ALGINATE AND GELATINE BLENDING FOR BONE CELL PRINTING AND BIOFABRICATION

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ABSTRACT
In order to develop suitable materials for biofabrication, the current work focused on the fabrication and characterization of alginate-gelatine matrices. Alginate-gelatine blends were prepared by mixing the stock solutions of alginate and gelatine in different ratios: 80/20 and 60/40. Films of these materials were chemical and mechanical characterised. To evaluate the cell behaviour of the different soft matrices, MG-63 osteoblast-like cells were employed. As a consequence, all investigated alginate matrices are suitable for cell encapsulation or rapid prototyping, promoting high cell viability. However, the most promising material is alginate blended with gelatin due to the extensive cell-matrix interactions and cell ability to migrate and proliferate through the hydrogel.

KEY WORDS
Alginate - gelatine blending, soft matrices, cell printing

1. Introduction

Soft matrices (hydrogel) have been widely used for many biomedical applications, including wound dressing, drug delivery and specially cell encapsulation and scaffold printing for tissue engineering [1]. One of the most promising biomaterial used for the printing process, is sodium alginate hydrogel, a linear polysaccharide derived from marine algae, composed of (1→4)-β-D-mannuronic acid and (1→4)-α-L-guluronic acid units arranged in blocks of poly(mannuronate), poly(guluronate) and altering units of both sugars [2]. Alginites are polysaccharides isolated from brown algae such as Laminaria hyperborea and Lessonia nigrescens found in coastal waters around the globe. The first characterisation of alginic acid was carried out by Stanford (patent 1881), who extracted it with sodium carbonate and then precipitated the alginite out of solution at low pH [3]. However, alginate is an inert biomaterial and does not promote cell attachment in its pure state. In an effort to find suitable biomaterial candidates for fabricating scaffolds, gelatine was chosen in this study because it is a derivative of collagen that is the major constituent of skin, bones and connective tissue. Gelatine does not exhibit antigenicity, and practically, it is one of the most convenient proteins to use, due to the low cost compared to collagen.

Cell printing is a technology for constructing bioartificial tissue or organs of complex three-dimensional (3D) structure [4]. Since cells are living materials, all fabrication processes should be performed under sterile and biocompatible conditions. There are several rapid prototyping (RP-) techniques available for biofabrication, e.g. dispense-plotting. This RP-technique was investigated and successfully applied to produce novel 3D-ceramic scaffolds with fully interconnected pore networks and highly controllable porosity and pore size [5]. This extrusion freeform fabrication technique is suitable for production of individually tailored scaffolds fabrication of tissues with complex geometries, as well to enable the production of constructs with spatial variations along multiple axes. The dispense-plotting technique includes a paste-like solution, which is extruded by air-pressure through a fine nozzle and deposited as rods. By using a 0/90° lay-down-pattern the 3D-scaffold is been produced layer by layer (Fig. 1).

Figure 1. Principle of the rapid prototyping technique dispense-plotting for the fabrication of cell-loaded scaffolds (modified [5]).
Therefore, the aim of the current study is to improve the material design by analysing cell response in vitro. Pure alginate was used as a base matrix and gelatine was added to improve its cell-adhesive properties. Alginate and gelatine were mixed by blending. Cell-material interactions were analysed by testing cell viability and morphology on 2D films and in printed 3D hydrogels, generated by the rapid prototyping technique dispense plotting.

2. Materials & Methods

2.1 Materials

Alginate (medium viscosity, M.W. 100,000-200,000 g/mol, Sigma-Aldrich, Germany) was dissolved in PBS (Phosphate Buffer Saline, Gibco, Germany) to obtain the hydrogel solution with concentration of 3.33% (w/v). The hydrogel solution was sterilized by ultrafiltration with 0.45 µm filters (Roth, Germany).

Gelatine (Type A, derived from porcine skin, suitable for cell study, Sigma-Aldrich) was dissolved in deionized water (Millipore, Germany) at 40 °C to a final concentration of 5% (w/v). The solution was sterilized by ultrafiltration with 0.22 µm filters (Roth).

Alginate-gelatine blends were prepared by mixing the stock solutions of alginate and gelatine in different ratios by vortexing followed by sonication at 40 °C. The final concentration of alginate was maintained at 2% (w/v), while concentration of gelatine varied from 0 to 2% (w/v). In the whole study pure alginate hydrogels were used as reference while gelatine could not be used as control due to have different gelation properties compared to alginate. The composition of different blends is shown in table 1.

Table 1. Composition of alginate-gelatin blend hydrogel materials

<table>
<thead>
<tr>
<th>Samples</th>
<th>Weight ratios</th>
<th>Added amount of blend components (ml)</th>
<th>Final concentration (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alginate</td>
<td>Gelatine</td>
<td>Alginate stock solution (3.33% w/v)</td>
</tr>
<tr>
<td>Alginate</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Alg:Gel (80/20)</td>
<td>80</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Alg:Gel (60/40)</td>
<td>60</td>
<td>40</td>
<td>6</td>
</tr>
</tbody>
</table>

2.2 Characterisation of the matrices

The films were made by casting the blend hydrogels of different ratios on glass petridish followed by crosslinking with 0.1 M CaCl₂ solution, which were then cut into strip shape of 35 mm × 2 mm × 1 mm dimension. Mechanical test of the strip shaped films of alginate-gelatine blend samples with different ratios was performed with INSTRON Testing Machine.

Chemical characterisation was also carried out using FT-IR (Fourier transform infrared spectroscopy) to determine the interactions (chemical bonding structure) between the alginate and gelatine components in the fabricated film.

2.3 Cell culture

To evaluate the cell behaviour of the different soft matrices, MG-63 osteoblast-like cells (Sigma-Aldrich, Germany) were used. The well-known MG-63 cells were used for initial testing biomaterials in the context of bone regeneration. MG-63 osteoblast-like cells were cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂ in DMEM (Dulbecco’s modified Eagle’s medium, Gibco, Germany) medium containing 10 vol.% fetal bovine serum (FBS, Sigma-Aldrich) and 1 vol.% penicillin/streptomycin (Sigma-Aldrich). Cells were grown to confluence in 75 cm² culture flasks (Nunc, Denmark), harvested using Trypsin/EDTA (Sigma), counted by a hemocytometer (Roth) and diluted to a concentration of 100,000 cells/ml culture medium. Afterwards, 1 ml of cell suspension was plated onto each surface investigated. After 48 h of incubation on the materials, cell distribution and cell morphology were analysed by light microscopy (LM, Primo Vert, Carl Zeiss, Germany) and fluorescence microscopy (FM, DMI 6000B, Leica Germany). For FM, live cells were stained with calcein and Höchst (2 μl calcein, 2.5 μl Höchst per 35 mm culture dish) and incubated for 20-30 minutes. Cells in 2D maintained their morphology for about 1 hour during imaging analysis.

Cell viability was measured using the WST-1 assay with a working concentration of 15 µl/ml WST-1 solution after 2 days of cell cultivation.

2.4 Fabrication of scaffolds

Cell-loaded scaffolds were fabricated using a plotting technique involving a bio-scaffold-printer (Gesim, Germany). The dispense-plotting includes a paste-like alginate-gelatine-cell solution, which was extruded by air-pressure through a fine nozzle and deposited as rods (Fig.
1) As this work is engaged with viable cells, the printer has to be placed in a sterile environment. In this study MG-63 osteoblast-like cells in a concentration of $1 \times 10^6$ per ml alginate solution were applied. By using a 0/90° lay-down-pattern the 3D-scaffold was produced layer by layer with a diameter of approx. 15 mm. Afterwards, biofabricated constructs were gelled with a 0.1 M sterile calcium chloride solution for 10-30 minutes (depending on the thickness). In the next step the constructs were transferred into cell culture media and cultured for over 21 days.

3. Results

3.1 Material characterisation

The stress-strain curves of alginate-gelatin blends of different ratios were shown in Fig 2. It was observed that the blend ratio of 80/20 of alginate and gelatine exhibited the highest elasticity and yield strength.

![Figure 2. Stress-strain curves of alginate and alginate-gelatin blends of different ratio.](image)

This result can be related with the interconnection between the chains of alginate and gelatine was higher in this blend material compared to other. It was revealed that the E-modulus of Alg:Gel (80/20) was comparatively much higher than pure alginate, presented in Table 2.

<table>
<thead>
<tr>
<th>Materials</th>
<th>E-Modulus [Nmm$^{-2}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wt% alginate</td>
<td>0.267±0.08</td>
</tr>
<tr>
<td>Alg:Gel (80/20)</td>
<td>0.440±0.1</td>
</tr>
<tr>
<td>Alg:Gel (60/40)</td>
<td>0.168±0.06</td>
</tr>
</tbody>
</table>

Table 2. E-modulus of alginate and alginate-gelatin blend hydrogels

FT-IR spectra of alginate and alginate-gelatin blends of all ratios exhibited the peaks at 1000 cm$^{-1}$, 1125 cm$^{-1}$ and 1240 cm$^{-1}$ region which confirmed the presence of guluronic acid, mannuronic acid and o-acetyl ester, the building blocks of alginic acid. The COO$^-$ asymmetrical stretching peak of alginate at 1594 cm$^{-1}$, which shifted to higher wavenumber due to influence of C=O stretch for amide I peak (1630 cm$^{-1}$) of gelatine in alginate-gelatin blend materials. This shifting confirmed the presence of gelatine in blend materials. However, the peak shifted to much higher for Alg:Gel (60/40) than other blends, this can be happened due to presence of higher amount of gelatine in Alg:Gel (60/40) than other. The peak at 1538 cm$^{-1}$ indicates N-H deformation for amide II corresponding to gelatine was also found in Alg:Gel (60/40).

![Figure 3. FT-IR spectra of alginate, gelatine and alginate-gelatin blends of different ratios.](image)

3.2 In vitro analysis

Morphology of MG-63 cells was also assessed by fluorescence microscopy of live cells (Fig. 4). Cells cultured on alginate displayed a mixed morphology of spread and round shape (Fig. 4, left). On blends with alginate: gelatine ratio of 80/20, cells are partially spreaded, formed cell rows (Fig. 4, center). Cell distribution on the films was not homogeneous. The increasing gelatine concentration in the blends lead to the formation of cell agglomerates and the remaining cells had mainly a round-shaped morphology. The majority of the cells have spindle-like or elongated triangle morphologies. However, a significant amount of cells displayed round-shaped morphology.

Cell viability for alginate-gelatin blends with different alginate: gelatine ratios have been tested on 2D films after 2 days of incubation (Fig. 5). Highest mitochondrial activity was measured on 80/20 films compared to pure alginate and also compared to the 60/40 blend material. An important observation was that since a high amount of cells were not attached to the substrate; they were removed through the washing steps, decreasing the overall number of cells involved in the measurement.
3.3 Scaffold fabrication and cell printing

The scaffolds produced by biofabrication had pore sizes of 300-500 µm and strut diameters of approx. 500-900 µm (Fig. 6). Calculated total porosity ranged between 37 and 44 vol%.

Cell staining revealed no toxic effect through the printing process to the cells (Fig. 7, left).

Regarding to the in vitro results with the tested matrices (Fig. 4 and 5) we present here only the 80/20 alginate-gelatine printed scaffolds. The FM-image of Fig. 7 left revealed that no toxic effect of the cell plotting process occurred on the cells. Furthermore, cells grew out of the hydrogel matrix and started to form their own tissue guided by the printed struts (Fig. 7 right). This cell response could be observed after 21 days of cultivation.

4. Conclusion

Designing materials that can promote cell adhesion and migration starts with the understanding of cell-material interactions in 2D. The results of this study show that the alginate/gelatin blend hydrogel of ratio 80/20 exhibits good cell adhesion property with MG-63, which makes it more suitable for biofabrication. With this technique printing on cell and tissue level seems to be a promising approach for the regenerative medicine.

Acknowledgement

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References


