Functionalization of tissue equivalents based on sodium alginate by human blood plasma

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Abstract. The work is aimed at the development of biodegradable porous frame materials that combine high biocompatibility and possess pro-angiogenic properties, that is, the ability to ensure the active germination of the vessels of the recipient tissue and, possibly, the formation of the de novo vascular bed. Approaches have been developed to create a two-layer matrix structure based on biopolymers - sodium alginate and collagen. The two-layer structure provides the functions of the skin - protective (from external environmental factors) and regenerative - restoration of epithelial tissues of the dermis. The article shows the functionalization of the polymer framework by growth factors — enriched and depleted human blood plasma in different concentrations from 5 to 25 masses. % Methods of functionalizing the polymer matrix were studied and the influence of the inclusion of growth factors on cyto- and biocompatibility, angiogenic properties was established.

1. Introduction
An increase in the number of patients with diabetes mellitus inevitably leads to a significant increase in patients with its late complications. In this regard, the modern conservative treatment of chronic wound defects of the lower extremities and the prevention of possible amputation remains relevant in modern medicine. The death of a large area of skin and the “taking” of skin for autotransplantation are accompanied by the development of infection and superinfection, loss of proteins, electrolytes, water, plasma, and pathological regeneration [1]. At present, it has become apparent that the best results in optimizing the processes of tissue defects reorganization allow using natural polymers capable of controlling the synthesis and orientation of fibrous structures [2]. The combination of such polymers with growth factors makes it possible to control the formation of granulations, accelerate the epithelization of skin defects, and obtain connective tissue that differs little from surrounding healthy skin. To create a complete bioengineered skin equivalent, a cell carrier is needed in which fibroblasts live, and multipotent stromal cells serve as the basis for keratinocytes forming a multilayer epidermis on their surface when cultured in vitro [3-5]. For such purposes, sodium alginate seems to be the most interesting as the main component, which has unique properties that allow it to form strong three-dimensional matrices of various structures and are widely used for the regeneration of other types of tissue. Recently, there have been many studies aimed at developing methods for the functionalization of materials for the regeneration of the skin [6]. The use of plasma rich in platelets of a patient (PRP) or, for medical reasons, a donor that contains all growth factors (PDGF, EGF, FGF, VEGF and TGF-b), is a promising opportunity for increasing angiogenic properties in wound healing [7].
Abbreviations: AG: sodium alginate, CL: collagen, MSC: mesenchymal stem cells, PKC: primary human epidermal keratinocytes, sadSC: subcutaneous adipose-derived stromal cells, sFb: primary human skin fibroblasts.

2. Materials and methods

2.1. Reagents
Sodium alginate (AG, CAS Number 9005-38-3), sodium lauryl sulphate (CAS Number 151-21-3), calcium chloride (CAS Number 10043-52-4) and magnesium chloride (CAS Number 7786-30-3) were purchased from Sigma-Aldrich (USA). Collagen (CL) (Moscow, Russia).

2.2. Получение трёхмерных тканевых эквивалентов.
The highly interconnected sponge scaffolds were fabricated from sodium alginate and collagen solutions by the freeze-drying procedure. Specifically, the AG solution (2%) was prepared by dissolving polymer powder in distilled water at RT using IKA EUROSTAR 40 digital stirrer (Germany) at 1000 rpm for 30 min. The CL solution (1%) was obtained from the prepared solution. Other solutions included sodium lauryl sulphate solution (surfactant, 10%), barium chloride solution (10%), calcium chloride solution (10%). The ratio of components used for production of different sponge scaffolds is listed in Table 1. The process of scaffold fabrication comprised five steps: 1) mixing solutions, 2) gelation and forming the cross-linked hydrogel, 3) freezing and drying by lyophilisation, 4) final cross-linking and 5) washing. Specifically, the relevant amount of alginate solution was mixed with collagen solution at 35°C for 30 min to form an unstabilized type of scaffolds. Then the 0.5% of calcium chloride solution was slowly added to this mixture and vortexes for 30 minutes at RT, and the surfactant solution was added and foamed for 10 minutes. The fixation temperature was -10°C for 2 h, -4°C for 1 h, -7°C for 10 h and -48°C for 12 h. Then, freeze-drying was carried out and finally, samples were cross-linked by Ba²⁺ in 30% EtOH. For freeze-drying procedure the vacuum dryer LS-1000 (Prointex-Bio, Russia) was used.

As a reference standard, a collagen spongy porous scaffold was prepared according to the generally accepted procedure consisting of cross-linking 1% colloidal acetic acid solution by 1 wt.% glutaraldehyde, 0.01 wt. % boric acid and 0.00015 wt. % of quinazole, fixation of structure and lyophilisation.

2.3. Research methods
The resulting materials were subjected to a comprehensive study using modern research methods. To study the microstructure of the obtained, on an alginate basis, multicomponent cellular carriers / matrices, scanning electron microscopy (TeScan Vega II SBU, Czech Republic) with local energy dispersive analysis (INCA) was used as the original method. Prior to imaging, the samples were sputter-coated with a 25 nm-thick gold layer to impart electrical conductivity to the specimen surface. Obtained images were used for assessment of scaffold porosity according to the formula:

\[ P = \left(1 - \frac{\rho_v}{\rho_t}\right) \times 100\% \]

where:
\( \rho_t \) - true density of the sample material in g/cm³;
\( \rho_v = \frac{m}{V} \) - the apparent density of the sample, where:
\( m \) - the mass of the sample, g;
\( V \) - the volume of the sample, cm³.

The true density (\( \rho_t \)) was determined by pycnometric method. The quantification of pore size and wall thickness was performed using ImageJ software. The tensile investigation of the specimens was performed according to ISO/DIS 527-3 standard. To assess the mechanical properties of the matrices, the strength characteristics tests in biological fluids at 37°C. For that five samples for each point were used. Tensile testing was carried out using an Instron ElectroPuls E-3000 testing machine (Bucks, UK) operating at a crosshead speed of 1 mm × min⁻¹ on Bio Bath. Statistical analysis was performed using SPSS software, version 17.0 (Statistical Package for Social Sciences, SPSS Inc., USA). To determine the phase and microstructural transformations of materials, a complex of studies of physicochemical...
properties was carried out infrared spectrometry (IR), energy dispersive analysis (EDA), viscosity of solutions, moisture permeability and water absorption, elasticity.

The primary cell cultures of skin dermal fibroblast (sFb), subcutaneous adipose-derived stromal cells (sadSC) and primary keratinocyte (PKC) were established from back skin explant of a healthy 16 year-old woman undergoing plastic surgery as previously described. Briefly the sadSC culture, the subcutaneous fat tissue has been separated from the skin and digested in 0.5 mg/ml collagenase type I solution for 30 min at 37°C in DMEM/F12 medium, washed twice and expended in the same medium supplemented by 10% FBS. For primary sFb culture, the skin was digested in 0.25% trypsin solution (Cat. No: 85450C, Sigma) in PBS at 4°C overnight to allow separation between dermal and epidermal skin layers. The dermis was further digested by collagenase type I and washed twice in DMEM/F12-10% FBS. Obtained cells were further cultured in the same medium. When confluent, the primary culture was trypsinized using 0.05% trypsin/EDTA (Cat. No: 25300062, Invitrogen) and subculture. The cells culture medium was replaced every 2-3 days.

The scaffolds biocompatibility was estimated as a function of scaffolds cytotoxicity and adhesiveness. To estimate the scaffolds cytotoxic effect to fibroblasts (sFb) and keratinocytes (PKC) the 24h-conditioned medium (CM) was used. The CM was obtained by incubation of each scaffold (d=5mm) in 1ml of DMEM/F12+10%FBS medium (for sFb and MSC) or KSFM for 24h under ordinary CO2 incubator condition. The sFb or sadMSC were plated at the density of 103 cells/w and PKC – at 2x103 cells/w in 96-well plates and cultured in sFb-sCM or in N-TERT-sCM correspondingly for short-term (24h) or long-term (96h). The cytotoxicity of scaffolds CM was assessed by FDA viability probe assay. The fluorescent metabolite FDA was measured using VICTOR X3 Microplate Reader (PerkinElmer) after administration of 0.5 μg/mL FDA (Cat. No: F7378, Invitrogen). To assess the vascularization capacity each type of sponge (diameter ~ 7mm, thickness ~ 1-1.5mm) was subcutaneously implanted through a small incision, in the humeral region of the back skin. The sponge implants was assessed after 10 days of implantation.

The obtained results were expressed as a mean ± standard deviation. All experiments were performed at least in triplicate. Non-parametric Kruskal–Wallis one-way analysis of variance by ranks was used for testing whether the samples originate from the same distribution (GraphPadPrism 5.0, GraphPad Software Inc.).

3. Results and discussion
Considering the differences in the structure of dermal and epidermal layers, we constructed a scaffold structurally close to the natural skin appearance. The spongy structure was made for the dermal layer, where the unitary fibroblasts are located in the thickness of the layer, and the film coating imitating the basement membrane for keratinocytes that form and multilayer epithelium.

The goal of this study was to assess cell adhesion capacity, biocompatibility and pro-angiogenic activity of AG-based matrices and thus to evaluate their suitability and efficiency as low-cost polymeric scaffolds of non-mammalian origin for skin bioengineering. Of all the crosslinking ions studied, only for Ba2+ cations, we were able to select conditions that allow obtaining biocompatible sponge carriers with the necessary strength characteristics. The use of Ca2+ and Mg2+ cations led to the formation of spongy matrices with a short lifetime, when cell structures were grown on them, and Sr2+ cations, providing the necessary strength, led to the production of low biocompatible with sFb and sadMSC. As a reference standard, a collagen spongy matrix was used, the formation of the porous structure was carried out according to the generally accepted procedure consisting in scaffold cross-linking by mixing of 1% acetic collagen solution with 0.01 wt. % glutaraldehyde, 0.01 wt. % boric acid and 0.00015 wt. % of quinazole, fixation of structure and lyophilization.
Figure 1. The microstructure of the obtained two-layer equivalent based on sodium alginate, cross-linked with barium cations at different magnifications.

Matrices obtained by the indicated method of functionalization of human blood plasma - depleted and enriched. Two approaches were used for this - vacuum infiltration and plasma incorporation into the initial hydrogel based on sodium alginate. Materials obtained using infiltration did not meet the stated goals and objectives of the study, since the structure is deformed under vacuum. Also, the amount of agent included is difficult to control, as is its output. In this regard, for further studies, model samples were obtained based on sodium alginate with plasma of various compositions included in the hydrogel - from 1 to 50 mass. %. It was shown that the inclusion of plasma does not have an important effect on the values of mechanical characteristics, as well as the porosity and microstructure of tissue equivalent samples (Table 1).

| Composition, % | AG 100 | AG \ PRP95 | AG \ PLP 95 |
|---------------|--------|------------|------------|
| Strength, MPa | 5.2    | 5.0        | 4.7        |
| Overall porosity (%) | 95±1 | 96±1 | 97±1 |
| Pore diameter, µm | 66±25 | 98±25 | 150±25 |

The effect of plasma concentration on cell proliferation in the developed materials was evaluated. It was found that the inclusion of plasma has an excellent positive effect on proliferation only until it is switched on within 25 masses. % An increase in plasma concentration leads to a significant deterioration in the properties of the matrix (see figure 2).

Figure 2. The effect of PRP content in the alginate matrix on the proliferation of human skin cells (48 hours in culture). sFb - human skin fibroblasts, PKC - primary keratinocytes of human skin.
In the course of the work, evaluation experiments were also carried out on the biocompatibility and cytotoxicity of matrices functionalized by human blood plasma and without matrices. It was found that the introduction of plasma into the tissue equivalent based on sodium alginate increases the proliferation and adhesive ability of cells, increases the biocompatibility of the material. Such studies suggest that functionalization will also successfully affect wound healing.

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