Amphiphilic Membranes with Controlled Mesh Dimensions for Insulin Delivery +

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SUMMARY: A series of amphiphilic networks (membranes), consisting of hydrophilic poly(*N,N*-dimethylacrylamide) (PDMAAM) main chains crosslinked by hydrophobic telechelic polyisobutylene di- and trimethacrylates (MA-PIB-MA or Ø(PIB-MA)$_3$) have been synthesized and used for the preparation of thin-walled tubules suitable for the immunoisolation of porcine islets. The molecular weight cut-off (MWCO) ranges, insulin and glucose diffusion coefficients and permeabilities of various membranes have been determined. The molecular weight of the PDMAAM moiety between two hydrophobic crosslinking points ($M_{c,hydrophilic}$) controls permeability, which in turn can be controlled by synthesis conditions. The strengths and elongations of water-swollen membranes crosslinked with Ø(PIB-MA)$_3$ are superior by a factor of about two to those prepared with MA-PIB-MA. Based on the values from these experiments, a well-defined membrane prepared with Ø(PIB-MA)$_3$ was selected and used to encapsulate porcine islet cells. Gratifyingly, the encapsulated islet cells remain functional and viable, and cells within the tubule release insulin upon glucose challenge. These in vitro experiments are sufficiently promising to encourage us to continue our studies to develop a bioartificial pancreas.

Introduction

Our ultimate goal is the development of a bioartificial pancreas to provide long-term correction of blood glucose levels in individuals with type I diabetes. Our device is envisioned to consist of porcine islet cells enclosed in a synthetic precision-designed membrane that is flexible, biocompatible and permeable to glucose, insulin, and oxygen, but impermeable to immune cells and proteins with MW greater than ~60 000, e.g. immuno-

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globulins. The membranes are designed such that upon implantation, the encapsulated porcine islets are able to sense and regulate the glucose level in the blood of diabetic individuals and at the same time are isolated from the onslaught of the immune system. The implanted device remains functional for extended periods and can be readily removed.

Upon analysis of the requirements for an ideal immunobarrier membrane, we concluded that the synthesis of such a membrane was feasible by our recently discovered/patented "living" polymerization technique[1,2]. By this technique, we have synthesized polyisobutylene-based novel hydrophobic crosslinking agents, e.g., telechelic polyisobutylene di- and trimethacrylates (MA-PIB-MA and the three-arm star (Ø(PIB-MA)$_3$)[3,4], which have become key ingredients for our membranes. Immunoisolatory amphiphilic networks have been prepared by crosslinking water-soluble acrylate (e.g., *N*,*N*-dimethylacrylamide (DMAAm)) chains with MA-PIB-MA[3].

The tensile strengths of our water-swollen immunoisolatory membranes are ~0.5 MPa[5]. We theorized that the mechanical properties of the membranes could be improved by more efficient crosslinking, specifically by substituting the two-functional MA-PIB-MA with a three-functional crosslinking agent

Ø(polyisobutylene~~~~~O–CO–C(CH$_3$)=CH$_2$)$_3$

Ø(PIB-MA)$_3$

The micromorphology of these networks changes depending upon the medium with which they are in contact, i.e., water, hydrocarbon, or an amphiphilic solvent, e.g., tetrahydrofuran ("smart" networks)[3-7]. This conformational isomerization upon contact with various media is virtually instantaneous and may account for the biocompatibility of our networks. The hydrophilic polymethacrylate moiety in our networks gives rise to slipperiness while the hydrophobic PEB moiety ensures elasticity, softness, and strength.

Networks prepared with the Ø(PIB-MA)$_3$ crosslinking agent possess two kinds of crosslinks, i.e., a "homogeneous" and a "heterogeneous" crosslink, and are characterized by two M_c's. Figure 1 shows the scheme of such a network. The core of Ø(PIB-MA)$_3$ (i.e., the initiator fragment that remains in the three-arm star PIB) becomes a homogeneous crosslink point connecting only hydrophobic segments. In contrast, the incorporation of the Ø(PIB-MA)$_3$ in the growing PDMAAm chains creates heterogeneous crosslink points that connect

hydrophilic and hydrophobic segments. $M_{c,PIB}$ can be directly measured whereas $M_{c,PDMAAm}$ can be calculated as described[4].

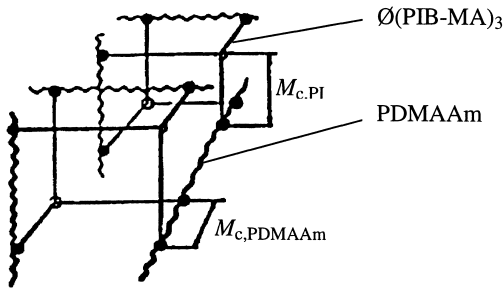

Figure 1: Microarchitecture of amphiphilic networks based on Ø(PIB-MA)$_3$. Straight line – PIB, wiggly line – PDMAAm, • homogeneous crosslink point, o heterogeneous crosslink point.

The properties of our amphiphilic networks have been characterized by a battery of techniques[3-16]. The hydrophilic/hydrophobic content of our network is preferentially 50/50, and the diameter of the hydrophobic domains is 2-8 nm[8]. These membranes can be formed into 3-5 cm long, 0.5 cm diameter tubules with very thin walls of defined thickness (0.15-0.2 mm). The rubbery tubules can be sealed with isobutyl 2-cyanoacrylate ("superglue") by crimping or by inserting a plug of the same amphiphilic material. The tubules are optically clear, heat-resistant and can be sterilized by autoclaving at 120 °C.

The biocompatibility of membranes prepared with MA-PEB-MA was demonstrated by in vivo implantation in rats for longer than 120 days and for those prepared with Ø(PIB-MA)$_3$ by implantation for 8 months[17]. The absence of inflammation and fibrous cell growth at the site of implantation, and lack of adherence or abnormal vascularization on or near the device was demonstrated by histology and scanning electron microscopy of explants. These membranes adsorb less fibrinogen, albumin and Hageman factor (factor XII) than glass, polyethylene or silicone rubber[7]. Reduced protein adsorption and cell adhesion also indicated hemocompatibility at blood-contacting surfaces[6,10].

The present research elucidates the molecular weight cut-off (MWCO) and hydrodynamic (or Stokes) radii (R_s) ranges of various membranes. The diffusional characteristics of glucose and

insulin through the membranes are also shown. Insulin production and release are demonstrated for a prototype artificial pancreas.

Experimental

Materials. Networks: The synthesis of the bi- and trifunctional initiators and chemicals and their purification have been published[8,18,19] . Islet cells: Hams FIO tissue culture medium (ICN), Liberase Pt collagenase (Boehringer-Mannheim, St. Louis, MO), F I hybrid Yorkshire Hampshire pig (Red Bam Veterinary Clinic, Oakland, NE), human insulin ELISA kit (Diagnostic Systems Laboratories, Webster, TX).

Network synthesis. The synthesis and functionalization of telechelic polyisobutylene di- and trimethacrylates (MA-PIB-MA and Ø(PIB-MA)$_3$) have been described[3-7,19-21]. The hydrophilic monomer, *N,N*-dimethylacrylamide (DMMAm, Aldrich), was copolymerized/crosslinked with MA-PIB-MA in THF solution. Active copolymerizing charges together with 2,2'-azobisisobutyronitrile (AIBN) were rotated in 20-25 cm long and ~4.0 mm inner diameter glass tubules at ~60 °C. The centrifugal force moves the active polymerizing charges to the wall of the revolving tube and thus tubular membranes can be prepared. The tubules were removed from the reactor, washed sequentially by hexanes, ethanol and distilled water, and conditioned with a physiological buffer solution. The inner diameter (~0.25 cm) and wall thickness (~0.02 cm) of the tubules were determined with a hemocytometer using a light microscope. The hydrophilic/hydrophobic ratios of the tubules were 40/60, 50/50, and 60/40 wt%.

Symbolism used. The amphiphilic networks are identified by a three-character symbol, e.g. A-l0-50 in which the letter refers to the hydrophilic component (A= *N,N*-dimethylacrylamide), the 10 refers to the molecular weight x 0.001 of the PIB component, and the 50 refers to the weight percentage of hydrophobic component in the membrane. Networks prepared by the use of the three-arm star crosslinker Ø(PIB-MA)$_3$ will be indicated by a star in the formula, e.g. A*-10-50.

Characterization. Tensile properties were obtained with an Instron 5567 tensometer with a 5-kg load cell and at a crosshead speed of 50 mm/min, with 30-mm clamp separation. Strain was measured with a video extensometer at a calculated strain rate of 1.67 min^{-1}. A minimum of three microdumbbells (ASTM D638-5) of networks containing 50 % PIB were tested and their averages are reported. Stresses and elongations were recorded for each sample.

Pore size diameter. The molecular weight cut-off (MWCO) and pore size ranges of the membrane tubules were studied by determining the permeability of a series of commercially available protein markers of known molecular weight and size, i.e., aprotinin (MW = 6 500, R_s = 1.5 nm), cytochrome C (MW = 12 400, R_s = 1.63 nm), carbonic anhydrase (MW = 29 000, R_s = 2.01 nm), and albumin (MW = 66 000, R_s = 3.62). Tubules were loaded with a mixture of four different proteins, and were sealed by crimping and cementing with commercially available cyanoacrylate. The protein-filled tubules were incubated in 5 mL of phosphate buffered saline at 37 °C with shaking, and aliquots of the surrounding buffer were removed at different times. The aliquots were then analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to identify the proteins that had permeated the tubules. The proteins were electrophoresed in tris-glycine 4- 20% bis-polyacrylamide gels[22] and then stained with Coomassie Blue.

Permeability and diffusion studies. Tubules of different composition were filled with sterile glucose (40 mg/mL) and insulin (40 mg/mL) solutions in phosphate-buffered saline, pH 7.4 (PBS). The tubules were sealed and then incubated with shaking at 37 °C in 5 mL of PBS. Aliquots were removed at different time intervals, and the concentration of glucose was determined calorimetrically by the glucose oxidase method[23] and that of insulin by the protein dye binding assay[24].

Encapsulated Porcine Islet Cell Device

Porcine islets. Porcine islets are being used by many groups of investigators as glucose-sensitive insulin-producing xenografts[25-29]. Porcine islets have a similar glucose set-point for production of insulin as humans and porcine insulin has been used extensively as replacement therapy for human insulin[30,31].

Source, purification and cultivation of islet cells. Fl hybrid Yorkshire Hampshire pigs were maintained on a defined diet until they grew to ~45 kg. The pancreases were then surgically removed to serve as the source of islets. The pancreas was minced and then digested using a specially formulated collagenase preparation optimized for porcine islet purification by Reach's continuous flow technique[32] , and the islets were purified by ficoll hypaque density gradient centrifugation. The islet cells were incubated and maintained at 37 °C in Hams FIO tissue culture medium.

In vitro testing. Islet cells were dispersed in 1% alginate solution in Hams FIO media, the suspension (~1.8×10^5 cells) was injected into previously autoclaved polymer tubules under

aseptic conditions. The ends of the tubule were crimped and sealed with cyanoacrylate. The device was equilibrated in low-glucose (50 mg/dL) Hams FIO medium for 40 min, baseline aliquots were obtained and then the device was shifted to high-glucose medium (300 mg/dL). Aliquots were withdrawn at different intervals for determination of insulin release into the surrounding medium. The tubules were placed in low-glucose content media after 2 h and additional aliquots were obtained. The insulin production was measured by ELISA.

Results and Discussion

Pore Size Determination

Networks prepared with MA-PIB-MA. The permeability of a series of tubules ranging from A-l0-50 to A-2.5-60 was compared (Table 1). A molecular weight cut-off range (MWCO) was obtained by determining which of a series of globular proteins was capable of permeating the tubule. This is the conventional parameter used to describe the pore size of membranes. We also assessed the permeability of the membranes with regard to the Stokes radius (R_s) of the proteins capable of passing into the surrounding medium. This parameter provides a better description of the shape and size of permeating molecules and hence, membrane porosity.

Table 1. Characteristics of Networks Crosslinked by MA-PIB-MA

Network	$M_{c,hydrophilic}$	Molecular Weight Cut-Off	Stokes Radii, R_s (nm)
A-10-50	5000	29000 - 66000	2.01 – 3.62
A- 10-60	3333	29000 - 66000	2.01 – 3.62
A-4.5-40	3375	29000 - 66000	2.01 – 3.62
A-4.5-50	2250	12400 - 29000	1.63 – 2.01
A-4.5-60	1500	12400 - 29000	1.63 – 2.01
A-2.5-40	1875	12400 - 29000	1.63 – 2.01
A-2.5-50	1250	6500 - 12400	1.50 – 1.63

The $M_{c,hydrophilic}$ correlates well with the permeability cut-off ranges of the membranes. The MWCO, or preferentially the R_s cut-off, is represented as a range because these membranes do not have well defined pores. The latter parameter can therefore be used to select the appropriate membranes for an insulin delivery biodevice. The membrane should provide optimal permeability to glucose (too small a molecule to be of concern) and insulin (MW

~5 700) but restrict the entry of immunoglobulins (> 60 000). With this criterion, membranes A-10-50, A-10-60 or A-4.5-40 may be appropriate to immunoisolate encapsulated islet cells but will allow optimal permeability of glucose and insulin. Membranes with $M_{c\text{-hydrophilic}}$ > 3000 were shown to have excellent permeability to glucose and insulin[14,20,21].

Networks prepared with Ø(PIB-MA)$_3$. The characteristics of networks prepared with Ø(PIB-MA)$_3$ are presented in Tables 2 and 3. While $M_{c,PDMAAm}$ decreases with increasing PIB content and increases with increasing PIB molecular weight, the crosslink density increases with increasing PIB content and decreases with increasing PIB molecular weight. Crosslink densities are shown in column 3 of Table 2. Permeabilities were checked using the same method and protein markers as in the case of networks prepared with MA-PIB-MA. Networks based on Ø(PIB-MA)$_3$ have higher crosslink densities than those based on MA-PIB-MA. As expected, the protein MWCO ranges of networks prepared with Ø(PIB-MA)$_3$ are lower than those made with MA-PIB-MA because of the above mentioned reasons, but they are still suitable for encapsulation of living cells for insulin delivery.

Table 2. Protein Permeabilities of Networks Crosslinked by Ø(PIB-MA)$_3$

Network	$M_{c,PDMAAm}$[a]	Crosslink Density[b] x 10^4 (mol/g)	MW Cut-Off	Stokes Radii, R_s (nm)
A*-4.5-40	2250	3.56	6500 – 12400	1.50 – 1.63
A*-4.5-50	1500	4.44	6500 – 12400	1.50 – 1.63
A*-4.5-60	1040	5.24	6500 – 12400	1.50 – 1.63
A*-10-30	7420	1.60	12400 – 29000	1.63 - 2.01
A*-10-40	5000	1.60	6500 – 12400	1.50 – 1.63
A*-10-50	3330	2.00	6500 – 12400	1.50 – 1.63
A*-10-60	2320	2.36	6500 – 12400	1.50 – 1.63
A*-15-40	7200	1.09	6500 – 12400	1.50 – 1.63
A*-15-50	5000	1.33	6500 – 12400	1.50 – 1.63
A*-15-60	3470	1.57	6500 – 12400	1.50 – 1.63

[a] Corrected by extraction results; [b] calculated, see ref. 4

Mechanical properties of networks. Water-swollen networks obtained with Ø(PIB-MA)$_3$ exhibit higher tensile strengths and greater elongations at comparable $M_{n,PIB}$ than those crosslinked by MA-PIB-MA (columns 4 and 6, Table 3). This must be due to the different swelling behaviors of the different network[6]. Due to their lesser or equivalent swelling in

water but higher crosslink densities, the networks prepared with the three-functional crosslinker have greater strength than those prepared with the linear MA-PIB-MA.

Table 3. Stress/Strain Properties of Selected Networks

Network	Crosslinker	Stress, MPa		Strain, %	
		dry	wet	dry	wet
A*-4.5-52	Ø(PIB-MA)$_3$	6.51	1.00	253	250
A*-10-53	Ø(PIB-MA)$_3$	6.03	0.84	314	268
A*-15-52	Ø(PIB-MA)$_3$	0.98	0.83	320	297
A-4.5-50	MA-PIB-MA	16.67	0.46	113	116
A-10-57	MA-PIB-MA	17.16	0.58	198	205

In vitro insulin production of encapsulated porcine islets

Because of their favorable diffusion characteristics and mechanical properties, membranes prepared with Ø(PIB-MA)$_3$ were selected for use in bioartificial devices. A device was prepared in which porcine islets suspended in 1% alginate were encapsulated in a A*-4.5-40 tubule. Earlier studies showed that encapsulated islets maintain viability and glucose-responsive insulin production in cell culture for longer than 4 months.

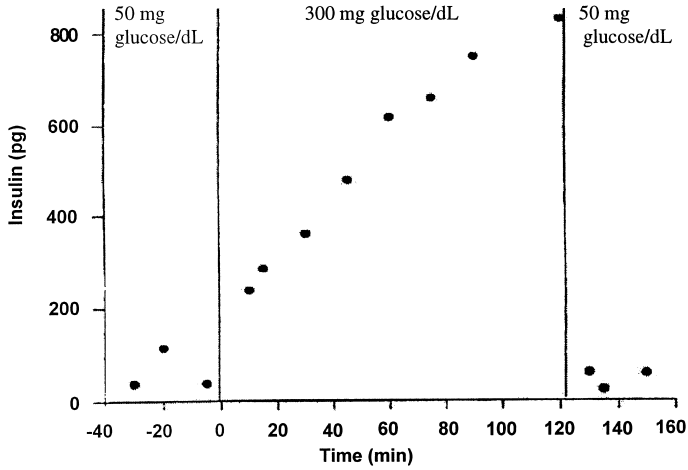

Figure 2. Glucose challenge of encapsulated porcine islet cells. Islet cells (~1.8 x 10^5 cells) were encapsulated in an A*-4.5-40 tubule. (The device was equilibrated in a low-glucose medium. Aliquots taken at different intervals were analyzed for insulin by ELISA.)

Figure 2 shows insulin release from the device after challenge with high-glucose (300 mg/dL) medium. Background levels of insulin production were detected in low-glucose (50 mg/dL) medium, before and after the high-glucose challenge. During the challenge, insulin release to the medium was approximately 6 pg per minute. This result demonstrates the efficacy of the device and encourages us to continue the development of an artificial pancreas for in vivo implantation into diabetic animals.

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