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Coupling of gelatin to inner surfaces of pore walls in spongy alginate-based scaffolds facilitates the adhesion, growth and differentiation of human bone marrow mesenchymal stromal cells

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Abstract We have developed a novel wide-pore scaffold for cell 3D culturing, based on the technology of freeze-drying of Ca-alginate and gelatin. Two different preparation methodologies were compared: (i) freeze-drying of Na-alginate + gelatin mixed solution followed by the incubation of dried polymer in saturated ethanolic solution of $CaCl_2$; (ii) freeze-drying of the Na-alginate solution followed by the chemical "activation" of polysaccharide core with divinylsulfone with subsequent gelatin covalent attachment to the inner surfaces of pore walls. The scaffolds produced using the first approach did not provide adhesion and proliferation of human bone marrow mesenchymal stromal cells (MSCs). Conversely, the second approach allowed to obtain scaffolds with a high adherence ability for the cells. When cultured within the latter type of scaffold, MSCs proliferated and were able to differentiate into adipogenic, osteogenic and chondrogenic cell lineages, in response to specific induction stimuli. The results indicate that Ca-alginate wide-pore scaffolds with covalently attached gelatin could be useful for stem cell-based bone, cartilage and adipose tissue engineering.

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1 Introduction

General strategy of modern regenerative medicine and tissue engineering involves isolation of cells from patient tissue biopsy, in vitro expansion of the cells followed by seeding them into a three-dimensional (3D) scaffold of predefined shape and size [1]. Mesenchymal stromal cells (MSCs) are one of the most promising cell types that can potentially be used for generating the engineered substitutes of connective tissue. MSCs have been shown to have high proliferative activity and ability to differentiate into multiple cell types [2, 3]; they are also relatively easy to isolate and expand. Protocols for differentiation of the expanded MSCs into adipogenic, osteogenic, chondrogenic and other lineages have been well established. Thus, the multilineage potential of MSCs could be used for the development of both uni- and multicomponent tissue substitutes [4–7].

The use of scaffold should both retain the cells within the defect site and promote tissue ingrowth and vascularization. Ideal scaffold should be biocompatible, non-immunogenic and (potentially) biodegradable; it should also provide optimal attachment, proliferation and differentiation of cells [8]. Optimal 3D growth and subsequent transformation of adhered cells are best achieved in solid scaffolds bearing a specific network of interconnected macropores. Various fabrication technologies have been applied for the preparation of porous scaffolds, such as salt leaching [9, 10], freeze-drying [11–13], cryotropic gelation [14, 15], electrospinning, etc. [16–18]. The cryogenic methods, freeze-drying and cryotropic gelation, utilize the ice crystals as porogens. Both cryogenic approaches are notable for their high potential to induce the interconnected gross pores in the resulting polymeric matrices [19, 20] and are most widely used techniques for the fabrication of macroporous matrices.

Many synthetic and natural polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), their copolymers (PLGA), collagen, gelatin, chitosan, agarose, alginate, etc. have been used upon the preparation of scaffolds for tissue engineering [8, 21–24]. Alginate has been safely used to deliver proteins and cells to patients, which makes the alginate-based scaffolds a highly attractive tool for clinical applications [25, 26].

Alginates do not contain bonds hydrolysable by human organism, but they are often used as resorbable biomaterials. This is achieved by the in vitro pre-formation of water-insoluble gels through ionic cross-linking of alginate chains with divalent cations such as Ca^{2+}, while the subsequent dissolution of these gels in vivo occurs via the ion-exchange of alginate-bound Ca^{2+} for monovalent cations, such as Na^{+}, K^{+}, NH_4^{+}, etc. The rate of the dissolution process can be considerably reduced by using additional chemical cross-linking of ionotropic alginate gel. Furthermore, water-soluble biopolymers can be attached to the alginate matrices by covalently coupling using glutaraldehyde, carbodiimide or carbonyl diimidazole [24]. This modification of scaffolds is needed to compensate for poor adhesive properties of alginate, which does not bind to any of the proteins expressed by animal and human cells, and assumes an incorporation of "recognizable" substances into the alginate matrix or their attachment to the alginate core. Several peptides (e.g., RGD [27]), proteins (collagen/gelatin, fibronectin, laminin, etc. [12, 28–30]) and polysaccharides (e.g., hyaluronates or heparin [31, 32] are known to provide strong cell adhesion and can therefore be used as "anchoring sites" on the alginate matrix. The choice of the coating depends on many factors, with the major one being the type of the cultured cells, as the properties of cell membrane determine its affinity to binding sites on the porous carrier. Cost and availability of the anchoring coating are important factors too, especially given that the scaffolds are deemed to be used routinely. Relatively cheap and readily available gelatin represents a balanced choice of anchoring coating molecule for a routine use.

The freeze-drying technology of alginate-containing scaffolds, as a rule, involves production of the ionically-cross-linked gel, which is followed by its freezing and lyophilization to generate wide pores in the scaffold [31, 33, 34]. This rather simple procedure has, however, some limitations. Firstly, initial concentrations of gel precursors, sodium alginate and respective calcium salt (e.g., chloride, acetate, etc.), must be sufficiently low to allow some time for preparation of a mixture and its molding into the desired shape. Increase of the concentrations above certain makes the process of cross-linking the alginate chains with Ca^{2+} proceed excessively quickly, which makes it difficult to prepare homogeneous feed and distribute it in appropriate mould for gelation. As a result, very inhomogeneous Ca-alginate gels are produced that consist of locally strongly cross-linked regions neighbouring with weakly cross-linked ones. The mechanical strength of this "inhomogeneous" alginate scaffold is significantly impaired, due to the presence of weakly-linked zones. Secondly, traditional Ca-alginate gels and scaffolds are insufficiently stable chemically, when exposed to aqueous media containing solutes capable of ion-exchanging or extracting Ca^{2+} from the Ca-alginate complexes (e.g. ions of alkaline metals, hydrophosphate or dicarboxylate anions, chelators, etc.). This exposure results in gradual solubilisation of the scaffold. Hence, increasing the mechanical strength and stability against the dissolution is critical for potential use of porous Ca-alginate-based scaffolds in tissue engineering.

This paper describes the preparation of a novel wide-pore alginate scaffold that contains mechanically incorporated or chemically coupled gelatine as an anchoring coating. The Ca-alginate-based gelatine-containing scaffolds were seeded with adult human bone marrow MSCs. Adhesion of the cells as well as their proliferation and differentiation during in vitro culture have been explored in order to reveal the most favourable variant of the scaffold.

2 Materials and methods

2.1 Preparation of scaffolds

The polymeric matrices for the wide-pore alginate-gelatin scaffolds were prepared in accordance with the method [35] as is schematically shown in Fig. 1. We tested two different methodologies of gelatin addition.

2.1.1 Alginate scaffolds with incorporated gelatin additives (Fig. 1a)

Gelatin from porcine skin, Type A, 300 Bloom (Sigma, USA) was dissolved in 1.5% (w/w) aqueous solution of sodium alginate (Manugel DMD, ISP, USA). The dissolution was assisted by heating the solution to +40°C. Three different concentrations of gelatin were tested: 0.125, 0.25, or 0.5%. The solutions were poured into 40-mm plastic Petri dishes (at the layer thickness of 2 mm) and quickly transferred into the chamber of FP 45 MH programmable cryostat (Julabo, Germany). The samples were kept at −15°C for 3 h, after which they were transferred into the Freezone 1L freeze-drier (Labconco, USA) and lyophilized for 24 h. The dry wide-pore alginate-gelatin disks were then immersed in the saturated ethanolic solution of $CaCl_2$ (Aldrich, USA) and kept there for 3 days with gentle shaking. After rinsing with ethanol the disks were stored in the 70% aqueous ethanol at 4–6°C prior to use. We refer to

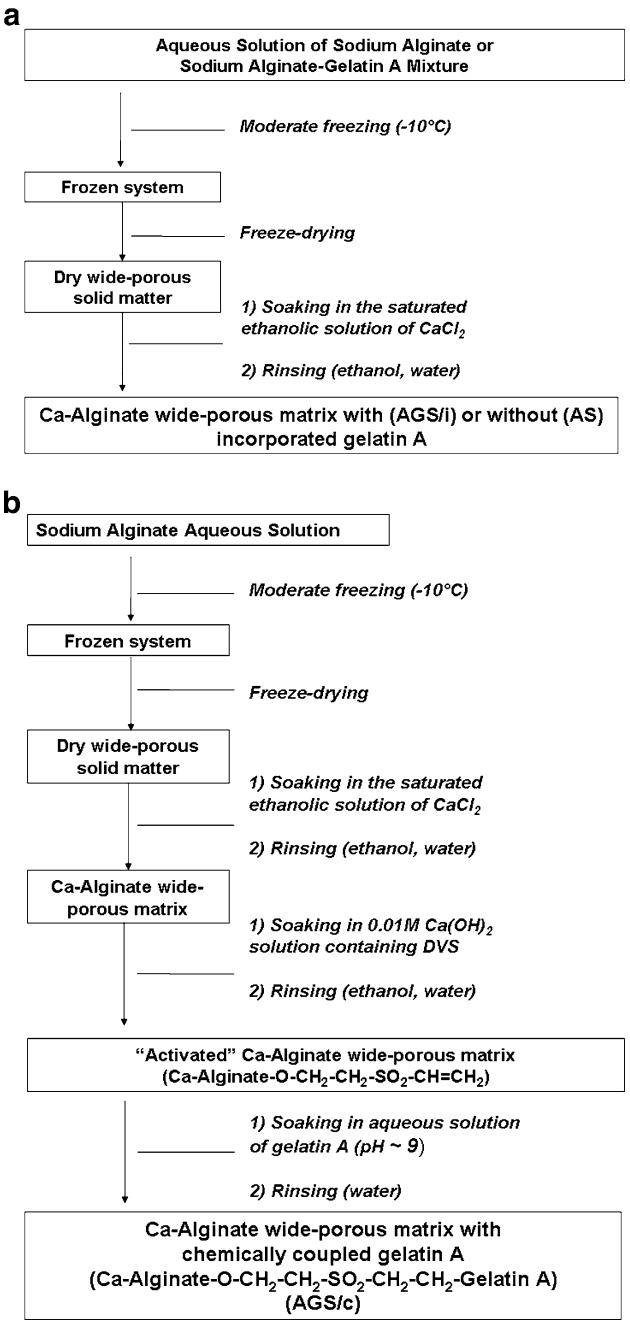

Fig. 1 Schematic diagram of the preparation of wide-pore alginate-gelatin scaffolds of the AS, AGS/i (**a**) and AGS/c (**b**) types

these alginate-gelatin matrices as AGS/i (Alginate-Gelatin Scaffolds with incorporated protein) with digital index indicating initial gelatin concentration (e.g., AGS/i/0.25).

2.1.2 Alginate scaffolds with chemically attached gelatin (Fig. 1b)

The alginate matrices were manufactured according to the same protocol as the one used for AGS/i, only excluding the addition of gelatin, which is referred to below as AS (Alginate Scaffolds). AS were soaked in an excess volume (100 ml per 3 items) of 0.01 N $Ca(OH)_2$ (Reakhim, Russia) aqueous solution for 30 min, followed by the addition of divinyl sulfone (DVS) (Sigma, USA) (0.76 ml per 100 ml of liquid phase) and gentle shaking for 3 h. The disks were next rinsed with pure water to achieve neutral pH and immersed in 1% (w/w) aqueous solution of gelatin adjusted to pH9 with $Ca(OH)_2$. The disks were gently shaken in the gelatin solution for 5 h at 40°C, then rinsed with warm water and stored in 70% aqueous ethanol at 4°C prior to use. We refer to these matrices as AGS/c (Alginate-Gelatin Scaffolds with chemically coupled protein). The amount of grafted gelatin ($\sim$0.3 mg per 1 g of wet matrix) was determined from the amino acid analysis after acid hydrolysis (5.7 N HCl, 105°C, 24 h) of a scaffold sample, similarly to as it was done for agarose-gelatin wide pore scaffolds [28].

2.2 Cells isolation and culture

Human bone marrow was aspirated from the iliac crest of healthy adult donors after receiving an informed consent, in appliance with Ethical Guidelines. The study was approved by the Institute for Problems of Cryobiology and Cryomedicine Ethical Committee for the usage of biological material for research purposes. Cells were flushed out from the bone marrow with α-Minimal Essential Medium (α-MEM) containing 50 units/ml penicillin, 50 μg/ml streptomycin and 0.2 mM L-glutamine, collected and centrifuged at 150×*g* for 10 min. Cell pellets were resuspended in the complete medium, which contained α-MEM, supplemented with 15% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, plated in T75 tissue culture-treated flasks (Nunc, USA) and grown at 37°C in a humidified incubator at 5% CO_2. After 48 h of culturing, the non-adherent cells were removed and fresh medium was added to the cells. After reaching subconfluence, cells were harvested by trypsinization and replated 1:3. Cells were cultured during four passages in total.

2.3 Flow cytometry analysis

Cells were directly stained using FITC- or PE-conjugated monoclonal antibodies (mAbs). The following mAbs were used: CD29-PE (Serotec, USA), CD34-FITC (Dako, Denmark), CD45-PE (Serotec, USA), CD73-PE (BD Biosciences, UK), CD90-FITC (Serotec, UK), CD105-FITC (Serotec). Briefly, trypsinized cells were resuspended in PBS and centrifuged at 200×*g* for 10 min. Pellets were resuspended in 50 μl of PBS and 5 μl of mAbs were added to each sample. After 30 min incubation in dark at room temperature, cells were washed twice with PBS and

resuspended for analysis. Flow cytometry was carried out on a FACSCalibur (BD Biosciences, UK) cytometer and the data was analyzed using WinMDI 2.8 software.

2.4 Scaffolds seeding

After the fourth passage, the cells were detached for the cell-seeding. Scaffold seeding was performed as described in [36] using simple system of two syringes connected with silicon rubber tube. Briefly, 5 mm in diameter disks of AS, AGS/i or AGS/c were placed in one syringe, with the complete filling of syringe inner diameter. Another syringe was filled with 100 μl of cell suspension (3×10^5 cells). Using gentle perfusion for 2 min, the scaffolds were filled with cell suspension and placed into the incubator for 2 h. After the incubation, the cell-seeded disks were removed from the syringe and placed in 24-well plates in complete medium for culturing.

2.5 Cell morphology and distribution analysis

Cell morphology within scaffolds after 7 days of culturing was estimated by staining of MSCs by fluorescein diacetate (FDA). Inclusion of fluorescent dye into cells was investigated by inverted confocal laser scanning microscope LSM 510 META controlled by LSM 510 ver. 4.2 software (Carl Zeiss, Germany).

Cell distribution within scaffolds was assessed by MTT (3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide) REDOX indicator. Briefly, after 7 days of culture cell-seeded macroporous alginate scaffolds were incubated during for 3 h at 37°C with 5% CO_2 in the complete culture medium, supplemented with MTT solution (Sigma-Aldrich, 5 mg/ml). After the removal of culture medium scaffolds were transferred into colorless PBS for further microscopic observations.

2.6 Scanning electron microscopy (SEM)

For SEM, the cell-seeded wide-pore matrices were fixed with 2.5% glutaraldehyde for 24 h at room temperature. After thorough washing with PBS, the cells that adhered to the scaffold were dehydrated in increasing concentrations of ethanol (40, 60, 80 and 100% for 10 min each) and allowed to dry on a clean Petri dish at room temperature. The surface and cross-section of the cell-adhered scaffold sections were observed by SEM (PEM-125K, Ukraine) after coating with silver layer.

2.7 Adipogenic differentiation

Adipogenic differentiation medium consisted of α-MEM supplemented with 10% FCS, 50 U/ml penicillin, 50 mg/ml streptomycin and 0.2 mM L-glutamine with the following adipogenic stimulants: 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma-Aldrich, St. Louis, MO), 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin, and 100 μM indomethacin (Sigma-Aldrich). Following 21 days of culture with adipogenic supplement, 2- and 3-D cultures were fixed in 4% buffered formalin for 30 min at 4°C and stained for 60 min with 1 μg/ml Nile Red solution (Sigma-Aldrich) diluted in PBS. After 60 min of incubation the cultures were thoroughly washed with PBS. In several experiments, the cultures were additionally stained for 10 min with DAPI (0.1 μg/ml, Sigma-Aldrich) and assessed by confocal microscopy (Carl Zeiss LZW 510).

2.8 Osteogenic differentiation

Osteogenic differentiation medium consisted of α-MEM supplemented with 10% FCS, 50 U/ml penicillin, 50 mg/ml streptomycin and 0.2 mM L-glutamine with osteogenic supplements: 20 mM ascorbic acid (Sigma-Aldrich), 10 mM β-glycerolphosphate (Sigma-Aldrich) and 1 μM dexamethasone (Sigma-Aldrich). Following 21 days of culture with adipogenic supplement, two- and three-dimensional cultures were fixed in 4% buffered formalin for 30 min at 4°C. Alkaline phosphatase expression was assessed using Fast Blue RR Salts/Naphtol kit (Sigma-Aldrich) during 30 min at room temperature in the dark. Extracellular accumulation of Ca^{2+} was assessed by von Kossa staining.

2.9 Chondrogenic differentiation

For chondrogenic differentiation, cell micromasses and cell-seeded alginate-gelatin scaffolds were cultured in chondrogenic differentiation medium consisting of α-MEM, supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin and 0.2 mM L-glutamine with chondrogenic supplements: 20 mM ascorbic acid (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin and 100 μg/ml sodium piruvate (Sigma-Aldrich), and 10 ng/ml TGF- β3. To make the micromasses, 10-μl droplets (containing 100,000 cells) were seeded in culture dishes in serum-free chondogenic medium and allowed to form cell aggregates during 6 h at 37°C. After that, chondrogenic medium was carefully added around the cell aggregates, and the cultures were kept in such conditions during 28 days with the medium being changed 2 times per week. After 28 days of culture, AGS or cell aggregates were fixed with 4% buffered formalin for 30 min at 4°C and 15 and 150 μm slides were prepared. Slides were stained with alcian blue (Sigma-Aldrich) for 1 min, followed by counter staining with Safranin for another 1 min.

2.10 Alamar blue assay

To determine the efficiency of scaffold modification and proliferation activity of MSCs within the AS, AGS/i or AGS/c, the Alamar Blue (AB, Serotec) assay was employed. Briefly, on the 1st and 7th day post-seeding, triplicate samples of scaffolds, containing MSCs, were incubated 2 h with the complete medium, containing 10% AB. The control cells (in the identical concentrations) were cultured in a monolayer and incubated with AB at the same time points. Reduced AB solution were collected with medium change and the fluorescence level of AB was assessed using microplate reader Tecan Genios (Tecan, Austria) with the excitation wavelength 550 nm and emission wavelength of 590 nm. Data were analyzed using XFluor4 software, blanked and presented as Relative Fluorescent Units per well.

2.11 Statistical analysis

Each experiment was performed in triplicate and repeated four times. Data was presented as mean ± SEM. Student's t-test was used for statistical analysis. Data was considered significantly different, if $P < 0.05$.

3 Results

3.1 Wide pore alginate/gelatin scaffolds

The diagrams in Fig. 1 show the sequences of preparative steps that were used in this study for the fabrication of wide-pore spongy carriers AS and AGS/i (Fig 1a) as well as AGS/c (Fig. 1b), that were subsequently examined as MSCs culturing environment.

Having employed the procedures described above, the respective scaffolds have been moulded into a shape of 2-mm-thick discs. In the case of AGS/i, various amounts (0.125–0.5%) of gelatin were mechanically incorporated into the bulk of pore walls of the heterophasic wide-pore material, while in the AGS/c case the "anchor" gelatin macromolecules were covalently bound to the inner surface of pore walls after the activation of the polysaccharide core of Ca-alginate network with divinylsolfone (DVS). Judging by the dry weight of polymers used for the preparation of the scaffolds, the overall content of gelatin was as follows: ~7.7% (AGS/i/0.125), ~14.3% (AGS/i/0.25), 25% (AGS/i/0.5) and ~0.2% (AGS/c). That is, the systems with the physically incorporated gelatin contained considerably (by a factor of 35–125) higher amount of the protein as compared to AGS/c scaffolds that carried only a small amount of gelatin covalently attached to the inner surface of the walls of macropores.

A wide-pore texture of ASs and AGSs was produced by the co-called cryostructuring technique [14], which uses the polycrystals of frozen solvent act as porogens. Of note is the fact that, the lower is the freezing temperature, the smaller crystals are, as a rule, formed. Thus, a certain optimization of the freezing conditions is necessary in order to adjust the pores in such polymeric matrices to desired dimensions, since the pore size in the range of 100–300 μm is usually required for culturing in 3D scaffolds, [23, 24, 37].

In the preliminary experiments, the screening of different temperature regimes was performed in order to achieve the desired extend of porosity. The temperature of about −10°C was found to be the most appropriate for the preparation of alginate-based sponges with required pore size (Fig. 2). Figure 2 demonstrates the wide-pore morphology inherent in such Ca-alginate-based cryostructurates fabricated in accordance with "freeze-drying—Ca-curing" scheme [35] depicted in Fig. 1. The structure somewhat resembles spider's web. This kind of morphology is frequently typical for the freeze-dried polymeric matrices, especially when freezing of initial aqueous solutions is carried out at moderate sub-zero temperatures, at which the branched ice crystals (snowflakes) are formed [38]. The evaluation of the size of the gross pores using AxioVision v.4.7 program showed that their average diameter was 106.2 ± 39.6 μm; i.e. wide enough for free penetration of MSCs upon their seeding into the scaffold.

3.2 Characterization of MSC

The defining characteristics of MSC are appropriate immunophenotype and the ability to differentiate along adipogenic, osteogenic and chondrogenic lineages in

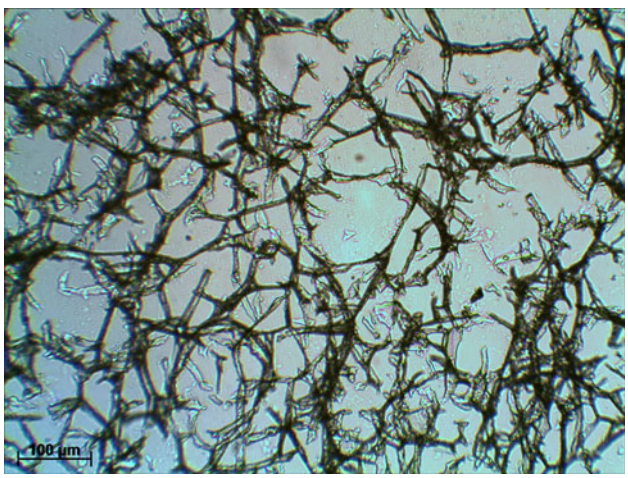

Fig. 2 Histological slide of alginate-gelatin scaffold

response to specific inductive stimuli. To confirm that isolated bone marrow fibroblast-like cells satisfy these criteria, their immunophenotype and multilineage differentiation potential were assessed. The immunophenotypic analysis of cells after 3–4 passages showed that they were 95% positive by CD29, CD73, CD90 and CD105 antigens and did not express hematopoietic markers CD34 and CD45 (Fig. 3). Such immunophenotype was described elsewhere for mesenchymal stromal cells [2, 3].

After 21 days of osteogenic induction, the cells acquired several properties of bone cells: they expressed alkaline phosphatase (Fig. 4a) and accumulated mineralized extracellular Ca^{2+} (Fig. 4b). In response to adipogenic stimuli, cells accumulated intracellular lipid droplets, which were positively stained by Nile Red (Fig. 4c). After 28 days of chondrogenic culture, cell microaggregates were analyzed using histochemical techniques. Cells were found capable to differentiate into chondrogenic lineage, which was confirmed by alcian blue/safranin staining (Fig. 4d).

Thus, fibroblast-like cells isolated from adult human bone marrow, that we used in this study, have all defining characteristics of mesenchymal stromal cells [2, 3].

3.3 Cell seeding into AS and AGS, growth and differentiation

After seeding the MSCs into the gelatin-free Ca-alginate scaffold (AS), no attachment and proliferation were indicated (Fig. 5). Two methods of Ca-alginate surface modification were assessed: direct incorporation of gelatin over the range of 0.125, 0.25 and 0.5% prior to freeze-drying (Fig. 1a) or chemical coupling of gelatin to the pore surfaces of the scaffold after respective chemical "activation" of the alginate matrix (Fig. 1b). To assess the efficiency of scaffold modification, MSCs were seeded and their metabolic and proliferation activity was determined by the AB assay on the 1st and 7th day (Fig. 5).

Figure 5 shows that mechanical incorporation of gelatin at different concentrations (AGS/i group) could not promote proliferation of MSCs within the scaffolds. However, chemical activation of the polymer matrix followed by attachment of gelatin molecules to the pore inner surface (AGS/c group) resulted in the 3.5-fold increase in AB fluorescence on the 7th day compared to 1st day, which indicates cell proliferation (Fig. 5). This parameter was significantly different ($P < 0.05$) compared to that of AGS/

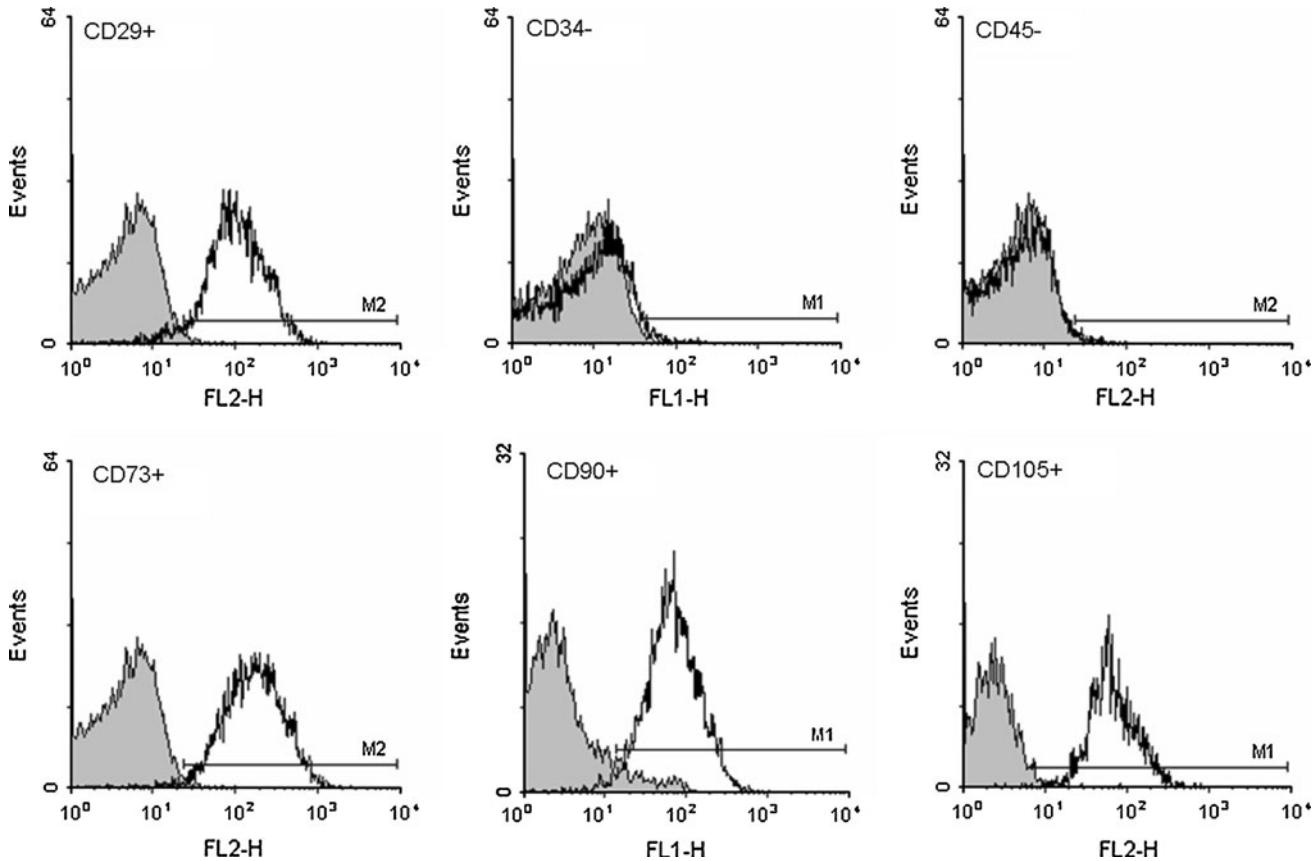

Fig. 3 Flow cytometry analysis of adult human bone marrow fibroblast-like cells

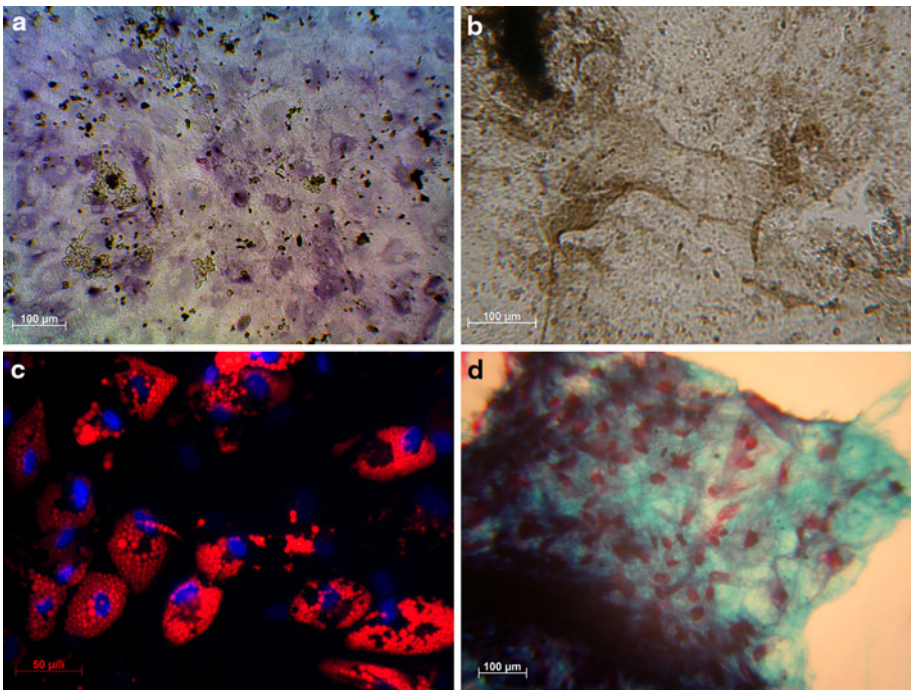

Fig. 4 Multilineage differentiation of MSCs in a monolayer culture: alkaline phosphatase expression (**a**) and matrix mineralization (**b**, von Kossa staining) in the osteogenic cultures; adipogenic differentiation (**c**, Nile red staining), chondrogenic differentiation (**d**, alcian blue/ safranin staining)

i group and did not differ from control MSCs, grown in monolayer (Fig. 5). Thus, chemical coupling of gelatin macromolecules to the surfaces of pore walls in alginate scaffold turned out to promote the main functions of MSCs such as adhesion and proliferation.

This data was further confirmed by investigation of the viability, morphological properties and distribution of MSCs within the scaffolds using the FDA and MTT redox dye staining. After 7 days of MSCs culture, within AS and all types of AGS/i scaffolds the formation of cell aggregates was observed, regardless of the amount of gelatin incorporated into the alginate matrix (Fig. 6a). Conversely, in the case of MSCs culturing within AGS/c, the cells had flattened fibroblast-like morphology (Fig. 6b). The analysis of the cell distribution within the scaffolds, prepared by MTT staining, showed uniform distribution of MSCs within the whole volume of AGS/c and almost complete filling the scaffolds with cells

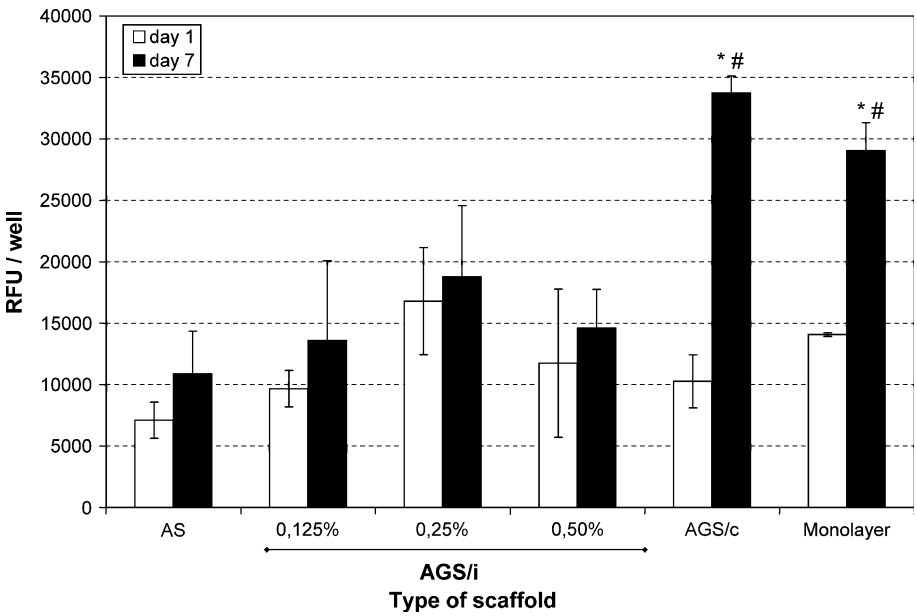

Fig. 5 Alamar blue assay of MSCs seeded into wide-pore Ca-alginate scaffolds modified by different methods. * Values are significantly higher versus that of AS and AGS/i groups ($P < 0.05$). # Values are significantly higher versus that measured on day 1 in the same groups ($P < 0.05$)

(Fig. 6d). At the same time a discrete distribution and poor extension of cells were observed within AS and AGS/i (Fig. 6c).

Normal adhesion and distribution of MSCs within AGS/c were confirmed by SEM observations (Fig. 6e, f). Cells appeared to be flattened with fibroblast-like morphology, growing and filling spaces within the outer and inner surfaces of pores (Fig. 6f).

It should be mentioned that during the whole cultivation period (7 days and longer) the scaffold did not change its integrity and strength, thus demonstrating good stability in the culture medium, which contained high enough (around 0.15 mol/l) levels of monovalent cations capable of, as it was already pointed out above, solubilizing common Ca-alginate matrices.

The next step of the study was to investigate the ability of bone marrow MSCs to differentiate in vitro into mesenchymal cell lineages during culture in three-dimensional modified alginate-gelatin carriers. During adipogenic differentiation, the accumulation of intracellular neutral lipids stained by Nile red in MSCs cultured within AGS/c was observed (Fig. 7a). Osteogenic differentiation capacity of MSCs after 3 week of induction in spongy scaffolds was confirmed by alkaline phosphatase expression (Fig. 7b). After 28 days of chondrogenic differentiation, cells accumulated extracellular matrix, which was positively stained by alcian blue (Fig. 7c, d). In the control group,no alcian blue staining was observed. Fig. 7d shows that cells did not only grow on the pore walls' surfaces of the scaffold, but also filled spaces between them.

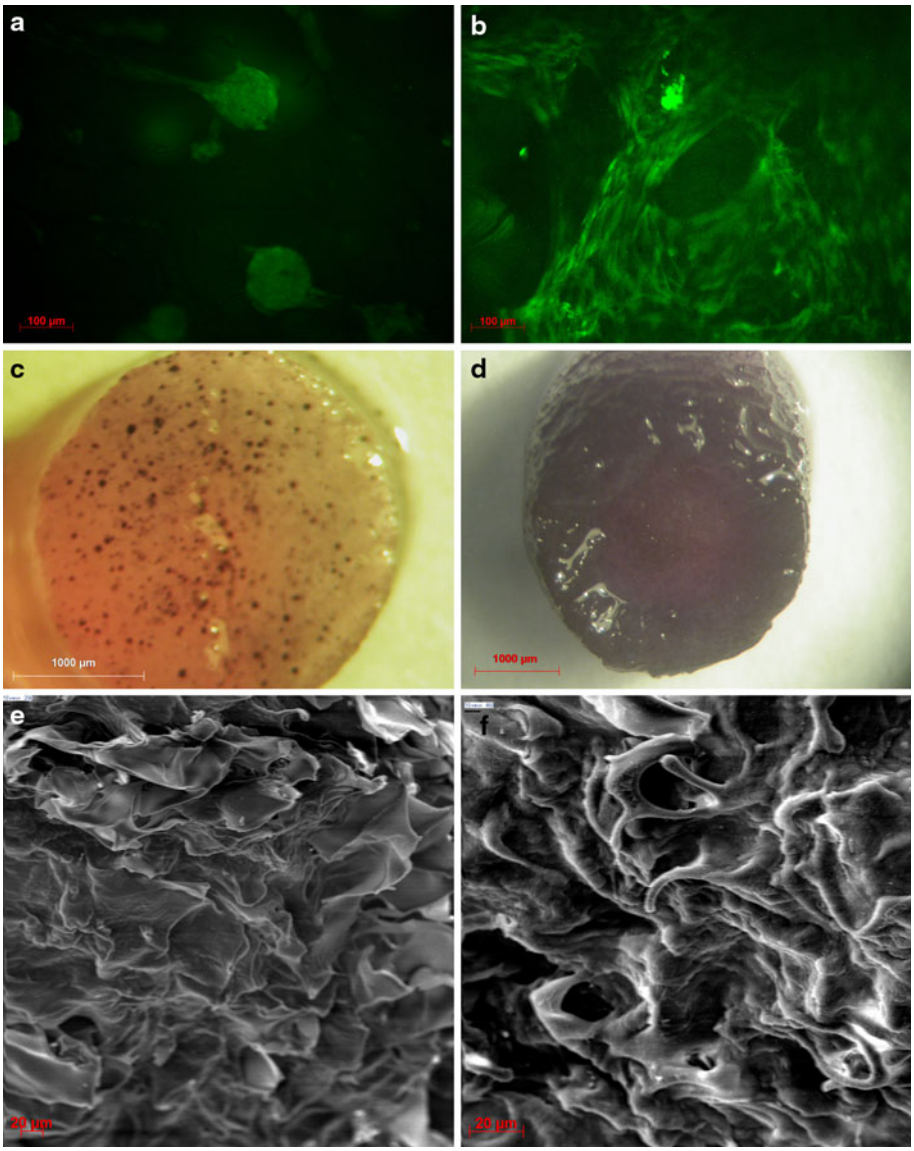

Fig. 6 Morphology and distribution of MSCs within matrices AS and AGS/i (**a**, **c**), compared to matrices AGS/c (**b**, **d**, **f**) on the 7th day of culture: **a**, **b**—FDA staining; **c**, **d**—MTT staining; **e**, **f**—scanning electron microscopy of the unseeded AGS/c (**e**) and MSCs-seeded wide-porous AGS/c matrix (**f**)

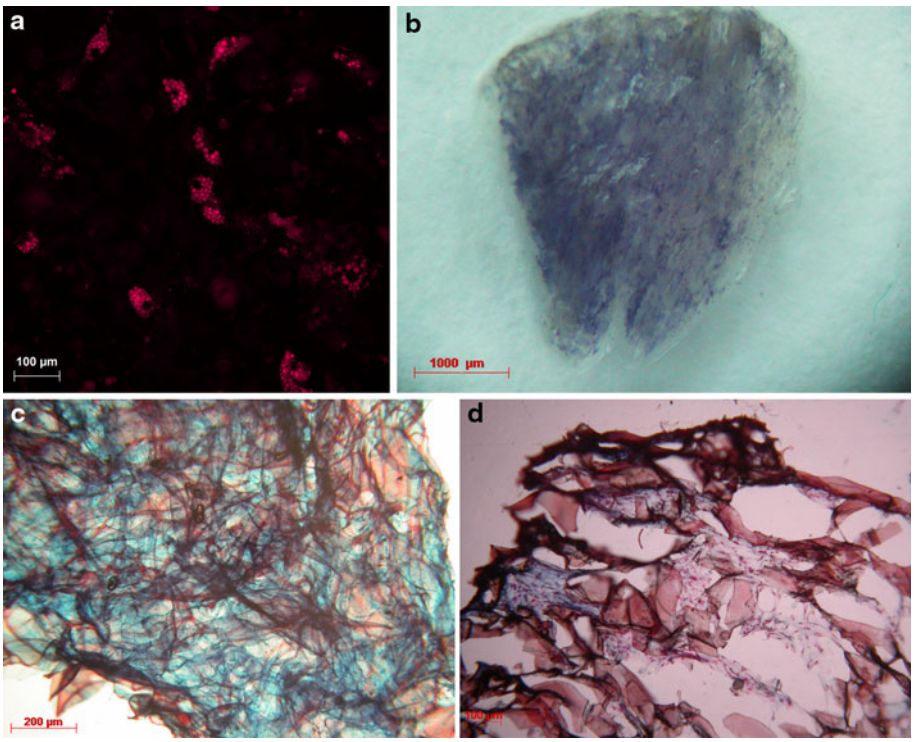

Fig. 7 Multilineage differentiation of MSCs within three-dimensional AGS/c: **a**—adipogenic differentiation (Nile Red staining); **b**—osteogenic differentiation (alkaline phosphatase expression); 150 µm (**c**) and 15 µm (**d**) sections of chondrogenically induced MSCs within AGS/c (alcian blue/safranin staining)

4 Discussion

Although many biopolymers are used for the fabrication of scaffolds for tissue engineering, alginate has a great promise due to confirmed biocompatibility [8, 23]. Various researches have investigated the ability of alginate-based matrices to act as a porous scaffold for tissue engineering [7, 33, 34, 39–41]. In this study we examined the behavior of human adult bone marrow mesenchymal stromal cells after seeding into wide-pore alginate scaffolds. Alginate-based porous scaffolds for cell culture are known to be commonly prepared through the freeze-drying procedure after mixing the solution of polymeric precursor (sodium alginate) with additives of respective salt containing cross-linking divalent cations (mainly, Ca^{2+}) capable of causing the ionotropic gelation [33, 42]. In this case, the stability of the scaffold in the culture medium is governed by the molecular properties of the alginate used; mainly, by its molecular weight and mannuronate/guluronate ratio, as well as by the amount of interchain salt links. If the quantity of Ca^{2+} ions incorporated into the 3D network is low, the resulting Ca-alginate matrix will rather quickly be dissolved in culture medium, due to the ion-exchange with Na^+ and K^+ ions routinely present in such media. On the other hand, if the concentration of calcium salt used for alginate curing is high, the Ca-alginate scaffold is sufficiently stable in the culture media, but the ionotropic gelation occurs too rapidly, thereby causing significant technical difficulties in the moulding the final scaffold into the desired shape.

Therefore we employed an alternative approach of preparation of Ca-alginate-based scaffolds–freeze-drying of alginate solution (or mixed alginate + gelatin solution) followed by ion-exchange of Na^+ for Ca^{2+} in the medium of a non-solvent (Fig. 1). This approach allows varying both the initial alginate concentration and the amount of Ca^{2+} ions incorporated in the wide pore matrix within broad limits [35]. This protocol allowed us to prepare wide-pore spongy alginate or alginate-gelatin carriers for subsequent culturing of MSCs.

After seeding the MSCs into unmodified alginate scaffold (AS), no cell adhesion and proliferation were observed. These results confirm the data reporting the absence of cellular recognition proteins on alginate matrices, which limits cell attachment to the natural polymer [12, 43–45]. Many authors improved attachment and proliferation of different animal and human cell lines within alginate scaffolds by either grafting peptide sequences onto alginate materials [45] or by incorporation of other substances such hyaluronic acid [31], β-TCP [12], gelatin [46], chitosan [47] or even by preparing multicomponent scaffolds, such HA-alginate-gelatin [7] or β-TCP-alginate-gelatin [6]. Lawson et al. [12] showed that alginate gels containing either collagen-1 or β-TCP were found to be capable of supporting human bone marrow stromal cell adherence and proliferation. Bernhardt et al. [7]

demonstrated high proliferation rate and osteogenic differentiation capacity of human bone marrow MSCs cultured within the alginate-gelatin-HA scaffolds. It has been shown that peptide-linked alginate scaffolds supported the adhesion and proliferation of seeded sheep preadipocytes and adipose tissue formation [48].

In the present study we used type A gelatin as an additive during formation of AGS in order to improve adhesive properties of wide-porous alginate scaffold. Gelatin is derived from collagen, but is less immunogenic compared to its precursor and has excellent biocompatibility, and gelatin hydrogels possess plasticity and adhesiveness. It has been shown by Yang et al. [46, 49] that gelatin-based matrices could be effective for MSCs adherence and proliferation. Hong et al. [50] have revealed the suitability of gelatin sponges for adipose tissue engineering. At the same time Kathuria et al. [51] showed that gelatin cryogels appeared to be very weak mechanically and could not even withstand its own weight, however the application of chitosan–gelatin scaffolds promoted good cell adhesion, proliferation and secretion of extracellular matrix in the cryogel matrices by fibroblast cell line Cos-7.

In our study, when the Ca-alginate scaffolds fabricated according to Fig. 1a, with addition of different (0.125, 0.25 and 0.5%) amount of gelatin (AGS/i scaffolds), were examined for MSCs culturing, no cell proliferation during 7 days of culture was observed. This fact can be explained by the hypothesis that mechanical incorporation of gelatin in the alginate bulk could provide very few number of gelatin adhesion molecules on the pore surfaces of alginate matrix ready to interact with cells.

Alternatively, when the gelatin-free Ca-alginate sponges were modified with DVS, followed by covalent coupling of gelatin molecules onto pore wall surfaces of the wide-pore material (AGS/c), the scaffolds exhibited good cell adherence. One of the features of the procedure implemented for the preparation of AGS/c matrices (Fig. 1b) was the application of $Ca(OH)_2$ instead of commonly employed $NaHCO_3$ [52] during the steps, when alkaline media were required for reactions to proceed (modification of the polysaccharide with DVS and chemical attachment of gelatin to the activated carrier). The $Ca(OH)_2$-containing solution was employed in order to omit dissolution of Ca-alginate matter under the reaction's pH. Such a modification of the reaction conditions allowed preparing the desired target scaffold—AGS/c.

As a result, after seeding of MSCs into modified AGS/c scaffolds, we observed normal attachment and morphology of cells, confirmed by SEM. At that, the cells cultured within AGS/c manifested improved proliferation activity assessed by Alamar blue assay. Further differentiation studies showed that MSCs preserved their multilineage differentiation potential, which was detected previously in monolayer culture.

5 Conclusions

We have assessed two protocols of manufacturing Ca-alginate-gelatin wide pore scaffolds using freeze-drying. The scaffolds were prepared and tested for culturing of human bone marrow mesenchymal stromal cells. The first protocol included a mechanical incorporation of gelatin (type A) in the alginate bulk via freeze-drying of initial mixed Na-alginate + gelation aqueous solution. The second protocol included four main steps: freezing of initial Na-alginate solution, ice sublimation from the frozen sample, curing the freeze-dried matter with calcium ions in polar organic medium with subsequent chemical coupling of gelatin to the wide pore matrix. Although both technical approaches resulted in fabrication of wide-pore scaffolds from Na-alginate solutions of any suitable concentration, the functional tests have revealed a marked superiority of the second protocol. The scaffolds obtained using this methodology possessed desired porous morphology and ability to adhere stromal cells, as well as facilitated chemical stability in the aqueous cultural media. The scaffold preparation technique based on covalent binding of gelatine not only promoted attachment and growth of human bone marrow mesenchymal stromal cells within the scaffold, but also helped preserving the main functional parameters of the cells, e.g. capacity to multilineage differentiation. The in vitro results indicate that the Ca-alginate wide-pore scaffolds with anchor gelatin covalently-attached to the surface of the pore wall can be useful for stem cell-based bone, cartilage and adipose tissue engineering.

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