Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 28 (2007) 1327–1345
Biomaterials
www.elsevier.com/locate/biomaterials

Physical and biological properties of barium cross-linked alginate membranes

Heiko Zimmermann[a], Felix Wählisch[b], Claudia Baier[b], Markus Westhoff[b], Randolph Reuss[b], Dirk Zimmermann[c], Marcus Behringer[b], Friederike Ehrhart[a], Alisa Katsen-Globa[a], Christoph Giese[d], Uwe Marx[d], Vladimir L. Sukhorukov[b], Julio A. Vásquez[e], Peter Jakob[f], Stephen G. Shirley[a], Ulrich Zimmermann[b,*]

[a]*Abteilung Kryobiophysik & Kryotechnologie, Fraunhofer-Institut für Biomedizinische Technik, D-66386 St. Ingbert, Germany*
[b]*Lehrstuhl für Biotechnologie, Biozentrum, Am Hubland, Universität Würzburg, D-97074 Würzburg, Germany*
[c]*Abteilung für Biophysikalische Chemie, Max-Planck-Institut für Biophysik, D-60439 Frankfurt, Germany*
[d]*Probiogen AG, Goethestrasse 54, D-13086 Berlin, Germany*
[e]*Departmento Biologia Marina, Facultad de Ciencias del Mar, Universidad Catolica del Norte, Coquimbo, Chile*
[f]*Lehrstuhl für Experimentelle Physik V, Am Hubland, Universität Würzburg, D-97074 Würzburg, Germany*

Received 25 September 2006; accepted 22 November 2006

Abstract

We describe the manufacture of highly stable and elastic alginate membranes with good cell adhesivity and adjustable permeability. Clinical grade, ultra-high viscosity alginate is gelled by diffusion of Ba^{2+} followed by use of the "crystal gun" [Zimmermann H. et al., Fabrication of homogeneously cross-linked, functional alginate microcapsules validated by NMR-, CLSM- and AFM-imaging. Biomaterials 2003;24:2083–96]. Burst pressure of well-hydrated membranes is between 34 and 325 kPa depending on manufacture and storage details. Water flows induced by sorbitol and raffinose (probably diffusional) are lower than those caused by PEG 6000, which may be related to a Hagen–Poiseuille flow. Hydraulic conductivity, L_p, from PEG-induced flows ranges between 2.4×10^{-12} and 6.5×10^{-12} m Pa^{-1} s^{-1}. Hydraulic conductivity measured with hydrostatic pressure up to 6 kPa is 2–3 orders of magnitude higher and decreases with increasing pressure to about 3×10^{-10} m Pa^{-1} s^{-1} at 4 kPa. Mechanical introduction of 200 μm-diameter pores increases hydraulic conductivity dramatically without loss of mechanical stability or flexibility. NMR imaging with Cu^{2+} as contrast agent shows a layered structure in membranes cross-linked for 2 h. Phase contrast and atomic force microscopy in liquid environment reveals surface protrusions and cavities correlating with steps of the production process. Murine L929 cells adhere strongly to the rough surface of crystal-bombarded membranes. NaCl-mediated membrane swelling can be prevented by partial replacement of salt with sorbitol allowing cell culture on the membranes.

Keywords: Hydrogels; Hydraulic conductivity; NMR-imaging; AFM; Topography; Cell culture

1. Introduction

Alginate gels are currently being employed and explored for a wide range of medical and pharmaceutical applications including cell microencapsulation, drug delivery, and tissue engineering. Examples are the encapsulation of islets of Langerhans and parathyroid tissue for the treatment of diabetes mellitus and hypoparathyroidism [1–8] and the encapsulation of human chondrocytes or mesenchymal stem cells for repair of cartilage defects [9–12]. Alginate-based wound dressings are increasingly viable alternatives to the traditional cotton or viscose gauzes [13,14] and membranes inoculated with cells are being explored for burn treatment. Alginate keeps wounds moist and fosters the formation of tissue [15]. Alginate may also serve as a cryoprotectant for single cells and, especially, for

*Corresponding author. Tel.: +49 931 888 4508; fax: +49 931 888 4509.
E-mail address: zimmerma@biozentrum.uni-wuerzburg.de (U. Zimmermann).

0142-9612/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2006.11.032

multicellular systems [16]. Alginate films on glass surfaces have been used for non-invasive cell guidance [6]. Additionally, alginate is an appealing material for the construction of ''biohybrid organs'' and ''micro-bioreactors'' because its hydrated three-dimensional network allows cells to adhere, spread, migrate and interact with other cells [6]. Although still in their infancy, micro-bioreactors on alginate membranes tackle many problems in regenerative medicine. An artificial lymph node can generate human monoclonal antibodies and test immune functions *in vitro* [17].

Alginate microcapsules or membranes for medical and pharmaceutical use must (i) preserve donor cell viability and function, (ii) prevent immunological response and (iii) have long-term integrity. Biocompatibility and mechanical and elastic properties depend, *inter alia,* on the algal source, processing methods, formulation and gelling conditions [16,18].

Extensive experience from 20 collection sites and unsuccessful trials of laboratory cultures has shown (see e.g. [19]) that the specific microenvironment and ecological history of the algal growth place is crucial for the reproducible manufacture of clinical-grade alginate. Microenvironments can vary on small scales (tens of meters) as a result of climatic and sea conditions. Characterisation of clinical-grade alginate should include high-resolution specification of the growth area as well as complete environmental and preparation data. These data, composition analysis, records of seasonal and annual biomass production and local data about the persistence and mortality of kelp populations are documented for *Lessonia nigrescens* and *Lessonia trabeculata* [20–22]. *L. nigrescens* from tidal zone of the Chilean coast is exposed to extremely high surf and its stipes are very elastic and flexible because they contain high-M alginates (about 60% mannuronate acid [23]). *L. trabeculata* is subtidal from depths between 5 and 30 m and its stipes are very stiff due to a high-G content (guluronate acid about 90% [24]).

The recent introduction of the ''crystal gun'' has considerably improved the cross-linking process of the alginate with Ba^{2+} ions because of uniform gelling [25–27]. These developments have resulted in a new generation of cell or tissue containing microcapsules made of 1:1 mixtures of high-G and high-M alginate that have functioned in rodent studies for more than a year without evoking any significant immunological response [8,16].

We demonstrate that these technologies can manufacture large, additive-free, biocompatible alginate membranes exhibiting the strength and elasticity required for wound dressing. In particular, protocols are given for the construction of (cell-seeded) alginate membranes that are highly stable and elastic in physiological solutions and also cell-permeable as required for micro-bioreactors. Although challenges remain, this body of knowledge facilitates implementation of the results described here into medical and pharmaceutical applications.

2. Materials and methods

2.1. Alginate

Alginate constitutes a family of unbranched anionic polysaccharides, mainly extracted from brown algae (*Phaeophyta*). It is composed of 1-4-linked α-L-guluronic acid and β-D-mannuronic acid arranged in homopolymeric (GGG-blocks and MMM-blocks) or heteropolymeric block structures (MGM-blocks). Ultra-high viscosity alginates of clinical grade were extracted from stipes of *L. nigrescens* and *L. trabeculata* growing at the Chilean coast close to Coquimbo (500 km north of Santiago de Chile). The algae were harvested directly from the sea, peeled and subjected to immediate antimicrobial treatment [16]. After harvesting, cutting and drying the stipe pieces, contaminants were physically removed from the surface. The large-scale technology for alginate extraction has been described elsewhere [16,18,19]. A 0.1% w/v solution of the ultra-high viscosity alginates (UHV-alginates) had a viscosity of 20–40 mPa s. Quality control of the purified alginates was performed using the assays developed by Leinfelder et al. [19].

2.2. Production of NT-alginate membranes

0.7% w/v purified UHV-alginates from *L. nigrescens* and *L. trabeculata* were dissolved in sterile 0.9% NaCl solution. 1:1 mixtures of *L. nigrescens* and *L. trabeculata* alginates (final composition: about 35% M and 65% G) were used if not stated otherwise. Screening experiments showed these ''NT-alginate'' membranes to have best mechanical stability and elasticity. In some experiments, up to 5% fetal calf serum (FCS; purchased from PAA, Linz, Austria) was added to the alginate mixture before cross-linking to test the effect of the protein on the swelling properties of the alginate [28].

The set-up for forming membranes consisted of a rectangular inner compartment of $52 \times 90\,mm^2$ whose thickness could be varied between 1.5 and 2 mm with spacers. This compartment was separated from two outer compartments (Fig. 1) by wetted filters (regenerated cellulose reinforced with non-woven cellulose; pore diameter 0.2 μm; Sartorius AG, Göttingen, Germany) supported by 10-mm thick ceramic plates (*Porolith*; average pore size 25 μm; Keramische Fabrik Meissen GmbH, Meissen, Germany). The inner compartment was filled with 8 or 12 ml of the 0.7% w/v alginate solution depending on the thickness of the spacer. Then isoosmolar 0.9% NaCl containing 20 mM $BaCl_2$ was added to the outer compartments. The compartments were set vertically to avoid the inclusion of air bubbles in the alginate matrix. The Ba^{2+} cross-linking proceeded for 2 or 15 h. The membranes were removed and their area determined. They were kept in distilled water (Ampuwa water; Fresenius Kabi, Bad Homburg, Germany) for at least 1 h before transfer into osmolyte solutions or into sorbitol-modified culture medium. The sorbitol-modified culture medium was made by 1:1 mixture of complete growth medium (CGM) and of an isoosmolar sorbitol solution. CGM consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 2 mM sodium pyruvate, 1% non-essential amino acids, $100\,\mu g\,ml^{-1}$ penicillin and $100\,U\,ml^{-1}$ streptomycin (PAA, Linz, Austria). The final FCS concentration (FCS Gold; PAA, Linz, Austria) of the modified growth medium was adjusted to 10%.

Some of these conventional alginate membranes were immediately bombarded with $BaCl_2$ crystals using the crystal gun technique introduced recently by Zimmermann et al. [16,24] for cross-linking microcapsules. The crystal gun was mounted on an XY plotter allowing local control of cross-linker concentration. Crystals (250–350 μm in diameter) were pre-heated at 150 °C for 3 h and stored under dry and sterile conditions. Membranes were bombarded for 45 s (corresponding roughly to 500–800 mg $BaCl_2$). Then the membranes were incubated further for 1 min in a 20 mM $BaCl_2$ solution before being transferred into distilled water for storage. Membranes of high hydraulic conductivity were made by introducing pores into the bombarded matrix with a cannula of tip diameter of 200 μm (so-called perforated membranes).