Society For Biomaterials

Considerations in binding diblock copolymers on hydrophilic alginate beads for providing an immunoprotective membrane

Milica Spasojevic,[1,2] Swapnil Bhujbal,[2] Genaro Paredes,[2] Bart J. de Haan,[2] Arend J. Schouten,[1] Paul de Vos[2]

[1]Department of Polymer Chemistry, Zernike Institute for Advanced Materials, University of Groningen, 9747, AG Groningen, the Netherlands

[2]Departments of Pathology and Laboratory Medicine, Section of Medical Biology, Division of Immunoendocrinology, University of Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands

Received 2 May 2013; revised 13 June 2013; accepted 28 June 2013
Published online 24 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34863

Abstract: Alginate-based microcapsules are being proposed for treatment of many types of diseases. A major obstacle however in the successes is that these capsules are having large lab-to-lab variations. To make the process more reproducible, we propose to cover the surface of alginate capsules with diblock polymers that can form polymer brushes. In the present study, we describe the stepwise considerations for successful application of diblock copolymer of polyethylene glycol (PEG) and poly-L-lysine (PLL) on the surface of alginate beads. Special procedures had to be designed as alginate beads are hydrophilic and most protocols are designed for hydrophobic biomaterials. The successful attachment of diblock copolymer and the presence of PEG blocks on the surface of the capsules were studied by fluorescence microscopy. Longer time periods, that is, 30–60 min, are required to achieve saturation of the surface. The block lengths influenced the strength of the capsules. Shorter PLL blocks resulted in less stable capsules. Adequate permeability of the capsules was achieved with poly(ethylene glycol)-block-poly(L-lysine hydrochloride) (PEG_{454}-*b*-PLL_{100}) diblock copolymers. The capsules were a barrier for immunoglobulin G. The PEG_{454}-*b*-PLL_{100} capsules have similar mechanical properties as PLL capsules. Minor immune activation of nuclear factor κB in THP-1 monocytes was observed with both PLL and PEG_{454}-*b*-PLL_{100} capsules prepared from purified alginate. Our results show that we can successfully apply block copolymers on the surface of hydrophilic alginate beads without interfering with the physicochemical properties. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 102A: 1887–1896, 2014.

Key Words: alginate, microencapsulation, diblock copolymer, physicochemical surface properties

How to cite this article: Spasojevic M, Bhujbal S, Paredes G, de Haan BJ, Schouten AJ, de Vos P. 2014. Considerations in binding diblock copolymers on hydrophilic alginate beads for providing an immunoprotective membrane. J Biomed Mater Res Part A 2014:102A:1887–1896.

INTRODUCTION

Encapsulation of mammalian cells is meant to protect tissue from the host immune system and to provide adequate conditions for functional survival of the immunoprotected tissue. Encapsulation provides a number of advantages. It avoids the application of immunosuppressive medication and it enables transplantation of not only allografts but also xenografts. The latter is a pertinent advantage as it solves the problem of donor shortage. As a consequence, the encapsulation concept has been proposed for the treatment of many endocrine diseases such as anemia,[1] dwarfism,[2] hemophilia B,[3] kidney,[4] and liver[5] failure, pituitary[6] and central nervous system insufficiencies,[7] as well as diabetes.[8]

Microencapulation of pancreatic islets in alginate–poly-L-lysine (PLL)–alginate capsules was first introduced by Lim and Sun in 1980.[8] However, a pertinent issue remains to be solved as reported graft survival rates vary considerable from several days to years.[9–11] These large variations of graft survival rates can be attributed to small lab-to-lab modifications of the encapsulation procedure such as different degrees of purity of alginates,[12–15] variations in the type of alginates[16–20] applied, and application of different polyamino acids.[12,20,21] Impurities, present in crude alginates can stimulate the immune system; therefore, they have to be thoroughly removed before application. Different types of alginate contain different quantities of the monomer units β-D-mannuronic acid (M) and α-L-guluronic acid (G). Alginates with a higher content of G-blocks provide stronger and more stable gels,[17,22–24] but after implantation high-G alginate–PLL capsules caused stronger immune response than capsules made of alginate with lower G content.[16,18,25] Also the type of polycation has been shown to be an essential factor in the success and failure of capsules.[21,23] The reason for application of polyamino acid is to decrease the permeability of the capsules and to improve their mechanical properties.[26,27] Different polyamino acids have been

Correspondence to: P. de Vos; e-mail: p.de.vos@umcg.nl

tested and PLL proved to be the best available option.[21] However, inadequately bound PLL can cause inflammatory reactions. Therefore, the last step in the encapsulation procedures is treatment of capsules with a diluted alginate solution to force unbound PLL in beta-sheets or alpha-helical cores with alginate.[28–31]

We hypothesize that all these variations in preparation, which are difficult to control, can be avoided by applying a masking layer of block copolymers on the surface of the capsules. However, this approach has not been applied on hydrogels before. In the present study, we describe the stepwise considerations for successful application of diblock copolymer of polyethylene glycol (PEG) and PLL on the surface of alginate beads. PEG is a water-soluble polymer, and therefore suitable for this application.[32,33] It has been known as a nontoxic and nonimmunogenic polymer approved by the US Food and Drug Administration.[34] Also, PEG polymers are known to prevent potential protein adsorption on the surface.[35–37] The binding of PEG-*b*-PLL diblock copolymers to alginate was accomplished through electrostatic complex formation. PLL blocks act as an anchoring block whereas PEG blocks should form an antifouling layer on the surface of the capsules. The binding process and the location of PEG blocks were investigated. Adequate permeability of the capsules has been demonstrated. A macrophage cell lines with a nuclear factor κB (NF-κB) reporter were applied to demonstrate the absence of immune activation of our alginate–PEG-*b*-PLL diblock polymer system.

MATERIALS AND METHODS

Materials

Intermediate-G sodium alginate was obtained from ISP Alginates (UK). PLL hydrochloride (Mn = 16,000 Da; Alamanda Polymers), methoxy-poly(ethylene glycol)-block-poly(L-lysine hydrochloride) [PEG_n-*b*-PLL_m; n = 22, 113, and 454; m = 10, 20, 50, 100, and 200; polydispersity index (PDI) = 1.2; Alamanda Polymers] were used as received. Streptavidin fluorescein isothiocyanate (FITC) and rabbit anti-PEG biotin were purchased from DakoCytomation (Denmark) and Bio-Connect B.V. (the Netherlands), respectively. Narrow-distributed pullulan standards with the molecular weight in the range of 180–805,000 Da were obtained from Gearing Scientific (Ashwell, Herts, UK). Thp1-XBlue-MD2-CD14 cell line, lipopolysaccharide from *Escherichia coli* K12 strain (LPS-EK Ultrapure) and QUANTI-Blue™ were purchased from InvivoGen (Toulouse, France).

Alginate purification

Crude sodium alginate was dissolved at 4°C in a 1-m*M* sodium *ethylene glycol tetraacetic acid* solution to a 1% solution under constant stirring, and successively filtered over 5.0, 1.2, 0.8, and 0.45 μm filters. Next, the pH of the solution was lowered to 2.0 under constant monitoring by addition of 2 *N* HCl plus 20 m*M* NaCl. During this procedure, the solution was kept on ice to prevent hydrolysis of alginate. The further lowering of pH was associated with gradual precipitation of alginate as alginic acid, which was subsequently filtered over a Buchner funnel (pore size 1.5 mm) to wash out nonprecipitated contaminants. To extend the wash out of nonprecipitated contaminants, the precipitate was brought in 0.01 *N* HCl plus 20 m*M* NaCl, vigorously shaken, and filtered again over the Buchner funnel. Then, proteins were removed by extraction with chloroform/butanol (4:1). This procedure was repeated three times. Next, the alginic acid was slowly dissolved by raising the pH to 7.0 by the slow addition of 0.5 *N* NaOH plus 20 m*M* NaCl. The obtained alginate solution was subjected to a chloroform/butanol extraction (three times) to remove those proteins that can only be dissolved in chloroform/butanol at neutral pH. The mixture was centrifuged for 3 min at 600*g*, which induced the formation of a separate chloroform/butanol phase which was removed by aspiration. The final step was precipitation of the alginate with cold ethanol. To each 100 mL of alginate solution, we added 200 mL absolute ethanol. After an incubation period of 10 min, all alginate had precipitated. It was filtered over the Buchner funnel and washed with cold absolute ethanol. Finally, the alginate was washed two times with diethyl ether and freeze-dried overnight.

Purified alginate was dissolved in 220 mOsm Ca^{2+} Krebs–Ringer–Hepes (KRH) at concentration 3.4% (w/v) and sterilized by filtration (0.22 μm).

Microencapsules formation

Capsules were produced according to a previously described procedure with some modifications.[38] Briefly, the 3.4% (w/v) sodium alginate solution was converted into droplets using an air-driven generator.[39] The diameter of the droplets is controlled by a regulated air flow around the tip of needle. Alginate droplets were transformed to rigid alginate beads by gelling in 100 m*M* $CaCl_2$ solution for at least 10 min. The beads were washed with KRH (containing 2.5 m*M* $CaCl_2$) for 1 min and subsequently coated with PLL (10 min, 0.1% PLL solution in 310 mOsm Ca^{2+}-free KRH) or with PEG_n-*b*-PLL_m. In case of PEG_n-*b*-PLL_m capsules the progression of copolymer adsorption on the surface of the capsules with time was studied. Therefore, the alginate beads were incubated for 5, 10, 20, 30, or 60 min in the block copolymer solution, washed three times with 310 mOsm Ca^{2+}-free KRH and stored in 25 m*M* KRH solution.

FITC labeling of microcapsules

Fluorescent labeling of microcapsules is a multiple step procedure. Primary antibody was added to a 10% solution of normal rabbit serum in phosphate-buffered saline (PBS). The optimal primary antibody concentration was tested and found to be when antibody was 500 times diluted. To stain end groups of PEG, 100 μL of this PBS solution was added to an Eppendorf cup with approximately 20 capsules and left to shake for 1 h at room temperature. The capsules were washed several times with PBS and subsequently incubated in PBS solution of streptavidin FITC (streptavidin FITC/PBS = 1/100) for 30 min in dark. Finally, the capsules were washed several times with PBS, brought on a glass slide and studied at room temperature on a Leica DM-RXA fluorescence

microscope (Hg lamp pf 100 V, 5(6)-FITC; FITC excitation wavelength 494 nm and FITC emission wavelength 518 nm).

Immune activation by capsules

Capsules were coincubated with a Thp1-XBlue-MD2-CD14 cell line. This cell line derives from the human monocytic THP-1 cell line. They were obtained by stable transfection of THP-1 cells with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene. The SEAP reporter gene is under control of a promoter inducible by the transcription factors NF-κB and activator protein 1. The cell line contains all the human Toll-like receptors (TLRs) and, upon TLR stimulation, THP-1 cells activate transcription factors. This leads to the secretion of SEAP which can be quantified by spectrophotometry.

THP1 cells were suspended in fresh RPMI 1640 medium at 1×10^6 cells/mL and plated in 96-wells plates. Each well was stimulated with either 30 PLL capsules or 30 PEG-*b*-PLL capsules made of purified or nonpurified intermediate-G alginate and cultured overnight at 37°C and 5% CO_2. Lipopolysaccharide from *E. coli* K12 strain was used as a positive control and RPMI 1640 culture medium was used as a negative control. Production of SEAP was quantified by using QUANTI-Blue™.

Permeability of the capsules

Permeability was assessed with an inverse size exclusion chromatography (ISEC) with pullulan standards and saccharose.[40] Microcapsules of 10 mL were loaded in a 10 mm × 250 mm glass column (Omnifit, Cambridge, UK). The glass column was attached to a Waters SEC set-up consisting of Waters 515 pump, Rheodyne Injector 7725i with 100 μL loop and differential refractive index detector Waters 2410. First, the column was equilibrated for 12 h. Subsequently pullulan solutions in 25 m*M* KRH (3 mg/mL) were injected into the column with a flow rate of 0.2 mL/min. The elution volumes obtained correspond to 50% of the area of each pullulan standard, and calculated partition coefficient was used to construct a calibration curve. Molecular weight cut-off (MWCO) was determined from the calibration curve as an exclusion volume. The differential pore size distribution was obtained as the first derivate of the Boltzmann fit.

Microscopy

The size of microcapsules was estimated by microscopy (optical microscope Kapa 2000, Kvant, Slovakia, equipped with color charge-coupled device camera, Mintron CC-63KW1P, Mintron, Malaysia, operated with software Prover Image Forge v1.1, Prover s.r.o., Slovakia).

Mechanical properties

The mechanical properties of microcapsules were quantified with a Texture Analyzer TA-2Xi (Stable Micro Systems, Godalming, UK) equipped with a force transducer of resolution 1 mN. The equipment consisted of a mobile probe moving vertically at a constant velocity. The mechanical stability of microcapsules was measured by compressing individual microcapsules. A microcapsule was placed on a plate and the probe was moved with a constant speed of 0.5 mm/s toward the microcapsule until the travelled distance had reached 98% of the initial distance between plate and probe. The force displacement data were recorded with a frequency of 100 Hz. The force (expressed in grams) exerted by the probe on the microcapsule was recorded as a function of the displacement (compression distance).

Statistics

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made with the Mann–Whitney *U* test. A p value <0.05 was considered statistically significant.

RESULTS

Binding of PEG-*b*-PLL diblock copolymers to the alginate gel was accomplished through ionic interactions between alginate and PLL-blocks. Positively charged ammonium groups of PLL blocks form a polyelectrolyte complex with negatively charged carboxyl groups of alginate (Fig. 1). This was only successful with alginates with sufficient MG blocks due to the fact that only MG blocks of alginate are flexible enough to interact with PLL and form a complex consisting of α-helical PLL core surrounded by superhelically orientated polysaccharide chains.[29,41,42]

A pertinent consideration in the binding of diblock copolymers to alginate hydrogels is the molecular weight (M_w) of the PEG blocks as well as the ability of PEG to interact with alginate. The M_w will determine whether the entire diblock copolymer penetrates into an alginate bead or only the PLL tail. When the length of the PEG block is high and when phase separation occurs, we expect that only the PLL blocks will penetrate into the surface of an alginate bead. In this case PEG blocks will stay on the surface of the capsules and form a new layer [Fig. 2(a)]. If PEG can interact with alginate or the length of PEG blocks is too small, PEG will also diffuse into an alginate bead [Fig. 2(b)]. PEG is a neutral polymer. Therefore, there are no ionic interactions between PEG molecules and alginate. PLL was applied as the binding tail in the diblock copolymer. It has been shown that PLL penetrates readily into an alginate bead.[43] Therefore, we assumed that PLL blocks behave in the same manner as PLL homopolymers wheras PEG should stay on the surface of the capsules and determine the surface properties. Two expected structures of PEG_n-*b*-PLL_m capsules are illustrated in Figure 2.

Determination of the PEG position and kinetics of adsorption

To visualize whether diblock copolymers were successfully adsorbed on the surface of alginate beads, we designed an assay in which an antibody directed against the end group of PEG block (methoxy group) was applied. This antibody normally diffuses through the calcium–alginate network. PLL capsules were used as negative control. The presence of green fluorescence on the observed PEG_n-*b*-PLL_m capsules showed a successful diblock copolymer adsorption with PEG blocks at the surface. We tested the PEG_n-*b*-PLL_m

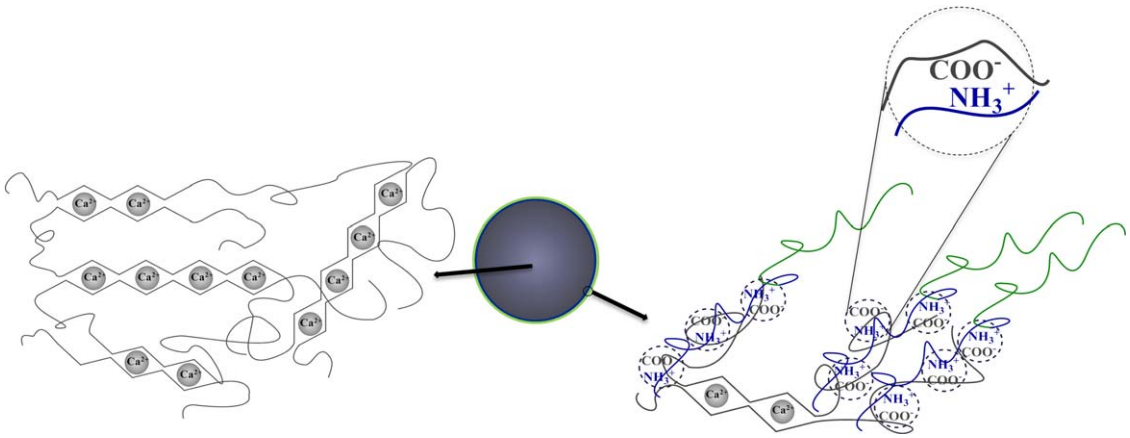

FIGURE 1. Schematic presentations of diblock copolymer binding on alginate beads. The binding of copolymer is achieved through ionic interaction between carboxyl groups of alginate and ammonium groups of PLL block. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

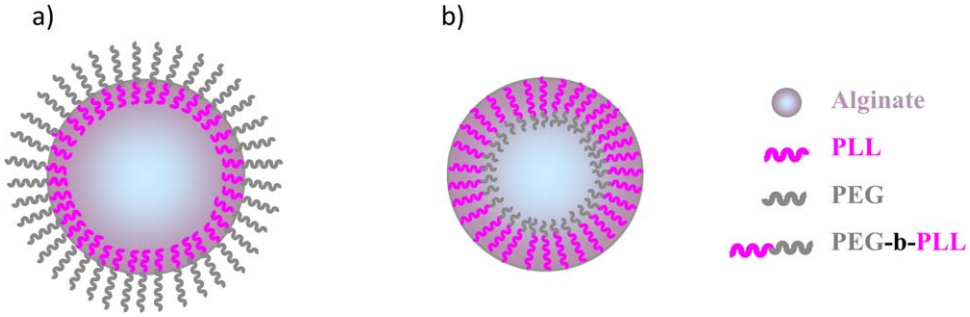

FIGURE 2. Schematics presentation of possible structures of PEG_n–PLL_m capsules. When the M_w of PEG is large enough PEG will not penetrate into the alginate network. Phase separation will occur forcing the PLL to penetrate and interact with alginate at the surface of the beads (a). When PEG chains are smaller they can penetrate into the network (b). This structures as well as intermixed conformations may occur. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

capsules with different lengths of PEG and PLL blocks (Table I). It has been found that the position of PEG blocks and the stability of the capsule are dependent on the length of diblock copolymers as well as the size of each block.

Fluorescence labeling of the capsules with short PEG and PLL blocks (PEG_{22}–PLL_{10}) showed penetration of the entire diblock copolymer inside the capsules [Fig. 3(a)]. When applying larger molecules, that is, PEG_{113}-*b*-PLL_{50} diblock copolymer, we found a high concentration of PEG blocks on the surface of the capsules [Fig. 3(b)].

PEG_n–PLL_{10} capsules (the capsules with the smallest PLL block; n = 113 and 454) cannot be used for these kinds of applications. The short PLL tail (PLL_{10}) did not cross-link the surface of the beads to a high enough extent as illustrated by the instability of the beads. They did fall apart before microscopical study could be performed.

Next, we studied the adsorption kinetics of PEG_{113}–PLL_{200}, diblock copolymer (Fig. 4). The adsorption starts immediately as we found that after 5 min approximately 50% of the saturation value was achieved. However, the adsorption growth slows down after this period and finally reaches a plateau after 30–60 min.

TABLE I. Diblock Copolymers PEG_n-*b*-PLL_m Tested in the Present Study

n	M_w(PEG) (kDa)	m	M_w(PLL) (kDa)	M_w(PEG_n-*b*-PLL_m) (kDa)
22	1	10	1.6	2.6
22	1	50	8.2	9.2
22	1	100	16	17
22	1	200	33	34
113	5	10	1.6	6.6
113	5	50	8.2	13.2
113	5	100	16	21
113	5	200	33	38
454	20	10	1.6	21.6
454	20	20	3.2	23.2
454	20	50	8.2	28.2
454	20	100	16	36
454	20	200	33	53

All diblock copolymers have narrow polydispersity (PDI = 1.10). N is the degree of polymerization (number of repeating monomeric units) of PEG blocks, M_w(PEG) is the molecular weight of the PEG blocks, m is the degree of polymerization (number of repeating monomeric units) of PLL blocks, M_w(PLL) is the molecular weight of PLL, and M_w(PEG_n-*b*-PLL_m) is the molecular weight of the entire copolymer.

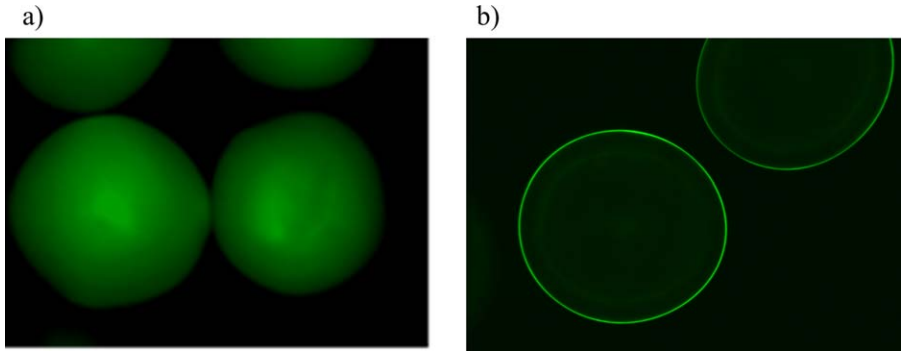

FIGURE 3. Fluorescence microscopy images of (a) PEG_{22}–PLL_{10} and (b) PEG_{113}–PLL_{50} alginate microcapsules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The adsorption kinetics was very dependent on the composition of the diblock copolymer applied. In some cases a maximum adsorption was not even achieved after 60 min (Fig. 5). When studying the PEG_{113}–PLL_y series, the size of the PLL blocks did not have a significant influence on the adsorption rate. PEG_{454}–PLL_{100} capsules demonstrated a higher fluorescence than PEG_{454}–PLL_{20} and PEG_{454}–PLL_{50} capsules. PEG_{454}–PLL_{200} capsules were not stable and fell apart even before microscopical observation.

NF-κB activation in THP-1 monocytic reporter cell lines induced by capsules in the presence and absence of diblock polymers

A major issue in bioencapsulation research is the presence of impurities in alginates that are not always efficiently removed by purification.[44] We tested the hypothesis whether applying diblock polymers can mask immunostimulation effects of impurities that stimulate monocytic cells via TLRs. To this end, we applied PEG_{454}-*b*-PLL_{100} capsules and

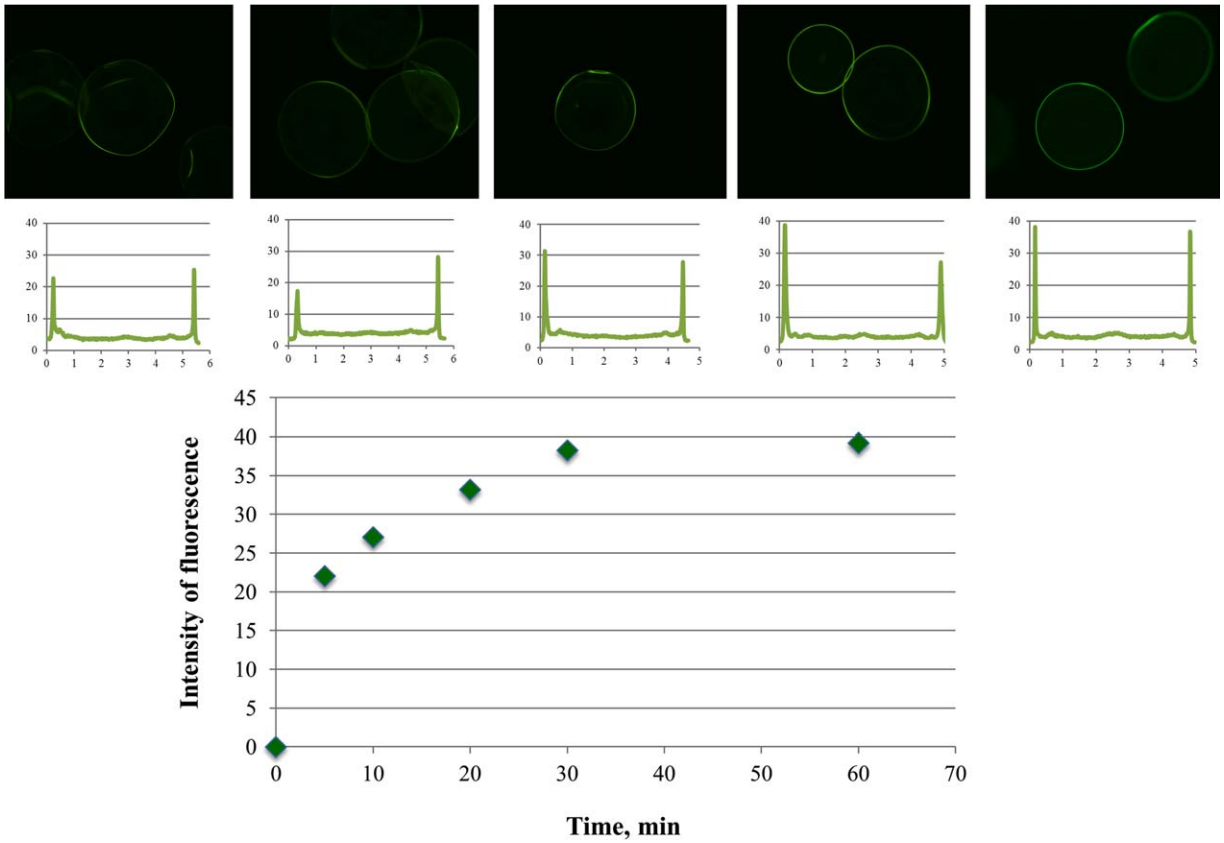

FIGURE 4. Fluorescence microscopy images of PEG_{113}–PLL_{200} capsules and their intensity profiles. During the capsule formation procedure the beads were exposed to the diblock copolymer solution for (a) 5 min, (b) 10 min, (c) 20 min, (d) 30 min, and (e) 60 min (top). The fluorescence intensity of the capsule surface as a function of polymer adsorption time (bottom). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

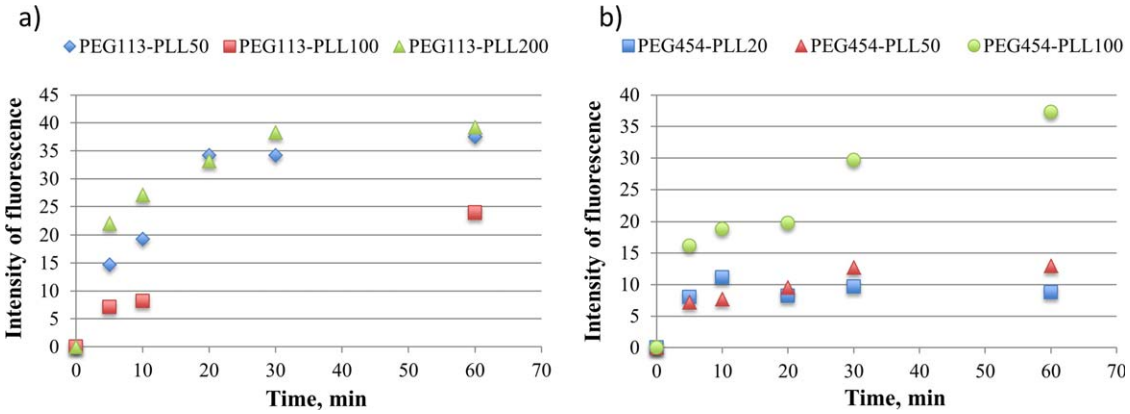

FIGURE 5. The adsorption profiles of PEG_{113}–PLL_y series and PEG_{454}–PLL_y series. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PLL capsules made of both purified and nonpurified intermediate-G alginate.

As shown in Figure 6, the diblock polymer PEG_{454}-*b*-PLL_{100} capsules was not able to reduce the immunostimulatory effect of impurities present in alginate. Our observations suggest that the impurities are soluble and diffuse out of the capsules as we found no adhesion of the THP-1 cell line on the capsules thus suggesting that there was no direct interaction between the cells and impurities on the surface of the beads. A positive finding is that the diblock copolymer coated capsules with purified alginates do not provoke any response of the THP-1 cell line.

Permeability and stability of the capsules

Fluorescence labeling experiments have shown that longer times (60 min) are required for reaching saturation of the capsules surface with PEG-*b*-PLL diblock copolymers. Therefore, we had to examine whether these prolonged times as well as diblock copolymers itself influence permeability and mechanical resistance of the capsules. Capsules should withstand shear forces during the implantation procedure. In addition the capsules should be resistant to various forces inside the peritoneal cavity.[12] Weak capsules affected by these forces can collapse and trigger enormous inflammatory reaction.[45] Therefore, proper mechanical stability of the capsules is essential for successful application. Mechanical stability of standard PLL capsules (alginate beads treated for 10 min with PLL) and PEG_{454}-*b*-PLL_{100} capsules (the capsules saturated with this copolymer) was evaluated using a texture analyzer. PEG_{454}-*b*-PLL_{100} capsules were chosen as they were stable and because long PEG chains are preferred for promoting PEG accumulation on the surface of the capsules. PLL capsules have shown to be slightly more stable than PEG_{454}-*b*-PLL_{100} capsules (68 ± 14 vs. 56 ± 16 g), but this did not reach statistical significance. This value is considered to be sufficient to withstand the above mentioned forces.[46]

Another important issue is permeability. Functional survival of encapsulated cells is dependent on the ability of the membrane to enable influx of molecules essential for cell survival as well as efflux of cellular products and waste material.[47–49] The membrane should also protect the encapsulated cells from the host immune system. Therefore, immunoglobulin G (IgG) as the smallest antibody should not be able to diffuse through the membrane. Permeability was assessed through the determination of MWCO of microcapsules. Standard alginate PLL capsules prepared by exposing alginate beads for 10 min to a 0.1% PLL solution have lower permeability (MWCO value of ~8 kDa determined for pullulan standards which corresponds to ~19 kDa for

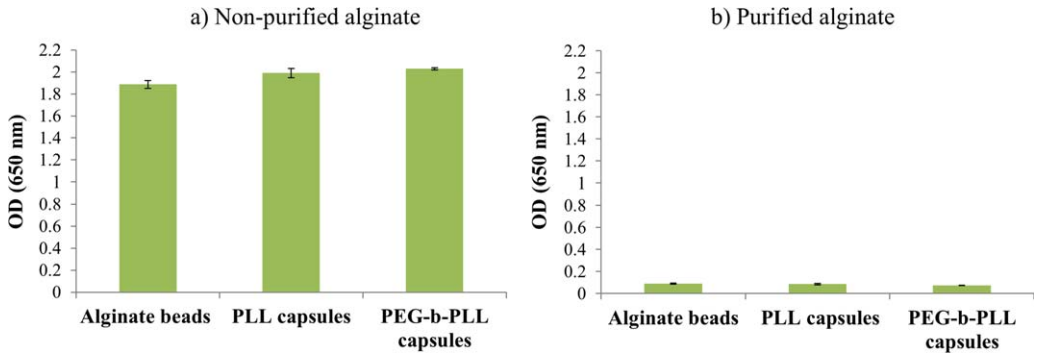

FIGURE 6. NF-κB activation of alginate beads, PEG_{454}-*b*-PLL_{100} capsules and PLL capsules made of nonpurified and purified intermediate-G alginates. Impure alginate coated with PLL or PEG_{454}-*b*-PLL_{100} was coincubated with THP-1 monocytes with a NF-κB reporter (left). The same procedure was applied with purified alginates (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Permeability, Mechanical, and Capsule Size of PLL-10 min and PEG_{454}-*b*-PLL_{100} 60-min Capsules

Microcapsule Type	Permeability MWCO (kDa)		Mechanical Properties (g)	Capsule Size (mm)
PLL_{100}, 10 min	~8	~19	68 ± 14	0.69 ± 0.02
$PEG_{454}PLL_{100}$, 60 min	~25	~98	59 ± 18	0.65 ± 0.02

Results are expressed as mean ± SEM for at least five experiments.

proteins; $p < 0.001$) than beads treated with PEG_{454}-*b*-PLL_{100} for 60 min (25 kDa determined for pullulan standards or 98 kDa recalculated for proteins). Although larger than the permeability of PLL capsules, this value is still acceptable since the smallest antibodies, IgG, have a molecular weight of approximately 150 kDa.[50] Therefore, PEG_{454}-*b*-PLL_{100} capsules represent a good barrier and a proper protection for encapsulated islets from the host immune system.

Capsule size and mechanical properties as well as permeability of both types of capsules are summarized in Table II.

DISCUSSION

A pertinent requirement is that capsule membranes for immunoisolation provide physicochemical stability, high biocompatibility, and adequate permeability. It has been repeatedly shown that multiple step coating with polyamino acids and alginate is far from easy.[12] Seemingly minor variations in the encapsulation procedure have a large impact on the capsule properties with extreme consequences for the enveloped cells. This is a major consideration for future application of the encapsulation as the low reproducibility is considered a major hurdle in clinical application of encapsulated cells to treat human disease.[12]

This study was undertaken as a first effort to overcome the complexity of the encapsulation procedure by proposing a simple one step procedure to create in a nonlaborious way a reproducible surface. As most studies with block copolymers have been performed with hydrophobic materials, we could not rely on published protocols.[51,52] Instead we had to test new procedures to demonstrate the efficacy of the membrane formation. As PLL has a high affinity for alginate, we applied this macromolecule as an anchoring block in our design. PEG blocks stay on the surface and form a polymer layer. This process was found to be highly dependent on the molecular weight of PLL. To understand this we have to elaborate on the binding process on bead surfaces (Fig. 7). After gelification of alginate beads in calcium, we apply a washing step in buffer with low calcium and containing high sodium.[17,53,54] This step is required to extract some calcium from the surface of the beads, which will subsequently be substituted by sodium.[17,53,54] Sodium will be electrostatically bound to the carboxyl groups of alginate. It has a lower affinity for the alginate than PLL. The PLL that is subsequently added is binding to constitutive alginate molecules in a highly cooperative manner to form a strong, rigid membrane. The degree of cross-linking determines the mechanical stability and permeability of the membrane.[8,12,26,27]

Our strategy is to create a block copolymer-based membrane that forms a so-called polymer brush. These brushes are end-attached polymer chains stretched away from the surface into the surrounding solution. These brushes have special properties that lead to diminished protein adsorption and cell adhesion.[55–57] To achieve that goal, we allowed long incubation times to have a high amount of block copolymer on the surface on the bead. This forces the PEG chains to stretch and form a brush. Unfortunately, standard, accepted technologies to demonstrate the presence of brushes such as ellipsometry[56–60] cannot be performed on microcapsules made of hydrogels. Up to now, we only have circumstantial proof that especially the long chain block copolymers do form brushes. This proof comes from our microscopical examination by immunofluorescence and by TEM (data not shown) that only the PEG layer is on the surface. We were not able however to exactly measure the thickness of the layer and to confirm whether this approaches the theoretical predicted thickness as applied for hydrophobic surfaces.[58] New technologies applicable for hydrogels have to be developed to achieve that goal.

As demonstrated in our study, capsules prepared with diblock copolymers with short PLL blocks, PEG_n–PLL_{10}, n = 113 and 454, are not forming stable membranes. This can be explained by the fact that these PLL chains are too small to form a dense network with alginate in the membrane.

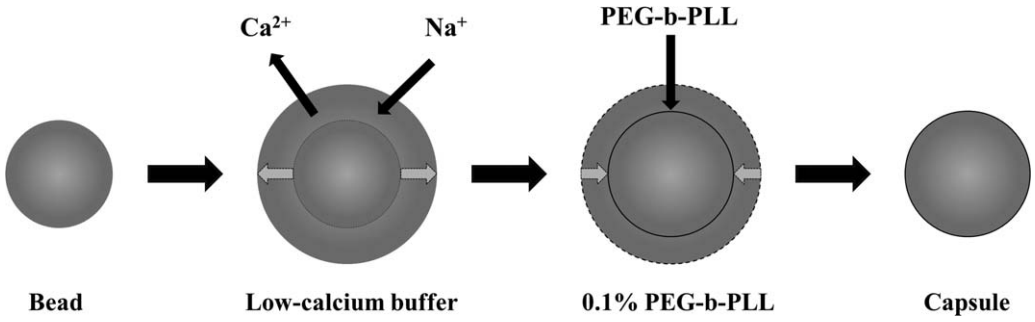

FIGURE 7. Schematic presentation of the capsules formation process. The bead swells due to substitution of calcium from the surface with sodium. Subsequently, added PLL has a higher affinity for the alginate than sodium and binds to constitutive alginate molecules.

Our data demonstrate that at least a PLL length of 9.2 kDa is required to achieve that goal. PEG lengths seem to be less important in this respect. However, when diblock copolymers with the smallest PEG block were applied, PEG_{22}–PLL_m, $m = 50$, 100, and 200, the capsules were more stable than PEG_n–PLL_{10} capsules. However, some stability issues such as deformation of the membrane were observed here as well. Thus, deformation of the spherical capsules probably occurs as a consequence of the osmotic pressure changes that the membrane cannot withstand.

Adsorption patterns of diblock copolymers are different from that of other polyamino acids applied for immunoprotection. Polyamino acids such as PLL and polyornithine typically need 10 min to provide a permeability to retain molecules larger than 120 kDa.[21,43,61,62] Such a long incubation period is desired since it allows for versatility and fine tuning of the membrane permeability for different applications.[27] On the other hand, due to larger chain lengths, the adsorption of diblock copolymers is slower and requires longer time periods. Also prolonged time of copolymer adsorption provides higher concentrations of neutral and biocompatible PEG on the surface of the capsules. More densely packed chains of PEG on the capsules surface will provide better antifouling properties. The adsorption profiles of PEG-*b*-PLL copolymers have shown a rapid adsorption in the first 5 min. Due to reduced numbers of available binding spots, the adsorption slows down and reaches a plateau value. We assumed that after 30–60 min, all carboxylic groups are occupied and therefore copolymers cannot be adsorbed anymore.

Capsules prepared with short PEG and PEG_{22}-*b*-PLL_y or short PLL and PEG_x-*b*-PLL_{10} were shown to be unstable during the staining procedure. Therefore, we decided to abandon these copolymers. Longer PEG blocks should provide better antifouling properties. The stability of the capsules is determined by the size of PLL blocks (longer PLL blocks provide more stable capsules). Despite long PLL blocks, PEG_{454}-*b*-PLL_{200} capsules were quite unstable. This is probably because the membranes were less elastic and brittle. Therefore, PEG_{454}-*b*-PLL_{100} is the best choice for the further investigations.

Diblock copolymers did not diminish the inflammatory effects of impurities in alginate. Such an effect might have been expected when the proinflammatory molecules are either too large to pass the diblock polymer membrane or when they were present on the surface. Our data however suggest that the contaminating, proinflammatory molecules are solutes that easily diffuse out since the monocytic cell line is not adherent to the capsules implying that direct interaction does not occur. As these contaminants are probably solutes smaller than the molecular diffusion limit of 120 kDa, not any type of coating will be able to avoid the release of these factors. As shown these solutes activate the monocytes in a NF-κB dependent fashion. A positive finding is that after purification this NF-κB activation does not occur anymore and both PEG_{454}-*b*-PLL_{100} capsules and PLL capsules made of purified alginates showed virtually no NF-κB activation [Fig. 4(b)].

Microcapsule membranes should also provide sufficient permeability. This is essential for functional survival of cells. Nutrients such as oxygen, nutrients, glucose and metabolic products should be able to freely diffuse in/out of a capsule, whereas antibodies and cytotoxic cells of immune system should stay outside the capsule. IgG is the smallest immunoglobulines responsible for the protection from bacterial and viral infections. They can interfere with functional survival of the encapsulated islets. Therefore, the permeability of the capsules should be tailored in such a way that approximately 150 kDa large IgG[50] cannot pass into the capsules and harm the islets. This is what we accomplished.

Different methods, such as confocal[63,64] or fluorescence microscopy,[64,65] UV spectrophotometry,[66,67] high-performance liquid chromatography (HPLC),[68] and ISEC,[27,40,50,69–72] reported in the literature, were used to assess permeability of the capsules. Determination of permeability of the standard alginate–PLL–alginate microcapsules by different laboratories using aforementioned techniques that gave significantly different, even contradictory results. These disagreements most probably arise from a lack of standardization of test solutes and measuring techniques and minor lab-to-lab differences in encapsulation procedure.[72] In our study, we used ISEC to assess permeability through the determination of MWCO of microcapsules. The MWCOs of PLL capsules and PEG_{454}-*b*-PLL_{100} capsules were found to be 8 and 25 kDa, respectively. These values were determined using pullulan standards. Pullulan is a polysaccharide and, in contrast to globular proteins, pullulan has a flexible coil conformation. Due to globular conformations, proteins with different molecular weights can have the same spatial dimensions whereas pullulan chains occupy more space with increasing molecular weight. This means that proteins will elute later than polysaccharide molecules of the same molecular weight.[69,70,72] Therefore, MWCOs obtained from pullulan standards are recalculated for proteins.[69,70,72] Although PEG_{454}-*b*-PLL_{100} capsules have quite high permeability (98 kDa, recalculated for proteins) in comparison to PLL capsules (19 kDa, recalculated for proteins), this value is acceptable for the application since it is lower than the size of the smallest IgG antibodies.

Sufficient mechanical stability is another important property of encapsulation systems. During the implantation procedure as well as inside the peritoneal cavity, the capsules are exposed to different forces. Therefore, a "certain" mechanical strength is required to withstand these stresses. This "certain" value is considered to be from a few grams to tens of grams per capsule.[12,46] The most commonly applied techniques to assess mechanical stability of the alginate-based capsules are osmotic pressure tests[71,73–75] and compression resistance test performed on a texture analyzer.[40,50,61,74] In our study, mechanical stability of alginate–PLL capsules and alginate–PEG_{454}-*b*-PLL_{100} capsules was examined using a texture analyzer. It has been shown that alginate–PEG_{454}-*b*-PLL_{100} capsules are somewhat weaker than standard PLL capsules (59 ± 18 vs. 68 ± 14 g). Nevertheless the rupture load from a few grams to tens of grams per capsule is in the sufficient range for intraperitoneal application.[12,46] Therefore, PEG_{454}-*b*-PLL_{100} diblock copolymer provides sufficient stability and strength to alginate capsules.

CONCLUSIONS

Successful surface modification of alginate beads with series of PEG-*b*-PLL diblock copolymers was confirmed by fluorescence labelling of methoxy end group of PEG blocks. The capsules prepared with diblock copolymers consisting of either short PEG or short PLL blocks were quite unstable during the labelling procedure. PEG_{22}-*b*-PLL_{10} diblock copolymer penetrates entirely into the alginate beads, whereas PEG_{454}-*b*-PLL_{200} adsorbs to alginate beads in low amounts creating weak capsules. Capsules with the other PEG-*b*-PLL diblock copolymers had a clear surface bound fluorescence indicating the presence of PEG block on the surface of the capsules. The progression of diblock copolymer adsorption to the alginate beads was followed through the increase in the intensity of the fluorescence with time. Longer times (30–60 min) are required to reach the maximum fluorescence intensity. When purified intermediate-G alginate was used for the preparation of the capsules, both PLL and PEG-*b*-PLL capsules caused minor activation of NF-κB. No beneficial effect of diblock copolymer was observed when capsules were made of nonpurified alginate. The capsules prepared with PEG_{454}-*b*-PLL_{100} have adequate permeability and mechanical stability comparable with PLL capsules.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Dr. Igor Lacik and Ing. Gabriela Kollarikova from Polymer Institute of the Slovak Academy of Sciences for performing mechanical stability and permeability measurements.

REFERENCES

1. Koo J, Chang TM. Secretion of erythropoietin from microencapsulated rat kidney cells: Preliminary results. Int J Artif Organs 1993; 16:557–560.
2. Chang PL, Shen N, Westcott AJ. Delivery of recombinant gene products with microencapsulated cells in vivo. Hum Gene Ther 1993;4:433–440.
3. Liu HW OF, Chang PL. Expression of human factor IX by microencapsulated recombinant fibroblasts. Hum Gene Ther 1993;4:291–301.
4. Cieslinski DA, David Humes H. Tissue engineering of a bioartificial kidney. Biotechnol Bioeng 1994;43:678–681.
5. Capone SH, Dufresne M, Rechel M, Fleury MJ, Salsac AV, Paullier P, Daujat-Chavanieu M, Legallais C. Impact of alginate composition: from bead mechanical properties to encapsulated HepG2/C3A cell activities for in vivo implantation. PLoS One 2013;8: e62032.
6. Colton CK. Implantable biohybrid artificial organs. Cell Transplant 1995;4:415–436.
7. Aebischer P, Goddard M, Signore AP, Timpson RL. Functional recovery in hemiparkinsonian primates transplanted with polymer-encapsulated PC12 cells. Exp Neurol 1994;126:151–158.
8. Lim F, Sun A. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908–910.
9. O'Shea GM, Goosen MF, Sun AM. Prolonged survival of transplanted islets of Langerhans encapsulated in a biocompatible membrane. Biochim Biophys Acta 1984;804:133–136.
10. De Vos P, De Haan BJ, Wolters GH, Strubbe JH, Van Schilfgaarde R. Improved biocompatibility but limited graft survival after purification of alginate for microencapsulation of pancreatic islets. Diabetologia 1997;40:262–270.
11. De Vos P, Andersson A, Tam SK, Faas MM, Halle JP. Advances and barriers in mammalian cell encapsulation for treatment of diabetes. Immunol Endocr Metabol Agents Med Chem 2006;6: 139–153.
12. de Vos P, Bučko M, Gemeiner P, Navrátil M, Švitel J, Faas M, Strand BL, Skjak-Braek G, Morch YA, Vikartovská A, Lacík I, Kolláriková G, Orive G, Poncelet D, Pedraz JL, Ansorge-Schumacher MB. Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials 2009;30: 2559–2570.
13. Tam SK, Bilodeau S, Dusseault J, Langlois G, Halle JP, Yahia LH. Biocompatibility and physicochemical characteristics of alginate–polycation microcapsules. Acta Biomater 2011;7:1683–1692.
14. Tam SK, Dusseault J, Bilodeau S, Langlois G, Hallé J-P, Yahia LH. Factors influencing alginate gel biocompatibility. J Biomed Mater Res Part A 2011;98A:40–52.
15. de Haan BJ, Rossi A, Faas MM, Smelt MJ, Sonvico F, Colombo P, de Vos P. Structural surface changes and inflammatory responses against alginate-based microcapsules after exposure to human peritoneal fluid. J Biomed Mater Res A 2011;98:394–403.
16. de Vos P, de Haan BJ, Kamps JA, Faas MM, Kitano T. Zeta-potentials of alginate–PLL capsules: A predictive measure for biocompatibility? J Biomed Mater Res A 2007;80:813–819.
17. De Vos P, De Haan B, Van Schilfgaarde R. Effect of the alginate composition on the biocompatibility of alginate–polylysine microcapsules. Biomaterials 1997;18:273–278.
18. de Vos P, Hoogmoed CG, Busscher HJ. Chemistry and biocompatibility of alginate–PLL capsules for immunoprotection of mammalian cells. J Biomed Mater Res 2002;60:252–259.
19. van Schilfgaarde R, de Vos P. Factors influencing the properties and performance of microcapsules for immunoprotection of pancreatic islets. J Mol Med 1999;77:199–205.
20. Orive G, Tam SK, Pedraz JL, Hallé J-P. Biocompatibility of alginate–poly-L-lysine microcapsules for cell therapy. Biomaterials 2006;27:3691–3700.
21. Ponce S, Orive G, Hernández R, Gascón AR, Pedraz JL, de Haan BJ, Faas MM, Mathieu HJ, de Vos P. Chemistry and the biological response against immunoisolating alginate–polycation capsules of different composition. Biomaterials 2006;27:4831–4839.
22. Thu B, Skjåk-Bræk G, Micali F, Vittur F, Rizzo R. The spatial distribution of calcium in alginate gel beads analysed by synchrotron-radiation induced X-ray emission (SRIXE). Carbohydr Res 1997; 297:101–105.
23. Thu B, Bruheim P, Espevik T, Smidsrod O, Soon-Shiong P, Skjak-Braek G. Alginate polycation microcapsules. I. Interaction between alginate and polycation. Biomaterials 1996;17:1031–1040.
24. Thu B, Bruheim P, Espevik T, Smidsrød O, Soon-Shiong P, Skjåk-Bræk G. Alginate polycation microcapsules: II. Some functional properties. Biomaterials 1996;17:1069–1079.
25. de Vos P, Spasojevic M, de Haan BJ, Faas MM. The association between in vivo physicochemical changes and inflammatory responses against alginate based microcapsules. Biomaterials 2012;33:5552–5559.
26. Leblond FA, Tessier J, Hallé J-P. Quantitative method for the evaluation of biomicrocapsule resistance to mechanical stress. Biomaterials 1996;17:2097–2102.
27. Robitaille R, Leblond FA, Bourgeois Y, Henley N, Loignon M, Hallé J-P. Studies on small (<350 μm) alginate–poly-L-lysine microcapsules. V. Determination of carbohydrate and protein permeation through microcapsules by reverse-size exclusion chromatography. J Biomed Mater Res 2000;50:420–427.
28. de Vos P, van Hoogmoed CG, van Zanten J, Netter S, Strubbe JH, Busscher HJ. Long-term biocompatibility, chemistry, and function of microencapsulated pancreatic islets. Biomaterials 2003;24:305–312.
29. van Hoogmoed CG, Busscher HJ, de Vos P. Fourier transform infrared spectroscopy studies of alginate–PLL capsules with varying compositions. J Biomed Mater Res A 2003;67:172–178.
30. de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 2006;27:5603–5617.
31. Tam SK, Dusseault J, Polizu S, Ménard M, Hallé J-P, Yahia LH. Physicochemical model of alginate–poly-L-lysine microcapsules defined at the micrometric/nanometric scale using ATR-FTIR, XPS, and ToF-SIMS. Biomaterials 2005;26:6950–6961.

32. Mahou R, Tran NM, Dufresne M, Legallais C, Wandrey C. Encapsulation of Huh-7 cells within alginate–poly(ethylene glycol) hybrid microspheres. J Mater Sci Mater Med 2012;23:171–179.
33. Werner M, Biss K, Jerome V, Hilbrig F, Freitag R, Zambrano K, Hubner H, Buchholz R, Mahou R, Wandrey C. Use of the mitochondria toxicity assay for quantifying the viable cell density of microencapsulated jurkat cells. Biotechnol Prog 2013. doi: 10.1002/btpr.1734. [Epub ahead of print].
34. Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs 2008;22:315.
35. Gon S, Fang B, Santore MM. Interaction of cationic proteins and polypeptides with biocompatible cationically-anchored PEG brushes. Macromolecules 2011;44:8161–8168.
36. Gon S, Bendersky M, Ross JL, Santore MM. Manipulating protein adsorption using a patchy protein-resistant brush. Langmuir 2010; 26:12147–12154.
37. Jönsson M, Johansson H-O. Effect of surface grafted polymers on the adsorption of different model proteins. Colloids Surf B: Biointerfaces 2004;37:71–81.
38. de Haan BJ, Faas MM, de Vos P. Factors influencing insulin secretion from encapsulated islets. Cell Transplant 2003;12:617–625.
39. De Vos P, De Haan BJ, Van Schilfgaarde R. Upscaling the production of encapsulated islets. Biomaterials 1997;18:1085–1090.
40. Vaithilingam V, Kollarikova G, Qi M, Lacik I, Oberholzer J, Guillemin GJ, Tuch BE. Effect of prolonged gelling time on the intrinsic properties of barium alginate microcapsules and its biocompatibility. J Microencapsul 2011;28:499–507.
41. Bystrický S, Malovíková A, Sticzay T. Interaction of alginates and pectins with cationic polypeptides. Carbohydr Polym 1990;13:283–294.
42. Bystrický S, Malovíková A, Sticzay T. Interaction of acidic polysaccharides with polylysine enantiomers. Conformation probe in solution. Carbohydr Polym 1991;15:299–308.
43. Strand BL, Morch YA, Espevik T, Skjak-Braek G. Visualization of alginate–poly-L-lysine–alginate microcapsules by confocal laser scanning microscopy. Biotechnol Bioeng 2003;82:386–394.
44. Dusseault J, Tam SK, Menard M, Polizu S, Jourdan G, Yahia L, Halle JP. Evaluation of alginate purification methods: Effect on polyphenol, endotoxin, and protein contamination. J Biomed Mater Res A 2006;76:243–251.
45. De Vos P, Wolters GH, Fritschy WM, Van Schilfgaarde R. Obstacles in the application of microencapsulation in islet transplantation. Int J Artif Organs 1993;16:205–212.
46. Lacík I. Polymer chemistry in diabetes treatment by encapsulated islets of Langerhans: Review to 2006. Aust J Chem 2006;59:508–524.
47. Colton CK, Avgoustiniatos ES. Bioengineering in development of the hybrid artificial pancreas. J Biomech Eng 1991;113:152–170.
48. Chang TM. Hybrid artificial cells: Microencapsulation of living cells. ASAIO J 1992;38:128–130.
49. Lacy PE. Treating diabetes with transplanted cells. Sci Am 1995; 273:50–51.
50. Rosiński S, Grigorescu G, Lewińska D, Ritzén LG, Viernstein H, Teunou E, Poncelet D, Zhang Z, Fan X, Serp D, Marison I, Hunkeler D. Characterization of microcapsules: Recommended methods based on round-robin testing. J Microencapsul 2002;19: 641–659.
51. Marra J, Hair ML. Interactions between two adsorbed layers of poly(ethylene oxide)/polystyrene diblock copolymers in heptane–toluene mixtures. Colloids Surf 1988;34:215–226.
52. Watanabe H, M. T. Measurement of forces in symmetric and asymmetric interactions between diblock copolymer layers adsorbed on mica. Macromolecules 1993;26:6455–6466.
53. De Vos P, De Haan B, Wolters GH, Van Schilfgaarde R. Factors influencing the adequacy of microencapsulation of rat pancreatic islets. Transplantation 1996;62:888–893.
54. de Vos P, van Hoogmoed CG, de Haan BJ, Busscher HJ. Tissue responses against immunoisolating alginate–PLL capsules in the immediate posttransplant period. J Biomed Mater Res 2002;62: 430–437.
55. Barbey R, Lavanant L, Paripovic D, Schuwer N, Sugnaux C, Tugulu S, Klok HA. Polymer brushes via surface-initiated controlled radical polymerization: Synthesis, characterization, properties, and applications. Chem Rev 2009;109:5437–527.
56. Cabane E, Zhang X, Langowska K, Palivan CG, Meier W. Stimuli-responsive polymers and their applications in nanomedicine. Biointerphases 2012;7:9.
57. Chen T, Amin I, Jordan R. Patterned polymer brushes. Chem Soc Rev 2012;41:3280–3296.
58. Chernyy S, Jensen BE, Shimizu K, Ceccato M, Pedersen SU, Zelikin AN, Daasbjerg K, Iruthayaraj J. Surface grafted glycopolymer brushes to enhance selective adhesion of HepG2 cells. J Colloid Interface Sci 2013;404:207–214.
59. Poelma JE, Fors BP, Meyers GF, Kramer JW, Hawker CJ. Fabrication of Complex Three-Dimensional Polymer Brush Nanostructures through Light-Mediated Living Radical Polymerization. Angew Chem Int Ed Engl 2013;52:6844–6848.
60. Zdyrko B, Luzinov I. Polymer brushes by the "grafting to" method. Macromol Rapid Commun 2011;32:859–869.
61. Thanos CG, Bintz BE, Emerich DF. Stability of alginate–polyornithine microcapsules is profoundly dependent on the site of transplantation. J Biomed Mater Res A 2007;81:1–11.
62. Tam SK, Bilodeau S, Dusseault J, Langlois G, Hallé JP, Yahia LH. Biocompatibility and physicochemical characteristics of alginate–polycation microcapsules. Acta Biomater 2011;7:1683–1692.
63. Vandenbossche GM, Van Oostveldt P, Demeester J, Remon JP. The molecular weight cut-off of microcapsules is determined by the reaction between alginate and polylysine. Biotechnol Bioeng 1993;42:381–386.
64. Vandenbossche GM, Van Oostveldt P, Remon JP. A fluorescence method for the determination of the molecular weight cut-off of alginate–polylysine microcapsules. J Pharm Pharmacol 1991;43: 275–277.
65. Halle JP, Bourassa S, Leblond FA, Chevalier S, Beaudry M, Chapdelaine A, Cousineau S, Saintonge J, Yale JF. Protection of islets of Langerhans from antibodies by microencapsulation with alginate–poly-L-lysine membranes. Transplantation 1993;55:350–354.
66. Goosen MF, O'Shea GM, Gharapetian HM, Chou S, Sun AM. Optimization of microencapsulation parameters: Semipermeable microcapsules as a bioartificial pancreas. Biotechnol Bioeng 1985; 27:146–150.
67. King GA, Daugulis AJ, Faulkner P, Goosen MFA. Alginate–polylysine microcapsules of controlled membrane molecular weight cutoff for mammalian cell culture engineering. Biotechnol Prog 1987;3:231–240.
68. Coromili V, Chang TM. Polydisperse dextran as a diffusing test solute to study the membrane permeability of alginate polylysine microcapsules. Biomater Artif Cells Immobilization Biotechnol 1993;21:427–444.
69. Briščová M, Petro M, Lacík I, Powers AC, Wang T. Evaluation of Microcapsule Permeability via Inverse Size Exclusion Chromatography. Analytical Biochemistry 1996;242:104–111.
70. Wang T, Lacik I, Brissova M, Anilkumar AV, Prokop A, Hunkeler D, Green R, Shahrokhi K, Powers AC. An encapsulation system for the immunoisolation of pancreatic islets. Nat Biotechnol 1997; 15:358–362.
71. Dusseault J, Leblond FA, Robitaille R, Jourdan G, Tessier J, Ménard M, Henley N, Hallé J-P. Microencapsulation of living cells in semi-permeable membranes with covalently cross-linked layers. Biomaterials 2005;26:1515–1522.
72. Powers AC, BrissovÁ M, LacÍK I, Anilkumar AV, Shahrokhi K, Wang TG. Permeability Assessment of Capsules for Islet Transplantation1. Annals of the New York Academy of Sciences 1997; 831:208–216.
73. Darrabie MD, Kendall Jr WF, Opara EC. Characteristics of Poly-L-Ornithine-coated alginate microcapsules. Biomaterials 2005;26: 6846–6852.
74. Orive G, Hernández RM, Gascón AR, Igartua M, Pedraz JL. Development and optimisation of alginate–PMCG–alginate microcapsules for cell immobilisation. Int J Pharm 2003;259:57–68.
75. De Castro M, Orive G, Hernandez RM, Gascon AR, Pedraz JL. Comparative study of microcapsules elaborated with three polycations (PLL, PDL, PLO) for cell immobilization. J Microencapsul 2005;22:303–315.