a specific surface of 0.5 m^2/g . The overall processing technique of the used protein foaming method and materials are described in an earlier publication [13]. The selected materials for cell culture investigations in general have interconnecting pores ranging from 20 μm up to 300 μm for Al_2O_3 and from 150 μm up to 800 μm for Hydroxyapatite.

Chemical modification of proteins

Chemical modification or derivatisation of proteins is a well-known process in food chemistry. It is known that an acetylation, succinylation or a glycation with sugars yields enhanced emulsifying properties or increased foaming ability [14, 15]. A chemical modification of Bovine serum albumin (BSA) was carried out in order to generate large pores that guarantee adequate permeability of the substrate. For glycosilation, 5 g of BSA are incubated with 0.25 M, or 0.5 M, glucose or maltose solution in a 250 ml phosphate buffer solution at pH 7.5. The incubation time was 10 days at 37 °C.

One milliliter toluol was added to the solution to prevent microbiological attack.

Acetylation of the BSA was carried out according to the Hoagland method [16]. BSA was added to a saturated sodium acetate solution at 25 °C. Consequent stirring dissolved the protein into the solution. Small increments (0.2 ml) of acetic anhydride (a total of 7.2 ml) were added and stirring of the mixture was carried out for 2 h.

After the modification of the proteins, the solutions were dialysed against distilled water and freeze-dried. The success of the dialysation was determined using enzymatic examination (for sugars) or pH testing (acetylation). The degree of derivatisation was determined using the mass examination method with matrix-assisted laser ionisation desorption time-of-flight mass spectrometry (MALDI/TOF-MS).

Synchrotron tomography

Tomographic imaging techniques allow for non-destructive three-dimensional imaging of the mass distribution of real objects. Absorption tomography is based on the detection of radiation attenuated by an object for many different angular positions. This results in a set of projections which are used for reconstructing a complete 3D-image [17, 18].

The measurements were carried out at the beamline of the Federal Institute for Materials Research and Testing (BAM) at the electron synchrotron BESSY [19].

The sample diameter was about 5 mm. The time of measurement for each tomogram was 1.5 h. An optical resolution of 3.512 μm was used.

Cell culture studies

Cells for culture on ceramic substrates were taken from four healthy donors involved in therapeutic programs for the treatment of tumor patients undergoing a hematopoietic cell transplantation. The donors underwent leukapheresis using a Cobe Spectra (Gambro BCT, Planegg, Germany) following standard procedures [6]. Informed consent was obtained from all donors according to institutional guidelines. The cells in the leukapheresis product were washed twice in phosphate buffer solution (PBS) and incubated with culture medium as previously described [20]. In the leukapheresis product, a percentage of 0.2%, 0.5%, 0.7% and 0.8% of cells were CD34 positive. Culture medium was Iscove's modified Dulbeccos's medium (IMDM, Gibco, Paisley, UK) containing 10% human AB serum (Sigma Taufkirchen, Germany), 100 ng/ml of SCF, 50 ng/ml of flt-3L, 8 ng/ml of IL-3, 20 ng/ml of

IL-6, 13.5 ng/ml of G-CSF (all cytokines: Pepro Tech/ Tebu, Offenbach, Germany) and 0.4 U of EPO (Cilag, Schaffhausen, Switzerland).

The ceramic specimens generated by using native BSA as foaming agent (Al_2O_3), whey protein (HA) as well as glass slides for controls were placed in 24 well plates (Nunc, Naperville, IL, USA) with culture medium 24 h before cell seeding. Cells were seeded at a concentration of 1×10^6 cells per ml, equivalent to five specimens per well. The cell suspension was poured directly onto the ceramic block or glass slides. The medium was changed one to two times per week. Cultivation time was 2.5 and 5 days in four experiments with a total of 147 specimens. After the culture period the specimens were removed, rinsed in PBS, fixed in cold acetone, dried and stained for morphology using Pappenheim or Giemsa stain, immunohistology with primary antibodies against CD34, CD45, CD68 with PG-M1, CD61 with Y2 51, Ret 40 for detection of glycophorin of erythrocytes and MPO7 for leucocytes. The antibodies were detected using the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method or by using the fluorescent dyes Carbocyanin 2 (Cy2) and Indocarbocyanin (Cy3). The vitality of cells was detected using fluorescein-diacetate and ethidiumbromide (FDA–EB).

Results

The microstructure of the foams developed by the use of chemically modified proteins displays differences in pore volume and pore size distribution (Fig. 1). Pores

of different size with varying degrees of interconnectivity were observed. The microstructure of ceramics generated by the use of chemically modified proteins shows an accrument of bubbles; conversion with sugars (glucose, maltose) particularly exhibits a change in pore size and the size of pore windows.

The influence of the chemical modification can be seen in Fig. 2a–c. The influence of the dispersing agent content can be seen in Fig. 2a, d. There is an increase in mean pore size but a decrease of pore numbers (down right). The amount of small pores ($<100 \mu\text{m}$) decreases as the big pores ($>150 \mu\text{m}$) increases, which could be due to thermodynamics equilibrium and consolidation conditions.

With regard to the microstructure of these samples, a diminishing of individual pores in the sintered stage and an increasing interpenetration of the pores was observed. The ceramic structure seems to become more aerated as the dispersing agent content is increased. This can be observed in both the sintered stage and the slurry stage.

In cell culture, surfaces are covered by cells and cell clusters in most cases (Figs. 3, 4). The cells display different morphologies and stainability in conventional staining methods. Immunostains indicate differentiation into different cell lines and stages of maturation. Identified are a few stem cells (CD34), clusters of erythrocytes and precursors positive for glycophorin with Ret 40, leukocytes positive for myeloperoxidase MPO7, cells positive for CD45 the leucocyte common antigen, thrombocytes and megakaryocytes positive for CD61 with y2 51, macrophages CD68 positive with PG-M1 disseminated on the surface and in pores. Generally speaking, more cells

Fig. 1 SEM images of alumina microstructures using different protein derivatives as foaming agent. (a) native BSA, (b) glycated with maltose, (c) acetylated (d) glycosylated

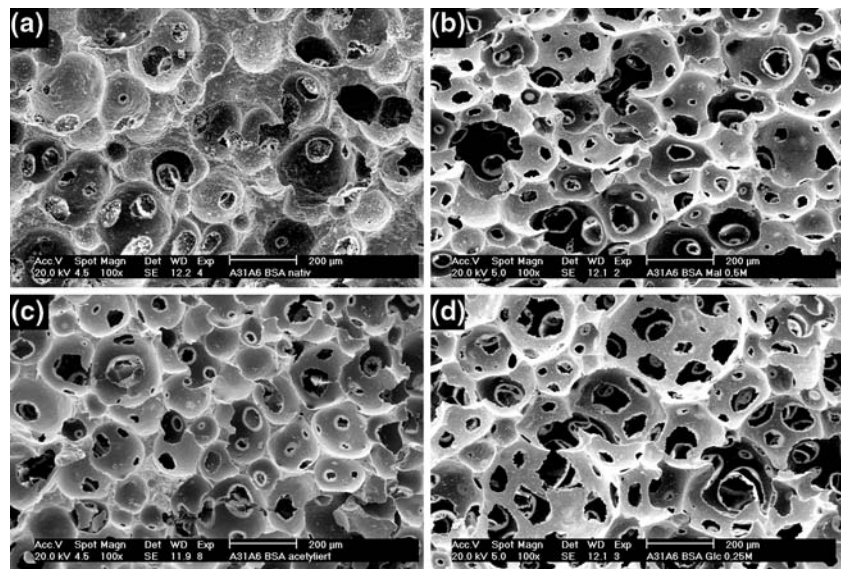


Fig. 2 3D images of sintered ceramic (a) BSA native, (b) BSA glycated with maltose, (c) BSA acetylated, (d) BSA native with dispersant, (top right) pore size distribution of ceramic foams by using modified proteins, (down right) influence of the dispersant on pore size distribution

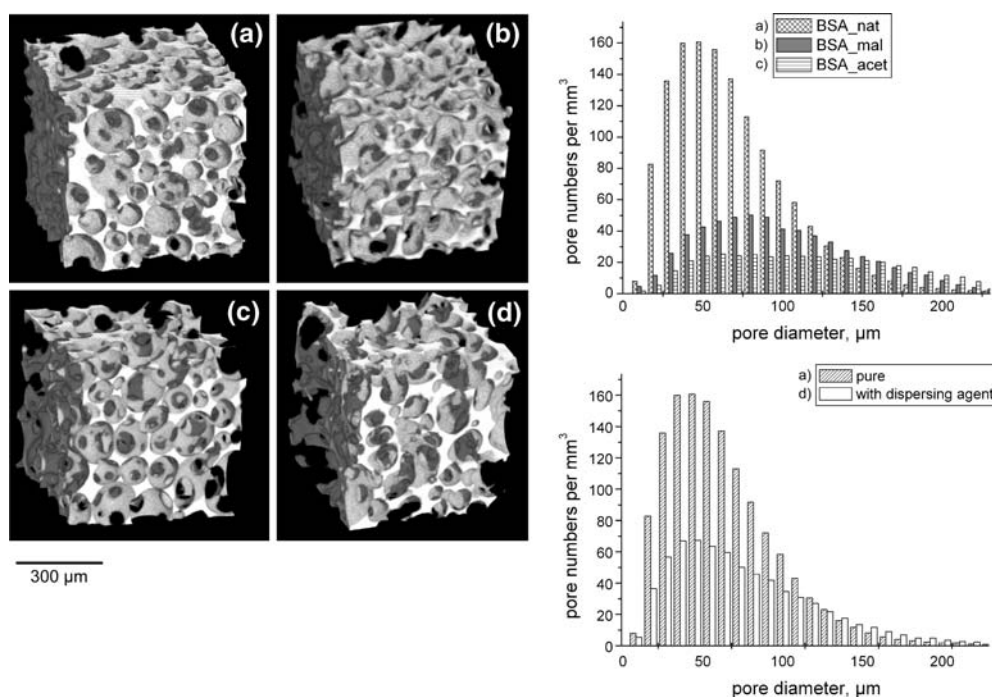
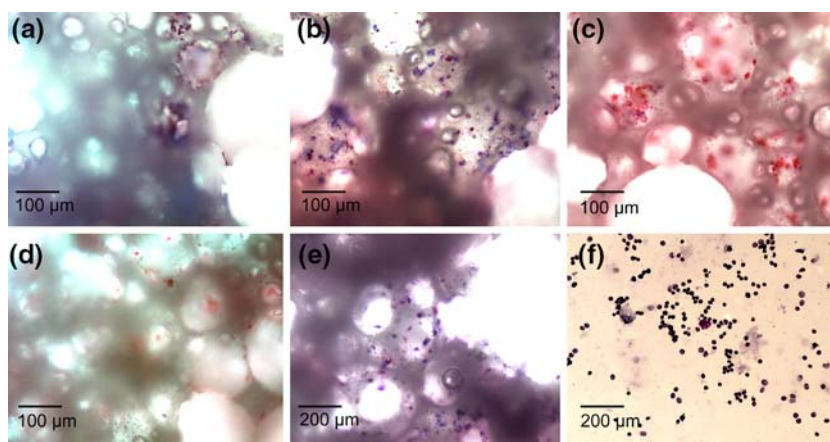


Fig. 3 Hpsc 5 days in culture on porous Al₂O₃ ceramic stained with APAAP for glycoprotein with Ret 40 in (a), CD61 with γ2 51 in (b), for myeloperoxidase with MPO7 in (c), CD45 in (d), for CD68 with PG-M1 in (e). Hpsc as seeded on day 0, Pappenheim stain (f). Stain of nuclei with Giemsa in (b) and (e)



can be observed on the Al₂O₃ substrate than on the HA substrate. A couple of samples display stellate cell forms indicating a pattern of attachment. Some pores exhibit cell clustering mainly with erythrocytes and leucocytes due to the favourable microenvironment. A few samples show cell debris in the center of the substrate material. At least five cell lines could be identified.

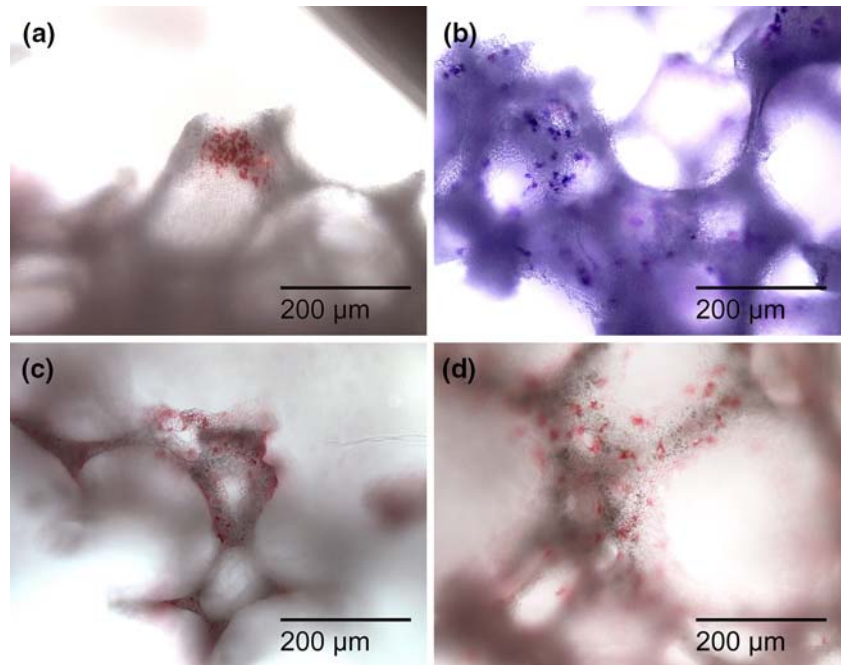
Discussion

The chemical modification of BSA indicates a potential method for changing the porosity of alumina and hydroxyapatite foams. The glycation of the protein with sugars in particular is a promising technique that

can be used to tailor the more or less statistical processing of the developing ceramic microstructure. This is caused by the advanced foaming and emulsifying ability of the protein and thus of the whole slurry. Not only the pore size but also the size of the interconnecting windows changed.

Above all, the enlargement of the pore size and the degree of the interconnectivity is thought to be helpful for alimentation of the cells during cultivation in order to build up cultivation setups of larger dimensions. Cell culture investigations on these scaffolds were not carried out down to the present day; therefore, nothing can be said about the advantages of greater interconnectivity. However, previous investigations revealed a need for adequate porosity and the size of pores as windows to ensure cultivation success; specifically, the

Fig. 4 Hpsc 5 days in culture on porous HA ceramic stained with APAAP for glycoporphin with Ret 40 in (a), Pappenheim in (b), CD61 with y2 51 in (c), for CD68 with PG-M1 in (d)



alimentation of cells in the centre of the 3D-substrate was suboptimal.

The influence of the dispersing agent is ambivalent because the enlargement of pore size and enhanced permeability are accompanied by a viscosity decrease of the slurry; thus, the processing time of the foam is abbreviated.

The potential for growth and differentiation of peripheral blood stem cells on ceramic substrate material with different pores sizes was shown. The formation of more than five cell lines indicates the potential of these three-dimensional ceramic materials for the cultivation of hematopoietic cells. However, there is a need for increased sampling for quantitative evaluation to confirm scalability. Furthermore, an additional permeation of the ceramic material is desirable with view to the built-up of a bioreactor device.

Conclusions

The development of ceramic foams with a wide range of porosity using the modification methods of the foaming agent is a promising step to deliver aligned cell culture substrates for the culture of hematopoietic cells. Substrate materials manufactured by unmodified proteins were able to provide support for human peripheral stem cells under cell culture conditions. After 5 days the cells showed proliferation and differentiation into more than five cell lines.

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