Materials Letters 184 (2016) 104–107

Contents lists available at ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/matlet

Electrolyte alginate/poly-L-lysine membranes for connective tissue development

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ARTICLE INFO

Article history:
Received 9 April 2016
Received in revised form
23 June 2016
Accepted 7 August 2016
Available online 8 August 2016

Keywords:
Alginate
Poly-l-lysine
Electrolyte membranes
Cell adhesion

ABSTRACT

The aim of this study was to change the surface of sodium alginate hydrogel by electrostatic binding of poly-L-lysine (PLL) in order to provide more advantageous conditions for connective tissue development. Its impact on L929 mouse fibroblast adhesion, morphology and viability was investigated. Analysis of the material microstructure has shown that performed modification increased surface roughness. It also altered the swelling properties of alginate hydrogel, resulting in less rapid water absorption. Mouse fibroblasts seeded on regular alginate and alginate modified with PLL exhibited different behaviour. The presence of PLL turned out to promote cell adhesion and F-actin spreading, resulting in significantly increased number of viable cells 96 h after seeding.

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1. Introduction

The increasing need for connective tissue regeneration has led to a rapid development of biodegradable and cell-compatible materials to serve this purpose. The surface design of scaffolds has attracted especially much attention and is investigated in order to provide most advantageous conditions for new tissue formation. Cell adhesiveness plays an important role in tissue development processes such as cell migration during embryogenesis and morphogenesis in response to particular extracellular matrices. Whether or not cell adhesion occurs onto a particular surface, or how it occurs, mainly depends on the chemistry and structure of the surface. Cell adhesion to protein-free surface appears to be a nonspecific adsorption phenomenon independent of the metabolic state or viability of the cells. Adhesion to protein coated surfaces mainly depends on cell physiology [1].

Alginates are algae-derived, linear polysaccharides consisting of β-D-mannuronic acid and α-L-guluronic acid monomers. They are considered to be biocompatible, non-immunogenic and have low toxicity. Forming an alginate hydrogel is most commonly performed by ionic cross-linking of aqueous alginate solution by divalent cations (i.e. Ca^{2+}), which are believed to connect guluronic blocks of adjacent polymer chains [2]. One of the main problems in employing alginate hydrogels as cell-delivering matrices is that they are unable to specifically interact with mammalian cells, what results in poor cell adhesion and thus low viability [2,3].

Poly-L-lysine (PLL) is widely used for coating cell cultureware due to its ability to improve cell adherence. As a multivalent cation, the polymer provides positively-charged sites to which negatively-charged sites on cells' surfaces can bind [4]. Another field, still extensively studied, in which both PLL and alginates are used, is cell encapsulation technology. Alginate capsules are coated with PLL in order to achieve stabilisation, control of porosity and controllable swelling properties [5–7]. What is more, it was discovered that the addition of PLL to the medium can stimulate chondrogenesis in cultures of chick limb mesenchymal cells [8].

In this study, positively-charged PLL was electrostatically bound to alginate hydrogel membranes. Many approaches to encapsulating cells in PLL coated alginate capsules have been reported, however, cells seeded on the membrane form of these materials haven't been examined yet [4–8]. The motivation for this work was to design the surface that provides beneficial environment for cells proliferation. Mouse fibroblasts, that synthesise the extracellular matrix were chosen for cell studies due to their excellent properties in connective tissue regeneration. Two approaches to studying the problem of fibroblasts adhesion have been presented: investigation of the morphology and adhesion of the cells to each other and to the surfaces, since both processes play crucial roles in tissue formation.

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http://dx.doi.org/10.1016/j.matlet.2016.08.032
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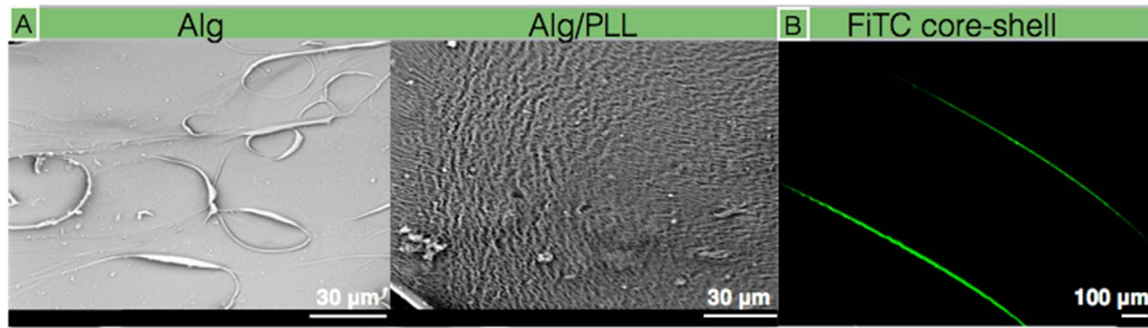

Fig. 1. (A) Microstructure of Alg (control) and Alg/PLL samples obtained by SEM, scale bars: 30 μm. (B) Confocal images of core-shell FITC fibre, scale bar: 100 μm.

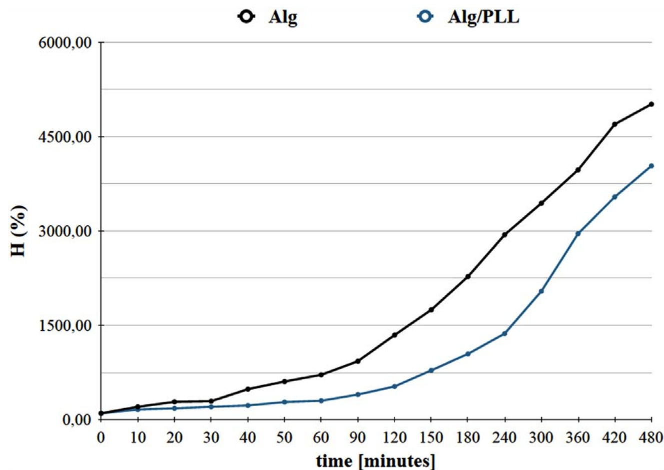

Fig. 2. Swelling isotherms of hydrogels for Alg (control) and Alg/PLL (poly-l-lysine coated) alginate samples.

2. Materials and methods

2.1. Materials

Sodium alginate Pronova UP LVG (guluronic acid content ≥60%, apparent viscosity 20–200 mPa s) was obtained from NovaMatrix, Norway. Poly-L-lysine (PLL 0.01% w/v), calcium chloride (anhydrous, Bioreagent, suitable for cell culture), phosphate buffer saline (PBS), albumin-fluorescein isothiocyanate conjugate from bovine (FITC) were purchased from Sigma-Aldrich.

2.2. Samples preparation

4% (w/v) sodium alginate solution in PBS was prepared. Alginate was then ionically cross-linked by simple spraying with 0.2 M $CaCl_2$ aqueous solution followed by immersing in 0.2 M $CaCl_2$ for 10 min. Samples used for cell studies were round membranes with 6 cm diameter and 0.3 cm height. Samples were formed in cell culture dishes. Solutions were sterilised using syringe-filters with 0,22 μm pore size (Millipore Express PES membrane, Millex-GP).

2.2.1. Alginate surface modification

2.2.1.1. Control group – basic ionic cross-linking. Samples were prepared as described above (see Section 2.2). Control scaffolds were indicated as Alg.

2.2.1.2. Electrostatic binding of poly-l-lysine. Samples cross-linked by 0.2 M $CaCl_2$ (see Section 2.2) were electrostatically bound to poly-L-lysine (PLL), by immersing in 0.01% PLL solution for 1 h. After this process, samples were immersed in PBS to remove excess reagent. Samples were indicated as Alg/PLL.

2.3. Samples characterisation

2.3.1. Surface visualisation

The surfaces of dehydrated successively in a concentration gradient of ethanol solutions (50%, 70%, 80%, 90% and 100%) samples were visualised by means of Phenom Scanning Electron Microscope (SEM) ProX desktop at 10 kV.

To investigate how much PLL attached to the 4% alginate hydrogel, 555 μm diameter alginate fibres were formed by extrusion through the needle, then spraying and dipping in 0.2 M $CaCl_2$ bath for 10 min. Prepared samples were immersed in 0.01% solution of fluorescein-labelled PLL (10 mg of FITC reagent for 1 mL of PLL solution) for 1 h. These samples were indicated as FITC. Fibres surface was visualised using Leica TCS SP8 Confocal Microscope.

2.3.2. Swelling behaviour analysis

The swelling behaviour of regular and modified alginate samples was characterised in order to compare their tendency to absorb water and therefore their stability in aqueous solutions. To perform this experiment, samples with dimensions of 3 × 3 × 3 cm were formed. Dehydrated samples were placed in PBS solution and weighed repeatedly for 8 h (every 10 min for the first hour, every 30 min for second and third hour and later every 60 min). The swelling degree at different times can be calculated from the following equation:

$$H(\%)=[(m_i - m_0)/m_0]*100\% \quad (1)$$

m_i – sample mass after soaking time, m_o – dry sample mass (before soaking)

2.3.3. Cell culture. Viability and adhesion analysis

L929 mouse fibroblasts were cultured in DMEM (high glucose, 10% FBS, 1% penicillin-streptomycin and 2,5 mM L-glutamine, Life Technologies) at 37 °C in 5% CO_2 atmosphere. Cells were seeded on previously prepared samples with 92,000 cells/cm^2 density.

Live-dead experiment was performed 24 h and 72 h after seeding, using acridine orange (AO) and propidium iodide (PI) (Sigma-Aldrich). Hydrogel constructs were washed in PBS, incubated in a solution of 5 μg/mL AO and 10 μg/mL PI for 5 min at 37 °C, then washed in PBS. Images were captured using Leica TCS SP8 Confocal Microscope.

Additionally, the number of viable cells was measured colourimetrically 96 h after seeding, using MTS Cell Proliferation Colorimetric Assay Kit (BioVision). Absorbance was recorded at 490 nm wavelength using Fluorostar Omega Spectrophotometer (BMG Labtech). Acquired absorbance signal (OD) is proportional to the number of viable cells. To obtain the background signal, absorbance of an alginate sample without cells was measured. Five samples per group were analysed and each value represents a mean of 3 replicates. Assay was performed according to the kit protocol.

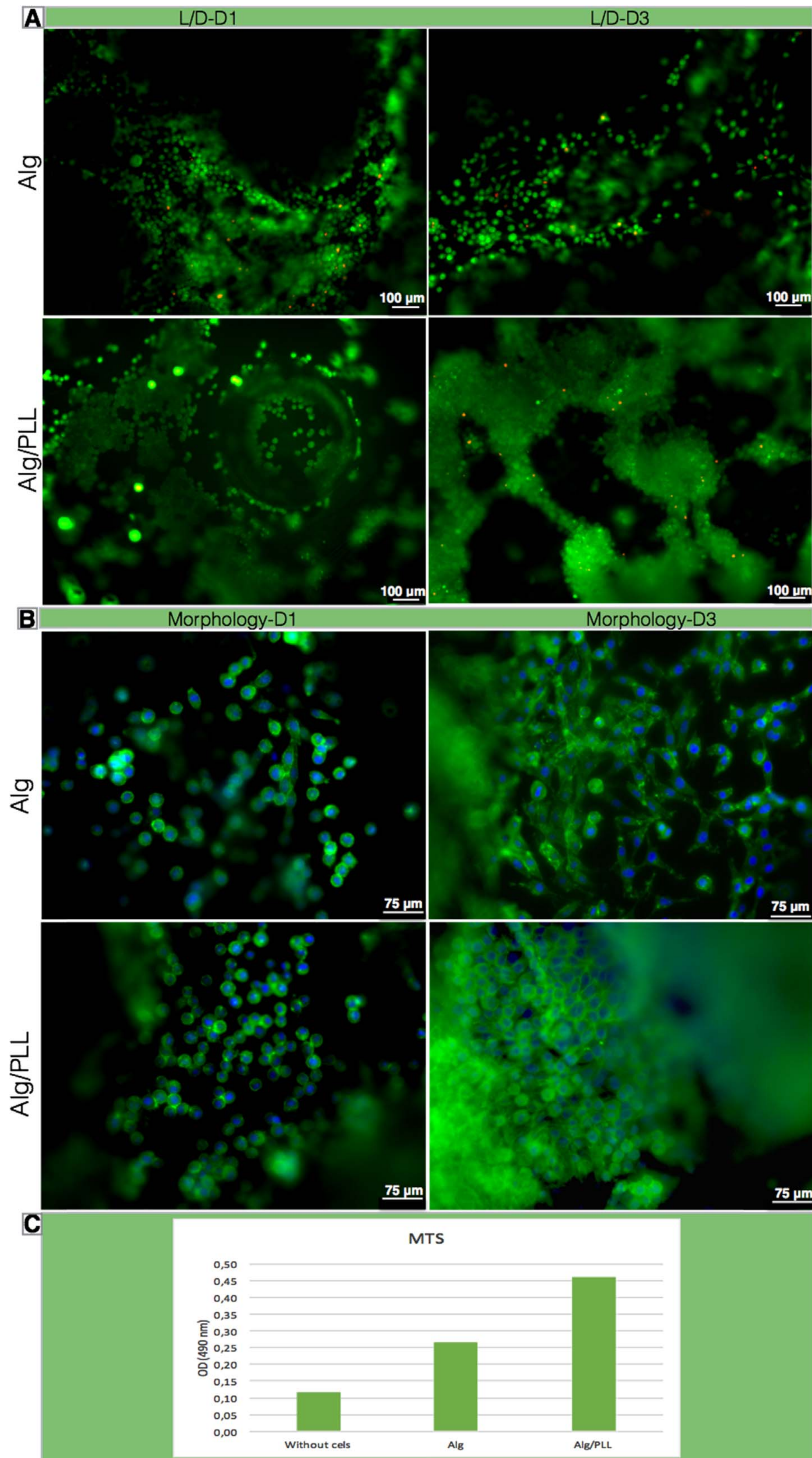

Fig. 3. (A) Viability of mouse fibroblast culture. L/D- live/dead images (D1, D3 – day 1 and 3, respectively), living cells appear green and dead cells appear red. Scale bars: 100 μm, (B) Morphology (D1, D3 – day 1 and 3, respectively) – cell morphology images, actin filaments were stained with Alexa Fluor 488 Phalloidine – shown in green, and nuclei were counterstained with DAPI – shown in blue. Scale bars: 75 μm, (C) MTS test performed after 72 h cell seeding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Cell adhesion was visualised 24 h and 72 h after seeding by F-actin and nuclei staining, using confocal laser scanning microscopy. Cells were fixed in formalin (10 min), permeabilized in 0.2% Triton X-100 (90 s) and blocked with 2% BSA (30 min), then incubated with fluorescently labelled Alexa Fluor 488 Phalloidin (Life Technologies) for 3 h to stain F-actin. Nuclei were stained by incubating for 5 min in 300 nM DAPI (4′,6- diamidino-2-phenylindole solution, Life Technologies).

3. Results and discussion

3.1. Surface visualisation

Presented below are the images showing alginate surface at 10,000 magnification (Fig. 1A). Microstructure of Alg (control) is generally smooth and has only a few cracks due to water evaporation, while in case of Alg/PLL the surface roughness visibly increased. It may possibly provide more advantageous environment for adherent cells.

The image of fluorescein-labelled PLL shell attached to the surface of 4% alginate hydrogel core is shown in Fig. 1B. A coating of approximately 8.21 μm was obtained by modification of 555 μm fibre lasting for 1 h.

3.2. Swelling behaviour analysis

Scaffolds for tissue implementation should precisely meet shape and size requirements. The relationship between soaking time and water absorption (expressed as swelling degree) for both types of alginate samples is presented in Fig. 2. Alg/PLL absorbs smaller amounts of water than Alg, so its mass rises less rapidly. This result confirms that coating with PLL decreases osmotic swelling and therefore stabilizes the sample in applied period of time.

3.3. Cell viability and adhesion analysis

Live/dead experiment results after 24 h (L/D-D1) and 72 h (L/D-D3) are presented in Fig. 3A. Both control (Alg) and modified (Alg/PLL) alginate samples show highly satisfactory proportion of live cells - only few dead ones can be found in the field of view.

The visualisation of F-actin spreading after 24 h and 72 h is presented in Fig. 3B. After 24 h there is no big difference between Alg and Alg/PLL – most cells have circular shape, F-actin is located around nuclei and contact adhesion with the surfaces occurs. But it can be observed, that fibroblasts seeded on Alg/PLL samples exhibit greater tendency to agglomerate. After initial cell-cell and cell-surface contact, a general reorganisation of the microtubules and microfilaments was developed. Filopodia (cytoplasmic extensions) are protruded from the cell and become attached to the surface. As result a progressive flattening of the cell on the surface and an increase in strength of cell attachment can be observed after 72 h. F-actin visibly spreads in both samples, what gives an indication of successful adhesion process. However, the process is more extensive in Alg/PLL - F-actin filaments produced by adjacent cells often overlap, cell-cell adhesion and the tendency to form compact agglomerates is enhanced, what results in tissue-like structure.

The number of viable cells 96 h after seeding was measured colourimetrically, using MTS reagent. Alg/PLL sample presented 2 times higher absorbance (OD signal) of live fibroblasts (Fig. 3C) compared to Alg. It can be assumed, that the presence of PLL stimulated cell adhesion, which is crucial to fibroblasts survival.

4. Conclusions

By modifying sodium alginate with poly-L-lysine, a material with altered microstructure can be achieved. It exhibits more advantageous swelling properties, very important feature in scaffold fabrication. Cell studies have shown, that such modified alginate promotes adhesion and F-actin spreading of mouse fibroblasts, what results in increased number of live cells 96 h after seeding. Moreover, on the PLL coated alginate surface strong cell-cell adhesion, which is crucial in tissue development process, was formed. Proposed electrolyte alginate/poly-L-lysine membranes may provide more advantageous conditions for connective tissue development.

Acknowledgement

This work was financed by The National Centre for Research and Development on the basic of a decision number Pol-Nor/202 132/68/2013.

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