

In vivo biostability of a polymeric hollow fibre membrane for cell encapsulation

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The biostability of poly(acrylonitrile-co-vinyl chloride) (P(AN/VC)) hollow fibre membranes was assessed in the rat peritoneal cavity over a 12 month period. The mechanical and chemical stabilities of the hollow fibre membrane (HFM) were characterized by measuring its tensile strength and molecular weight (by gel permeation chromatography) pre-implantation and post-explantation. The stability of the HFM transport properties was determined by molecular weight cut-off (MWCO) and hydraulic permeability (HP). Explanted HFMs were treated with 4 M NaOH to remove adsorbed protein before measuring mechanical, chemical and transport properties. The HFM was stable *in vivo* for at least 12 months: (i) weight average molecular weight (M_w) at $t = 0$ was $143\,000\text{ g mol}^{-1}$ (with a polydispersity index (PDI) of 2.3) and at $t = 12$ months it was $128\,000\text{ g mol}^{-1}$ (with a PDI of 2.8); and (ii) tensile strength at $t = 0$, 52 ± 2 mdyne, did not change significantly over time and was 46 ± 7 mdyne at $t = 15$ months ($P > 0.05$ by a two-tailed Student's *t*-test); and (iii) no significant differences, with respect to standard deviation, were observed in the transport properties: HP was $7.4 \pm 1.5\text{ ml min}^{-1}\text{ m}^{-2}\text{ mmHg}^{-1}$ at $t = 0$ and $7.5 \pm 1.5\text{ ml min}^{-1}\text{ m}^{-2}\text{ mmHg}^{-1}$ at $t = 12$ months, while MWCO (at 90% rejection) was initially $40\,000 \pm 8000\text{ g mol}^{-1}$ and then $54\,000 \pm 10\,000\text{ g mol}^{-1}$ at $t = 12$ months.

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The long-term chemical and mechanical stability of an implanted biomaterial is essential for its continued use *in vivo*. The biomaterial must be biocompatible, maintaining chemical and mechanical integrity, or degrade to known non-toxic compounds, such as with poly(lactic acid-co-glycolic acid) sutures. Although polyurethanes have been used successfully in numerous medical applications, some poly(ether urethane) pacemaker leads have degraded *in vivo*, prior to the lifetime of the pacemaker, rendering it an unsuitable application^{1,2}. Polyurethanes have since received the attention of several industrial and academic researchers who have developed *in vitro* stability studies to gain a better understanding of polyurethane *in vivo* stability. The major mechanisms of degradation are auto-oxidation, hydrolysis, mechanical loading and mineralization. Degradation may affect the biostability of an implanted material, and can be catalysed by metal ions or enzymes³. The *in vivo* environment represents the ultimate test for material stability by providing a complex host tissue reaction to the implanted material which is difficult to

reproduce *in vitro*. For example, macrophages may produce superoxides that result in oxidative degradation of the biomaterial. Controlled *in vitro* studies allow elucidation of the mechanisms of degradation^{4,5} and have been used to develop new biomaterials⁶. *In vitro* stability results are valued in their relevance to those *in vivo*.

In this study, the long-term stability of poly(acrylonitrile-co-vinyl chloride) (P(AN/VC)) hollow fibre membranes (HFMs) was studied *in vivo* for 12 months. The HFMs were assessed for stability in terms of bulk molecular weight by gel permeation chromatography and mechanical strength by tensile strength. The transport properties of the HFM were determined by measuring hydraulic permeability and molecular weight cut-off, which reflect changes in the pore size. The properties of the HFMs were compared to those of the starting material to assess stability. To our knowledge, this is the first *in vivo* biostability study of P(AN/VC); however, P(AN/VC) has been used in haemofiltration membranes⁷, certain hybrid artificial pancreas applications⁸ and central nervous system (CNS) encapsulation devices^{9,10}.

P(AN/VC) HFMs are used in cell encapsulation applications to provide immunoisolation, thereby allowing cell transplantation to allogeneic and xenogeneic hosts (i.e. cross-species transplantation).

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The porous HFM allows the transport of both cell secretory products out of and nutrients and oxygen into the device while restricting transport of the host's immune system. The mechanical, chemical and transport stabilities of the P(AN/VC) *in vivo* is important for continued immunoisolation, diffusive transport and safe use of the device. While there have been no indications of P(AN/VC) degradation *in vivo*, determining its stability allows its continued use. P(AN/VC) HFMs have provided continued immunoisolation to encapsulated cells for 6 months *in vivo*, indicative of its stability therein^{11,12}. In the event of degradation, the HFM may not continue to isolate the encapsulated cells and thus expose them to the host's immune system, resulting in device malfunction. Degraded P(AN/VC) HFM may be undesirable in the host.

The immunoisolatory device consists of cells suspended in a hydrogel matrix and encapsulated within an HFM, the ends of which are sealed. It is implanted into the CNS for the treatment of neurological disorders. For example, calf adrenal chromaffin (CAC) cells, which may release analgesics for the treatment of chronic pain, are immobilized in a matrix, calcium-cross-linked alginate, which provides an even distribution of cells¹³. The sealed HFM, containing cells and alginate, is implanted in the CNS, where the cell secretory products are delivered. In this study devices were prepared excluding the cells. Although cells within the device may contribute to membrane stability, implantation in the peritoneal cavity provides a more stringent environment than the CNS in terms of biomaterial stability for cell encapsulation.

EXPERIMENTAL DETAILS

Methods

Membrane preparation

P(AN/VC) membranes were prepared at CytoTherapeutics with an outer diameter of $\sim 900\mu\text{m}$, a wall thickness of $\sim 100\mu\text{m}$ and selectively permeable skins on both the inner and outer surfaces. The P(AN/VC) used was a random copolymer. The fibres were cut to $\sim 2.5\text{cm}$ lengths and sterilized in sterile-filtered 70% ethanol (Sigma, NJ, USA) overnight and then rinsed with sterile Hanks balanced salt solution (HBSS; BioWhittaker, Walkersville, MD, USA) three times and held overnight.

Capsule preparation

Sodium alginate powder (ultrapure grade, Pronova, Norway), a polysaccharide derived from seaweed, was sterilized by ethylene oxide and then dissolved in a sterile solution of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES)-buffered HBSS. Approximately 8–10 μl of sterile sodium alginate solution were injected into the hollow fibre membrane. The capsule was then immersed in a 1% solution of calcium chloride (Fisher, Fairlawn, NJ, USA) for $\sim 10\text{min}$ at room temperature to cross-link the alginate within. Cross-linked alginate provides structural support to the device implanted *in vivo*. The capsule was sealed by application of polyacrylate glue

(Albestick, UK) to the ends of the HFM. The capsule was rinsed in HBSS, HL-1TM serum-free medium (Hycor Biomedical Inc., Irvine, CA, USA) and then held in fresh HL-1TM at 37 C prior to implantation.

Implantation and explantation of encapsulated fibres

At implantation, Lewis strain rats (100–120g) were anaesthetized by i.p. injection of Nembutal[®] (0.1 ml per 100g body weight) in the peritoneal cavity. The abdominal region was shaved, swabbed with alcohol and then Wescodyne[®]. Using a sterile surgical set-up, the animal was draped and an incision of approximately 5 cm in length was made through the skin along the midline. An incision (approximately 3.5 cm long) was made into the muscle wall at the midline. Holding one side of the muscle wall with tissue holders, 10 capsules were tucked vertically between the muscle wall and the bowel at the furthest possible distance from the incision. Care was taken to prevent contact between the capsule and the incision site. The muscle wall was sutured using 6.0 EthilonTM (Ethicon, NJ, USA) sutures. The skin was closed using 9 mm wound clips. Each animal was numbered and held for 6, 12 or 15 months.

At explantation, the animals were anaesthetized and prepared for surgery as described above. Incisions were made at the midline and the skin was folded back to both left and right sides. An incision was made through the muscle wall at the midline. The animal was draped and the two sides of the muscle wall were held to their respective sides using mosquito clamps. The peritoneal cavity was gently probed to locate the capsules. The capsules were transferred to sterile HBSS. A total of 50 capsules were removed from the intraperitoneal sites of five rats (i.e. 10 capsules per rat) after each time point: 6, 12 or 15 months. After explantation of the capsules, the animals were killed by lethal injection of Nembutal[®].

Treatment of fibres post-explantation

Upon explantation, the seals of the capsules were removed with a razor blade. The open-ended fibres were immersed in a 50 mM solution of sodium citrate (Sigma, St. Louis, MO, USA), overnight at room temperature, to decross-link the sodium alginate and allow it to diffuse out of the fibre. The fibres were then rinsed three times in sterile HBSS at room temperature. The HFMs were immersed in 4 M NaOH (Fisher, Fairlawn, NJ, USA) for 8 h at 60 C, after which the HFMs were rinsed five times with water prior to characterization. The HFMs were glycerinated by immersion in 25% (w/v) glycerol in water overnight and dried first by blotting and then in air overnight.

Validation of post-explantation fibre treatment

In order to ensure that the sodium hydroxide was the optimal post-explantation treatment, four other treatments were explored for their protein-removing abilities. HFMs explanted after 1 month *in vivo* were treated with either (i) 1% sodium dodecyl sulphate (SDS; Life Technologies, Gaithersburg, MD, USA) in water for three 1 h washes, with a water rinse after each hour, at 37 C; (ii) 0.5% sodium dodecyl sulphate,

0.5 mg ml⁻¹ proteinase K (SDS/proteinase K; Boehringer-Mannheim, Germany) in water for three 1 h washes, with a water rinse after each hour, at 37°C; (iii) 1% Triton X-100 (Sigma, St Louis, MO, USA) in 0.1 M Tris-HCl, pH 8, for three 1 h washes, with a water rinse after each hour, at 37°C; (iv) 4 M NaOH for three 1 h washes, with a water rinse after each hour, at 37°C; or (v) 4 M NaOH for one 8 h wash at 60°C with five water rinses afterward. An extensive analysis was conducted to select and validate the appropriate method for treating the HFMs post-explantation.

Characterization of fibres

The HFMs (*in vivo* versus $t = 0$) were characterized by (i) gel permeation chromatography (GPC); (ii) tensile strength; (iii) hydraulic permeability (HP); and (iv) molecular weight cut-off (MWCO).

Gel permeation chromatography

Ten fibres were dissolved in 1 ml of dimethyl sulphoxide (DMSO; HPLC grade, Aldrich, NJ, USA) to prepare a 0.2–0.3% (w/v) solution. The solution was filtered through a 0.22 µm poly(vinylidene fluoride) filter and then heated for approximately 10 min. A 100 µl sample of dissolved fibre was injected and assayed on the organic (DMSO, 80°C) mobile phase GPC using polysaccharide standards (Polymer Labs, UK) and a Waters 410 refractive index detector. The weight average molecular weight (M_w) and the polydispersity index (PDI) of the sample are reported relative to polysaccharide standards. PDI is the ratio of M_w to number average molecular weight (M_n).

Tensile strength

The ends of dry glycerinated fibres were mounted into the tensile test instrument (Liveco Vitrodyne V1000, Burlington, VT, USA). Five fibres were exposed to tension at a constant strain rate of 100 µm s⁻¹ using a 2 kg load cell (Huntleigh Technology, UK). The force at break of the fibres was measured in grams and converted to mdyne by gravity multiplication. Student's *t*-test was used to determine significance based on 95% confidence level.

Hydraulic permeability

Three fibres were potted into 2-cm-long polypropylene cartridges (Cole Parmer, Niles, IL, USA) using 5 min epoxy (Devcon, Danvers, MA, USA), then deglycerinated for 20 min using 18.2 MΩ deionized water. The hydraulic permeability was determined by measuring the permeate volumetric flow rate at a transmembrane pressure of 5 psig (≈34.5 kPa). The hydraulic permeability was calculated as

$$HP = \text{flux}/(\Delta p \times \pi D l) \quad (1)$$

where HP is the hydraulic permeability (ml min⁻¹ m⁻² mmHg⁻¹); flux is the filtrate flux (ml min⁻¹); Δp is the transmembrane pressure (mmHg); D is the fibre inner diameter (m); and l is the fibre length (m).

Molecular weight cut-off

After the hydraulic permeability measurement, the sieving properties of the HFM were determined using

the same fibre cartridge and replacing the water recirculation bath with a polydisperse (10³–10⁶ g mol⁻¹) dextran solution. A dextran rejection curve was generated from the filtrate and reservoir concentrations as a function of molecular weight, determined from gel permeation chromatography^{14,15}. To minimize hydrodynamic effects on the rejection curves, luminal flow rates were set to establish a wall shear rate of 2000 s⁻¹ and the permeate flow was set to minimize concentration polarization¹⁶. The percent rejection (%R) for each molecular weight fraction was calculated from the filtrate (c_f) and reservoir concentrations (c_r) as

$$\%R = \left(1 - \frac{c_f}{c_r}\right) \times 100 \quad (2)$$

The MWCO of a fibre is defined here as the dextran molecular weight at 90% rejection.

RESULTS AND DISCUSSION

Numerous studies have been performed on the biostability of polyurethanes where *in vitro* models have been shown to correlate with *in vivo* results. Such models describe different degradation mechanisms, including environmental stress cracking², metal ion oxidation¹⁷, enzymatic degradation¹⁸ and electrostatic ion/polymer interaction¹⁹. The surface morphology and oxidative environment have been shown to affect polymer stability and, more specifically, the soft segment ether linkage has been shown to be susceptible to oxidative cleavage. Unlike polyurethanes, P(AN/VC) has a hydrocarbon backbone (see Figure 1) which is expected to be more stable to oxidative attack. In addition, the cell-encapsulated devices are ultimately implanted within the CNS, where mechanical stress is minimized.

P(AN/VC) HFMs are used to encapsulate cells for the treatment of neurological disorders. Implants of HFM-based capsules in the CNS are size-constrained by the animal species studied. Because the rat brain limits the HFM length and thus the proceeding characterization, the peritoneal cavity was chosen as the implantation site. The P(AN/VC) capsules were thus exposed to the chemically and mechanically stressful environment of the peritoneal cavity. Capsules explanted from the peritoneal cavity were often kinked even when filled with cross-linked alginate. In this regard, the stability of the P(AN/VC) HFM was assessed under more stringent conditions than those to which it would normally be exposed.

Determining the most effective protein removal treatment post-explantation

Like other materials exposed to the *in vivo* environment, P(AN/VC) HFMs adsorb proteins and other organic molecules non-specifically. In order to adequately assess the properties of the HFM, the adsorbed proteins must be removed. For example, adsorbed proteins have been shown to affect the MWCO of fibres¹¹. A number of reagents were investigated with the aim of removing adsorbed

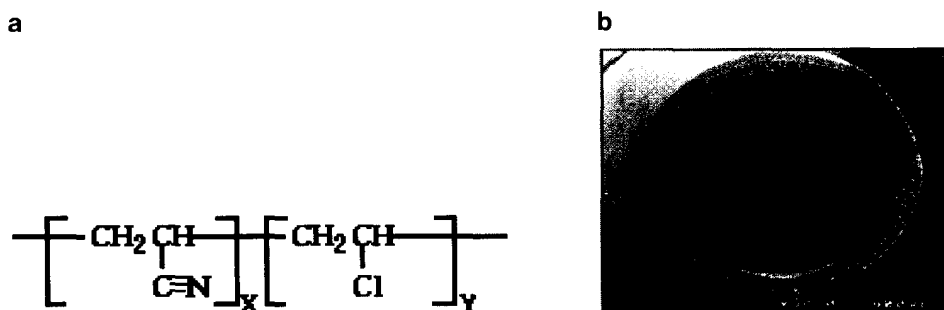


Figure 1 Poly(acrylonitrile-co-vinyl chloride): **a**, chemical formula (random copolymer); **b**, scanning electron micrograph of hollow fibre membrane having two selectively permeable skins (original magnification $\times 50$).

material without affecting the properties of the P(AN/VC) HFM. The effectiveness of a given reagent was determined by (i) the amount of protein detected by the bicinchoninic acid kit (BCA, Pierce, Rockford, IL, USA) or (ii) the hydraulic permeability measured post-treatment relative to control fibres: i.e. non-protein-exposed.

The amount of protein present on an HFM post-explantation was compared to that on HFMs pre-implantation (controls) and on HFMs post-explantation and post-treatment. For example, using the BCA assay, fibres post-explantation from the peritoneal cavity of rats had $162 \pm 22 \mu\text{g cm}^{-2}$ of adsorbed proteins. Fibres pre-implantation had no detectable protein present, nor did fibres post-explantation and post-NaOH treatment (4 M, 60 C, 8 h). In contrast to NaOH, trypsin was ineffective at removing proteins; explanted fibres showed $153 \pm 32 \mu\text{g cm}^{-2}$ of adsorbed proteins following exposure to trypsin. It is important to note that the calculated surface area of the fibre is nominal, based upon the fibre's gross dimensions ($A = \pi l(\text{o.d.})$), and did not include the additional surface area provided by the membrane's pores. The amounts of protein adsorbed, normalized by the surface area, were thus artificially high.

HP is a sensitive technique to measure effect of protein adsorption on the membrane transport. Protein adsorption decreases the effective pore radius, reflected in a decrease in the HP. In addition to indicating the effectiveness of a given 'protein-removal' treatment, HP provides information on the bulk transport properties. A successful protein-removing technique will result in similar HP values for explanted and control fibres. *Table 1* summarizes the HP measurements for a given treatment of explanted fibres. The fibres characterized in the protein-removal assay share similar properties with, but are not identical to, those HFMs studied in the biostability assay.

The results of *Table 1* indicated that treatment with SDS or Triton X-100 alone was insufficient for protein removal. Treatment with SDS/proteinase K or sodium hydroxide was more effective for protein removal, where the 4 M NaOH treatment for 8 h at 60 C was most effective and that with SDS/proteinase K was most variable. NaOH-treated fibres (4 M, 60 C, 8 h) were effectively cleared of protein as determined by BCA assay and HP measurements.

The slight increase in HP of P(AN/VC) after exposure

Table 1 Hydraulic permeability of P(AN/VC) fibres: comparison of control fibres with 1 month explanted fibres post-protein-removal treatment (mean \pm s.d.)

Protein-removal treatment ^a	HP ($\text{ml min}^{-1} \text{mmHg}^{-1} \text{m}^{-2}$) ($n = 4$)
SDS 1% 37 C, 3 h	0.5 ± 0.3
SDS/proteinase K 37 C, 3 h	3.1 ± 1.9
Triton 1% 37 C, 3 h	0.3 ± 0.2
4 M NaOH 37 C, 3 h	3.6 ± 0.9
4 M NaOH 60 C, 8 h	5.9 ± 0.2
Control glycerinized	4.9 ± 0.6

^aSee Methods section on 'validation of post-explantation fibre treatment' for details.

to NaOH warranted investigating other membrane properties. P(AN/VC) HFMs were assayed by tensile strength, dextran MWCO and GPC before and after exposure to NaOH. Neither tensile strength, dextran MWCO, nor M_w and PDI (from GPC) changed significantly after exposure to NaOH. For example, the tensile strengths ($n = 5$) were 57 ± 2 mdyne and 52 ± 2 mdyne before and after NaOH treatment, respectively. The MWCO at 90% rejection was unchanged at $37\,000 \text{ g mol}^{-1}$ and the M_w and PDI were unchanged pre- and post-NaOH treatment. The NaOH treatment regime effectively removed protein from the membrane without significantly changing the membranes' properties as determined by BCA, GPC, tensile strength, HP and MWCO.

In vivo stability of P(AN/VC)

The stability of P(AN/VC) HFMs was determined by comparing membrane and material properties before implantation and after explantation over a 12 month period. The stability of P(AN/VC) *in vivo* was assessed by measuring, over time, (i) the weight average molecular weight and polydispersity index by GPC; (ii) the tensile strength at break; and (iii) the transport properties of the membrane, assessed by MWCO and HP.

The weight average molecular weights (M_w) and the PDI of P(AN/VC) hollow fibre membranes were determined for explanted fibre samples and compared to those of fibres pre-implant ($t = 0$). The determinations were made of 10 fibres dissolved in DMSO at each time point. At $t = 0$, the P(AN/VC) had an M_w of $143\,300 \text{ g mol}^{-1}$ and a PDI of 2.3. After 12 months, *in vivo*, the M_w and PDI were $128\,400 \text{ g mol}^{-1}$ and 2.8,

respectively. The large polydispersity of the P(AN/VC) material renders the difference in $143\,300\text{ g mol}^{-1}$ and $128\,400\text{ g mol}^{-1}$ insignificant. Such changes in M_w are within the scatter of the measurement and, in fact, variations in M_w of $\pm 10\%$ are routinely observed on determinations from the same material. The M_w values and PDIs of the P(AN/VC) HFMs were indicative of *in vivo* stability.

The tensile strengths of HFMs post-explantation were compared to those of fibres pre-implantation ($t = 0$) (see Table 2).

The initial ($t = 0$) tensile strength of HFMs was 52 ± 2 mdyne and the tensile strength at 15 months was 46 ± 7 mdyne; the tensile strength changed insignificantly by a two-tailed Student's *t*-test ($P > 0.05$) over time *in vivo*. At 12 months, the tensile strength of HFMs was apparently decreased with respect to that at $t = 0$. The abnormally low tensile strength was attributed to kinks present in the fibre length, which were not present at $t = 6$ and 15 months. To determine the effect of 'kinking' on fibre tensile strength, it was found that the kink provided a locus of failure for fibres in tension and that 'kinked' fibres had approximately half the tensile strength (38 ± 6 mdyne) of non-kinked fibres (64 ± 2 mdyne) ($n = 6$).

The hydraulic permeability and convective MWCO of HFMs pre-implantation and post-explantation are compared in Table 3.

The hydraulic permeability of the fibres pre-implantation was $7.4\text{ ml min}^{-1}\text{ m}^{-2}\text{ mmHg}^{-1}$, and did not change with respect to the standard deviation over time up to 12 months *in vivo*. The MWCO of the fibres was also constant with respect to the standard deviation at $40\,000\text{ g mol}^{-1}$ up to 12 months *in vivo*. Figure 2 shows the complete dextran rejection profile pre-implantation and post-explantation. These rejection profiles are generated from the average of the cartridges, each of which contains three fibre segments. Error bars are shown for the pre-implantation rejection profile, and represent the standard deviation of the cartridge mean. The rejection profiles

Table 2 Tensile strength pre-implantation ($t = 0$) and post-explantation ($t = 6, 12, 15$ months) of P(AN/VC) hollow fibre membranes (mean \pm s.d.)

Time (months)	Tensile strength ($n \geq 5$) (mdynes)
0	52 ± 2
6	45 ± 8
12	25 ± 8^a
15	46 ± 7

^aDamaged fibres broke at kink.

Table 3 P(AN/VC) hollow fibre membranes compared by hydraulic permeability (HP) and molecular weight cut-off (MWCO) (mean \pm s.d.)

Time (months)	HP ($\text{ml min}^{-1}\text{ m}^{-2}\text{ mmHg}^{-1}$) ($n = 3$ fibres)	MWCO (g mol^{-1}) (90% rejection) ($n = 3$ fibres)
0	7.4 ± 1.5	$40\,000 \pm 8000$
6	6.3 ± 1.5	$52\,000 \pm 10\,000$
12	7.5 ± 1.5	$54\,000 \pm 10\,000$

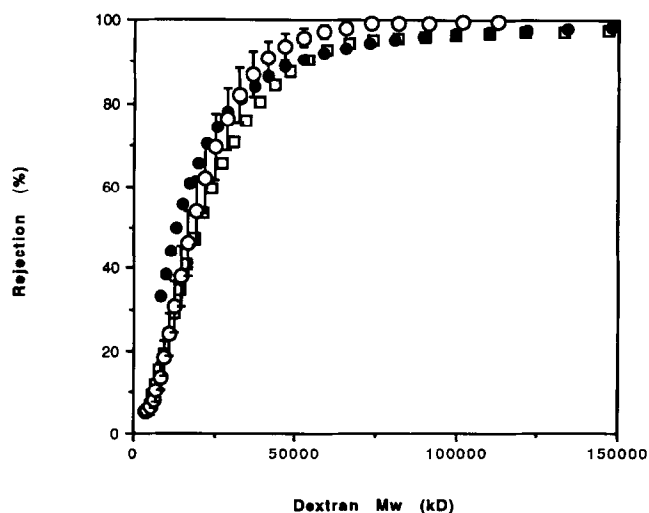


Figure 2 Molecular weight cut-off data of fibres (○) pre-implantation and post-explantation: (●) 6 months; (□) 12 months. % Rejection versus dextran molecular weight.

remain unchanged with respect to the standard deviation over time up to 12 months.

The MWCO data with polydisperse dextran indicated that the HFMs shared similar transport properties pre-implantation and post-explantation to $t = 12$ months.

This consistency of convective transport properties over time reflects the stability of the pore structure in the P(AN/VC) membranes. The hydraulic permeability and dextran rejection curves represent the pore size and pore size distribution of the HFMs.

Changes in either of these two measurements would have indicated either a collapse in the pore size or the creation of large pore defects. The structural integrity of the P(AN/VC) membranes with time *in vivo* is indicative of the molecular stability of P(AN/VC).

The analytical techniques used to characterize the bulk properties of the membrane indicated that *in vivo* fibres were stable over time for at least 12 months. M_w and PDI (determined from GPC) of *in vivo* fibres were similar at 6 and 12 months to those of fibres at $t = 0$; the tensile strengths of the *in vivo* fibres were maintained with respect to zero time point fibres; and the hydraulic permeability and MWCO of *in vivo* fibres changed insignificantly from $t = 0$ to $t = 12$ months. These results support the continued use of P(AN/VC) as a biomaterial for immunoisolation and xenogeneic transplantation.

ETHICAL REVIEW

All animal tests were performed in accordance with written protocols that had been approved by the CytoTherapeutics Inc. Institutional Animal Care and Use Committee.

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