Fig. 1. Effect of fetal calf serum (FCS) on transfection efficiency in COS-1 cells obtained with trimethylated chitosan oligomers/DNA complexes at different weight/weight ratios. Values are mean±S.D. (n = 3).

References


VII. Tissue Engineering

COLLAGENOUS POROUS STRUCTURES FOR THE DEVELOPMENT OF AN ARTIFICIAL SMALL DIAMETER BLOOD VESSEL

L. Buttafoco, P.J. Dijkstra, A.A. Poot, I. Vermees, J. Feijen
Institute for BioMedical Technology (BMTI), Polymer Chemistry and Biomaterial Group, Dept. of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

Introduction

Repairing blood vessels affected by atherosclerosis is still a partially unsolved issue, in particular when dealing with grafts having a diameter smaller than 6 mm [1]. Some of the problems encountered are limited donor availability and thrombosis, together with blood-material interactions, which start almost immediately when a foreign material is implanted in the body [2]. Since synthetic materials often stimulate the formation of thrombotic occlusion and intimal hyperplasia [3], natural materials such as collagen were introduced. Our aim is to build a three-layered tubular structure, eventually implantable in humans, where collagen, one of the most abundant proteins of the extracellular matrix, is chosen as the main constituent of the media layer. In the blood vessel wall fibre forming species of collagen, like type I collagen, are in fact particularly abundant. Besides this, collagen is a suitable scaffold for the proliferation and growth of smooth muscle cells (SMC) whose seeding is needed for the development of those extracellular matrix components which will form the new blood vessel. On the other hand, collagen has a certain degree of antigenicity, which must be taken into consideration when dealing with tissue engineering applications. In order to decrease this antigenicity, collagen is crosslinked. In this work, the porosity of collagenous tubes, prepared by freeze-drying, and the effect of different crosslinking methods on the morphological characteristics of the pores are analysed. Pores are in fact needed in order to achieve a homogeneous distribution of SMC in the tube itself.
Experimental methods

Type I insoluble collagen (1 g) derived from Bovine Achilles Tendon (Sigma, St. Louis, MO) is swollen overnight in 0.05 M acetic acid solution (50 ml) at 4°C. The mixture is dispersed with 50 g of crushed ice for 4 min in a Philips Blender and then homogenised for 15 min at 4°C using an Ultra-Turrax T25 (IKA Labortechnik, Staufen, BRD). The resulting suspension is poured in a mandrel such as the one shown in Fig. 1, before being frozen. Freezing is performed at different temperatures (−5°C, −15°C, −18°C, −22°C, −35°C and −196°C). The samples are then freeze-dried overnight.

Crosslinking

Collagen tubes are crosslinked using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Crosslinking is carried out in a buffer of 2-morpholinoethane sulfonic acid (MES) 0.05 M at pH 5.5, using a molar ratio of NHS/EDC = 0.4.

Collagen tubes are crosslinked also by using diamines as crosslink spacers. The diamines used as crosslinking spacers are: 4,7,10-trioxa-1,13-tricanediamine (TTDD) and poly(propylene glycol)-bis-(2-aminopropyl ether) (J230). With TTDD the reaction is performed in a 0.1 M MES buffered solution (pH = 5.4) containing 0.53 g TTDD/g collagen. After 30 min, 1.15 g EDC and 0.28 g NHS per gram of collagen are added and the reaction is performed overnight. With the Jeffamine J230, instead, collagen is added to a buffered (0.1 M MES, pH = 5.4) 0.062 M J230 solution. After 30 min, 5.75 g EDC and 1.38 g NHS are added per gram of collagen and crosslinking is performed overnight. In both cases the samples are rinsed in abundant water in order to stop the reaction and then freeze-dried.

All these reactions are performed exactly according to the same procedure, the only difference being that 40% EtOH (v/v in water) is used as solvent instead of water.

Determination of free amine group content

The number of free amine groups present in the (crosslinked) collagen tube is determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the following procedure.

Samples of approximately 2 mg are incubated for 60 min in a solution of NaHCO₃ (pH = 7.7; 4%wt). Then, TNBS (0.5%wt) solution is added and the mixture is incubated at 30°C for 4 h. All the samples are gently blotted on filter paper and transferred to a new tube for analysis. Here they are hydrolysed at 60°C for 90 min and 2 ml of HCl 6 M is added. After dilution with 8 ml of MilliQ water and cooling to room temperature, the absorbance at 345 nm is measured using a Varian Cary 300 Bio. The intensity of the absorbance is correlated to the number of free amino groups by applying the Law of Lambert and Beer, using the molar extinction coefficient of trinitrophenyl lysine (14.600 l/(mol cm)). Water is used as a reference material.

Shrinkage temperature

The shrinkage temperature of (crosslinked) collagen tubes is measured using differential scanning calorimetry (DSC 7 Perkin Elmer, Norwalk, CT). Samples of approximately 7 mg are swollen in 50 μl of PBS overnight in high-pressure capsules. Samples are heated from 20°C to 90°C at a heating rate of 10°C/min. A sample containing only 50 μl of PBS is used as a reference. The onset of the endothermic peak, which indicates the denaturation of the triple helix of collagen, is recorded as the shrinkage temperature.

Characterisation of the porous structures

The morphology of (crosslinked) collagen tubes is examined using scanning electron microscopy (SEM). Collagen tubes are freeze-dried before analysis, sputter coated with gold and examined using a Hitachi S-800 field emission scanning electron microscope (Hitachi, Tokyo, Japan). Figure 1 shows a polycarbonate mandrel used for the formation of porous structures. The inner rod is either made of glass or of Teflon. The outer diameter is 9 mm, the inner diameter can be easily varied, but till now 4 mm is being used.
Table 1
Influence of the freezing temperature on the morphology of the porous structures

<table>
<thead>
<tr>
<th>Freezing temperature (°C)</th>
<th>Pore size (µm)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5</td>
<td>Height = 480</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>-196</td>
<td>/</td>
<td>92</td>
</tr>
</tbody>
</table>

Japan) at varying acceleration voltages. The average size of the pores is evaluated from the height and width of ten pores selected at random. The porosity (P) of the structures obtained is calculated from the following formula (d = density):

\[ P = 1 - \left(\frac{d_{\text{sample}}}{d_{\text{ref}}}\right) \]

Results and discussion

The porous collagen tubes obtained by freeze-drying the collagen suspension have been analysed by SEM. Results obtained in terms of porosity of the structure and size of the pores are reported in Table 1. As expected, the size of the pores decreases with decreasing temperature. At lower temperatures the freezing process is faster and water crystals do not have enough time to increase in dimensions. When the tubes are frozen at -196 °C, the structure collapses completely, the tubular geometry is not retained and a very open fibrillar structure is formed, instead of the more well defined pores observed at higher temperatures (Fig. 2). In contrast, the degree of porosity is not influenced by the freezing temperature and only at -196 °C a small variation is noticed. It is also possible to notice that, while the cross section and the outer surface of the tubes are porous, the inner surface is closed in most cases, without any pore. This can be due to the attachment of the tube to the inner rod during the freeze-drying. On the other hand, after crosslinking also the outer surface changes into a sort of shell where no pores can be observed. Performing the same reaction in an aqueous or in an EtOH environment does not lead to any considerable variation in terms of shrinkage temperature or number of amino groups left. The only exception is given by the samples crosslinked with J230, which appears to be more sensitive to the slight pH change in the two environments (Fig. 3). Differences in the final

Fig. 2. Cross sections of porous collagenous structures obtained by freeze-drying at -15 °C (A,B) and at -196 °C (C,D). The tubular structure is not retained at the lowest temperature. The magnifications used are 15× (B and D), 100× (A), 1000× (C).
morphology can be noticed with every sample. Samples crosslinked in 40% EtOH retain the porosity in the cross section more than the ones crosslinked in an aqueous environment and have a more homogeneous structure. All the samples, after crosslinking, are characterised by a considerable shrinkage, especially in terms of wall thickness. From the observation of the images obtained a certain degree of interconnectivity among the pores can be assumed.

Conclusions

Freeze drying can be used as a method to obtain porous structures from insoluble collagen with a certain degree of interconnectivity. These pores are needed to allow homogeneous diffusion of SMC and regeneration of the blood vessel. Variation of the crosslinking conditions further tailors the morphology of the tubes thus obtained. In particular, the formation of a smooth shell inside the tube can be useful when a monolayer of endothelial cells is to be created.

Acknowledgements

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References


SURFACE MODIFICATIONS OF PEOT/PBT COPOLYMERS FOR THE IMPROVEMENT OF BONE MARROW STROMAL CELL ATTACHMENT

M.B. Claase, M.B. Olde Riekerink, D.W. Grijpma, J. Feijen
Department of Polymer Chemistry and Biomaterials and Institute for Biomedical Technology (BMTI), Faculty of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands
S.C. Mendes, J.D de Bruijn
Isotis NV, P.O. Box 98, 3720 AB Bilthoven, The Netherlands

Introduction

The response of bone to injury is to regenerate bone tissue and then remodel the newly formed bone in the direction of local stresses [1]. The mechanical function of bone, once lost by injury or other means, can only be regained by restoring skeletal continuity at the location of