Cell growth improvement on the poly (lactic acid) surface with spray deposited separate native collagen fibrils

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***Abstract*— In this paper, a method of preparation of stable dispersion of individual collagen fibrils acceptable for spray deposition was proposed. Proposed method preserves the native structure of collagen fibrils that was confirmed by atomic force microscopy. It was shown that covering even a small percentage of the poly (lactic acid) surface with collagen fibrils has a more significant effect on cell growth than hydrophilicity factor. Poly (lactic acid) surface modified with deposited collagen fibrils demonstrates cells grow rate equal to the tissue culture plastic.**

Keywords*— *collagen, fibril, polylactic acid, biopolymers, cell growth, spray deposition, biomedical applications

I. Introduction

Biocompatible coatings are currently actively developed for surgery application for improving integration of various kinds of implants in the human body. However, materials commonly used as biocompatible coatings, for example poly (lactic acid) (PLA), are often hydrophobic, have weak durability and low cell proliferation on the surface compared even to the glass surface. Increasing of surface hydrophilicity and coating with fibronectin and other biomolecules can significantly improve cell attachment to the surface and provide cell growth improvement, compared to the unmodified surface [1].

Collagen addition to the coating composition along with fibronectin also significantly accelerates cell growth [2] improving the integration of implants in comparison with pure PLA or unmodified biocompatible materials, but collagen is much cheaper than fibronectin that makes it much more promising material. Biocomposites production by chemical bonding of its components with cross linking agents or chemical modification of any of the components, have some disadvantages. The presence of a residual amount of cross-linking agents and changes in the structure of collagen is among them. Therefore, the development of special methods of biocomposite processing is required, especially when natural biopolymers (peptides, polysaccharides, etc.) are used [3].

Besides that, collagen is one of the intercellular matrix base material and thus its presence is important for growing of most types of connective tissue cells. Acceleration of tissue growth is particularly important for implants intended to advance osteoblast proliferation and bone tissue regeneration respectively. For this purpose, an addition of collagen to calcium phosphate coating, which is widely used in stomatology and orthopedics, can increase not only durability, but significantly improve primary cell attachment to composite implant surface [4]. Cell proliferation and adhesion are strongly influenced by methods of producing and treatment of collagen. For example, PLGA/collagen composite material obtained with using collagen in acid solution has not demonstrated significant improvement of cell growth on its surface [5]. Water-based collagen solution can be obtained by using acid medium that significantly simplifies its processing, but strongly modifies its structure and properties. It leads to the changes of its interaction with cells and its uselessness and even toxicity because of cell growth suppression on such surfaces [6]. Similar cell growth rate slowing occurs after collagen heat treatment and usage of hydrolyzed collagen [7].

As can be seen from the above, to get maximum possible cell growth improvement (in order of natural growth rate, i.e. without growth factor addition and cell morphology change) the presence of undamaged collagen fibrils with native structure in composition or onto surface of biocompatible coatings is required. Most suitable materials for modification are bioplastics that can form coatings of various thickness on surfaces of various shape and also implants with suitable shape with controlled resorption rate [8].

The main target of the current research is to develop a method of collagen fibrils water dispersion preparation preserving collagen native structure. Additional target was to modify PLA surface with obtained fibrils by spray deposition to increase the number of attached cell and proliferation

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activity, and compare the efficiency of collagen modification of PLA with PLA hydrophilized by plasma treatment.

II. MATERIALS AND METHODS

"VT-Pro" bovine fibrillar collagen was purchased from JSC "Verhnevolzhskiy tannery" (Russia). Deionized water (Milli-Q grade) was used as a solvent. Collagen was dispersed in water at an initial concentration of 16 mg/ml. Ultrasonic bath was used for dispersion homogenization at a power not exceeding 60 mW/ml. Dispersion was centrifuged at ~ 1000 g. Resulted dispersion concentration was estimated by photometry at wavelength 320 nm.

Collagen fibrils dispersion was spray-deposited on the pre-cleaned cover glasses with a size of 24 × 24 mm and analogous glasses covered with a PLA layer (as previously described [9]) by own designed automatic pneumatic spray unit. Dispersion deposition was carried out iteratively with a solution consumption about 0.1 ml/cm^2, air pressure 2 atm. and at substrate temperature 45 °C. Spraying process parameters were optimized to evaporate solvent from the volume of separate microdroplets distributed over the substrate, and to achieve complete solvent evaporation between the spraying iterations. That allows to avoid the "dry" and "wet" spraying regimes, when lack or in contrary excess of the solvent on the substrate surface leads to dispersed material redistribution resulting in non-uniform deposition of the material [10,11]. Modification of PLA films was carried out by RF Ar plasma treatment with low power density (about 2 times less compared to literature sources [12]) to decrease of the initial hydrophobicity of PLA, which limits cells attachment, spreading and growth on its surface.

Obtained coatings were characterized by atomic-force microscopy (AFM) ("Solver PRO", NT MDT Ltd, Russia) and water contact angles (WCA) measurements by horizontal optical microscope and specialized software.

The study of cell growth was carried out on four types of substrates: pre-cleaned cover glass (control), cover glass with a PLA layer before (PLA) and after plasma treatment (p-PLA) and on a PLA layer with additionally deposited collagen fibrils on its surface (Cp-PLA). Normal human embryonic fibroblasts (HEFs-T) from the cell culture collection of the N.F.Gamaleya National Research Centre for Epidemiology and Microbiology (Moscow, Russian Federation) were cultured with a seeding density of 6.4·10^3 cell/cm^2. Cultivation, control of cell seeding density and amount of grown cells estimation by MTT assay were carried out in accordance with the procedures described previously [6]. To estimate cell number after culturing by the Scepter Millipore automatic cell counter (Merck KgaA, Germany), cells were dissociated and deattached from the surface of the samples identical to the ones used for MTT assay by trypsin-Versene solution treatment and resuspended cells. Doubling time was calculated based on the obtained cells number.

III. RESULTS AND DISCUSSION

The initial dispersion contains a lot of large conglomerates (resulting in height differences of film more than 2 μm after drop drying on substrate) that limits application of spray deposition, decreases films homogeneity and material distribution uniformity on substrate.

To obtain a dispersion containing separated collagen fibrils with a diameter less than 100 nm, which can provide optimal surface morphology for cell growth [13], ultrasonic treatment was performed. However, along with individual fibrils (40-80 nm in diameter), a large amount of medium-sized conglomerates and low molecular weight fraction (LMWF) were observed (Fig. 1a,b). Pores in deposited layer on flat cover glass indicate the presence of a LMWF based continuous layer with thickness about 20 nm.

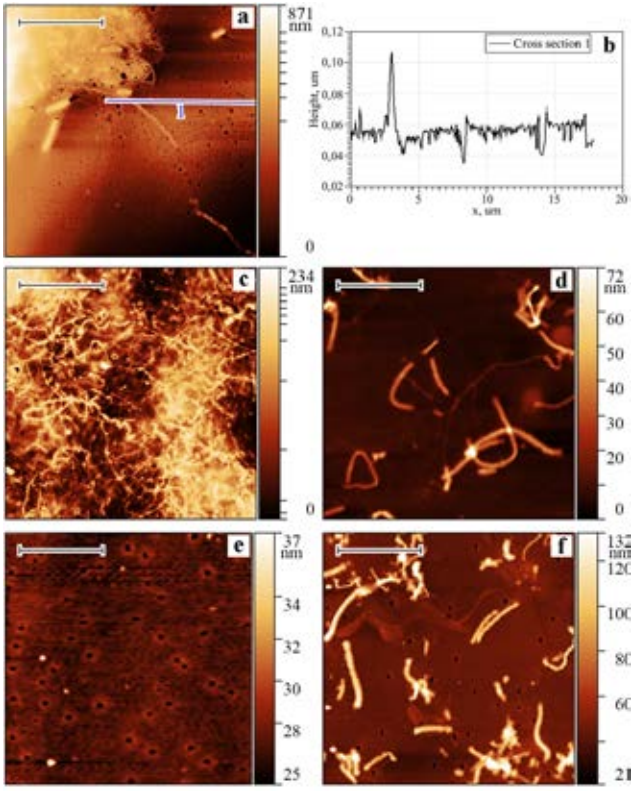

Fig. 1. AFM images of substrate surfaces: cover glass with collagen without (a) ((b) presents cross section from pointed line 1) and with (c) sedimentation performed, and spray-deposited dispersion that additionally centrifuged (d); PLA before (e) and after collagen fibrils spray deposition (f). The scale bar is 10 micrometers.

Sedimentation after ultrasonic treatment was performed to remove conglomerates that were not separated during ultrasonic processing, to obtain a more stable collagen dispersion (sedimentation time more than 2-3 hours, initial one was less than a minute) (Fig. 1c). Then dispersion was centrifuged to separate the collagen fibrils from the LMWF presents in the solution.

Obtained stable dispersion, containing predominantly collagen fibrils, was spray-deposited onto cover glass (Fig. 1d). As result collagen fibrils were uniformly distributed on the substrate surface. Residual content of LMWF is also observed on the cover glass, most apparently on the boundaries of the dried microdroplets. The resulting pattern is due to a better redistribution of LMWF in water compared to collagen fibrils.

Dispersion obtained by the method described above was spray-deposited on a PLA layer (AFM image of PLA surface before deposition is presented in Fig. 1e). The surface of the cover glass had a roughness less than 1-2 nm, while the formed PLA layer had thickness of about 200 nm, roughness of about 3 nm and pores with a diameter of about 0.5–2 µm. Separated collagen fibrils with retained structure can be observed uniformly distributed on PLA surface (Fig. 1f).

Wave-like repeat pattern with periodicity at 50-65 nm characteristic to native structure of fibril can be observed on AFM images (Fig. 2a), which is consistent with known data [14]. For better visualization of the periodic structure, additionally to the topography, the AFM phase shift signal (Fig. 2b) is presented. This data confirms the fact that the fibril structure does not undergo significant changes during spray deposition.

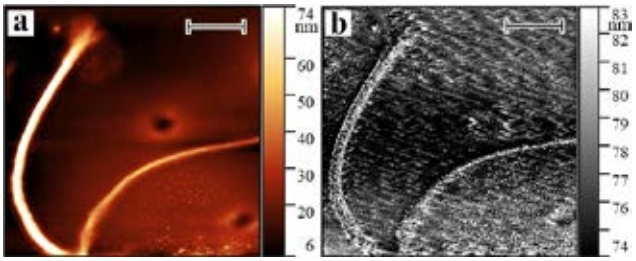

Fig. 2. High resolution AFM image of separated collagen fibrils demonstrating their native structure with wave-like repeat pattern at height (a) and phase shift (b) signals. The scale bar is 1 micrometer.

In order to determine the degree of influence of hydrophilicity as well as the modifications of the surface of the PLA by plasma treatment and collagen fibrils deposition on cell growth, WCAs of the coatings were measured (Fig. 3a) and also HEF-T cells were cultivated for 48 hours. MTT assay was carried out after cultivation for samples on cover glasses that were separately transferred to the empty wells of the sterile well plate for addition of dimethyl sulfoxide. MTT assay was also carried out for well plate in which the cultivation was performed to evaluate amount of cells that were grown on the culture plastic. The results of the MTT assay are presented in Fig. 3b. Evaluation of a cells number by the optical density (OD) of the specimens resulted in almost the same value for both control sample, and for culture plastic (TCPS).

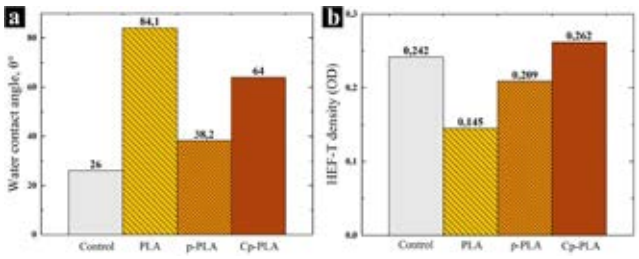

Fig. 3. Bar graphs of WCA of different substrates (a) and MTT assay results of HEF-T cultivation presented in optical density units. Bar graph labels correspond to investigated specimens.

The amount of grown cells on p-PLA was significantly less compared to the glass, despite of the relatively high hydrophilicity of PLA after plasma treatment. At the same time, Cp-PLA being less hydrophilic demonstrates higher amount of cells than p-PLA, and even slightly higher than control glass (i.e. higher than the TCPS), that is rarely achieved by PLA modification [12]. The greatest interest is that even the small percentage of PLA surface covered with collagen fibrils (0.7–1.5 %) significantly changes both hydrophilicity (from 38° to 64°) and cell growth (Fig. 3a,b).

It is should be noted, that cells have not formed continuous layer after culturing even on control glass and Cp-PLA surface. Surface occupied by the cells was less than 50 %, therefore cell growth was not limited by absence of unoccupied surface, needed for cell migration and growth. A doubling time was evaluated by comparison of the seeded cell density and the number of grown cells. Taking into account areas of the samples and well plates, a doubling time was estimated to be ~16 hours for a Cp-PLA sample (slightly less than for control sample), which is comparable to the doubling time for normal human fibroblasts [15].

IV. CONCLUSIONS

Low power ultrasonic treatment, followed by sedimentation for phase separation and centrifugation allows to obtain stable collagen dispersion, containing native collagen fibrils with a diameter about 40-80 nm and a length up to 15 µm. Obtained dispersion meets the requirements of spray-coating process, that was successfully used for substrate coating with uniformly distributed separated collagen fibrils. PLA surface modified with even low amount of collagen fibrils demonstrates a significant cell growth improvement, even in comparison with the surface of plasma treated PLA with higher hydrophilicity. Achieved cell growth activity on PLA surface covered with collagen fibrils does not concede to tissue culture plastic surface. The proposed technique can be applied for surface modification of a wide range of implants to accelerate of connective tissues regeneration.

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