Adsorption of human immunoglobulin to implantable alginate-poly-L-lysine microcapsules: Effect of microcapsule composition

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Abstract: Alginate-poly-L-lysine-alginate (APA) microcapsules continue to be the most widely studied device for the immuno-protection of transplanted therapeutic cells. Producing APA microcapsules having a reproducible and high level of biocompatibility requires an understanding of the mechanisms of the immune response towards the implants. Here, we investigate the adsorption of immunoglobulins (IgG, IgM, and IgA) onto the surface of APA microcapsules *in vitro* after their exposure to human serum and peritoneal fluid. Immunoglobulins (Ig) are considered to be opsonizing proteins, thus they tend to mediate inflammation when adsorbed to foreign surfaces. Ig adsorption was monitored using direct immunofluorescence. The amount of Ig adsorbed to the microcapsule surface was not significantly influenced by the guluronic acid content nor the purity level of the alginate, although microcapsules of intermediate-G purified alginate corresponded with the lowest adsorption levels. Ig adsorption was negligible when the poly-L-lysine membrane was omitted, suggesting that positive charges at the microcapsule surface are responsible for binding Ig. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 89A: 609–615, 2009

Key words: protein adsorption; immunofluorescence; biocompatibility; alginate; microcapsules

INTRODUCTION

Alginate-based microcapsules are commonly used for the immuno-protection of transplanted therapeutic cells. The biocompatibility of the encapsulated cell system, including the microcapsule itself, continues to be a subject of concern.[1,2] In the 1990s, the development of fibrotic tissue around the implanted microcapsules was considered to be one of the major obstacles hampering the progress of encapsulated cell transplantation[3]; fibrosis arising from the normal wound-healing process was stated to hinder the diffusion of cell nutrients and therapeutic products across the capsule membrane. Moreover, fibrosis was frequently associated with graft failure. More recently, it was demonstrated that, by using optimal biomaterials and fabrication methods, it is feasible to produce alginate-poly-L-lysine-alginate (APA) microcapsules that induce minimal fibrosis *in vivo*, even 2 years after implantation in rats.[4] Despite this encouraging step forward, the ultimate goal of these research efforts, i.e. permanent survival of the encapsulated cellular grafts, was unfortunately not achieved as graft survival varied between 4 and 6 months.

During recent years it has become more accepted that the immune response against the encapsulated cell system is far more complicated than initially assumed. The complexity of this response partially explains the wide range of biocompatibility that the microcapsule system has displayed in the literature. The fact is activation of the immune system starts with the mandatory surgery to implant the microcapsules, that is before the implant is even introduced into the body. More precisely, injury to vascularized connective tissue caused by the incision induces an immediate inflammatory response associated with the influx of inflammatory cells and plasma proteins, as well as the release of bioactive factors such as cytokines and nitric oxide.[5,6]

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An important and likely event that can decide the ultimate biocompatibility of the microcapsule is the adsorption of opsonins onto its surface immediately upon its implantation. In general, the surface adsorption of opsonins is necessary for immune cells to recognize a pathogen or biomaterial as being "foreign" and to subsequently attack it. To date, no one has verified experimentally whether this adsorption occurs in the case of microcapsules designed for cellular therapy. The immunoglobulins IgM, IgA, and especially IgG have the ability to opsonize foreign bodies, thus their capacity to adsorb to microcapsules is of interest when investigating their biocompatibility. IgG and IgM are particularly effective for complement activation, while IgG and IgA can mediate phagocytosis, though IgA is generally restricted to mucosal secretions.[7–11]

In the present study, we investigate the adsorption of immunoglobulin (Ig) to the surface of APA microcapsules *in vitro*. This was done for microcapsules of different compositions.

MATERIALS AND METHODS

Study design

The adsorption of human immunoglobulins IgG/IgM/IgA to the surface of APA microcapsules *in vitro* was monitored using direct immunofluorescence. Ig adsorption was achieved by incubating microcapsules in serum and/or peritoneal fluid (PF). These fluids were selected in order to simulate the *in vivo* situation where microcapsules come into contact with both blood and PF during implantation into the peritoneal cavity, i.e. the conventional transplantation site. Subsequent to incubation, the protein-covered microcapsules were rinsed then immersed in a solution containing a fluorescently labeled antibody to human immunoglobulin (Ig). The extent of Ig adsorption was measured in terms of fluorescence intensity. Ig adsorption levels to APA microcapsules composed of alginates varying in chemical composition (i.e. high vs. intermediate guluronic acid content) and in purity level were compared because these properties of alginate have a significant influence on the *in vivo* bioperformance of the microcapsules. Furthermore, to investigate the relative influence of the alginate matrix and the poly-L-lysine (PLL) membrane on Ig adsorption, the adsorption to complete APA microcapsules and to calcium alginate beads (having no PLL membrane) was compared.

Materials

Intermediate-G sodium alginate (Keltone® LVCR, International Specialty Products Corp, UK) having 40% guluronic acid (as specified by the manufacturer) and high-G sodium alginate (Manugel® DMB, International Specialty Products Corp, UK) having 50% guluronic acid (as specified by the manufacturer) were used for microcapsule fabrication. The alginates were purified using previously described methods.[12]

The alginates were dissolved in a 220 mOsm Ca^{2+}-free Krebs-Ringer-Hepes (KRH) solution consisting of 90.0 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, and 25.0 m*M* Hepes. Alginates were dissolved at a concentration of 3.4% w/v for the unpurified intermediate-G alginate, 3.5% w/v for the purified intermediate-G alginate and 1.9% w/v for each of the high-G alginates. These concentrations provided alginate solutions of appropriate viscosity for microcapsule fabrication. All alginate solutions were sterilized by filtration (0.22 μm).

PLL hydrochloride (Sigma-Aldrich, USA) having a molecular weight of 22,200 (as specified by the manufacturer) was used to form the microcapsule membrane.

For protein adsorption studies *in vitro*, human serum isolated from whole blood was donated by healthy volunteers. Human PF was obtained from a male donor within 12 h of his decease. For the experiments, only the PF supernatent was used.

To detect the immunoglobulin adsorbed to the microcapsule surface, a fluorescein isothiocyanate (FITC)-conjugated polyvalent antibody to human IgA/IgG/IgM developed in rabbit (Sigma-Aldrich, USA) was used.

Microcapsule fabrication

Microcapsules were produced using an air-driven droplet generator following previously described methods.[13] Alginate solution was extruded from a 23G needle using a syringe and a co-axial air stream to produce droplets. The alginate droplets were immersed in a 100 m*M* $CaCl_2$ solution and allowed to gel for 5 min after extrusion of the last droplet (the extrusion process lasted 2–4 min). Gelled calcium alginate beads measured 650–675 μm in diameter. To form the microcapsule membrane, the calcium alginate beads were rinsed and then immersed for 10 min in a PLL solution that consisted of PLL dissolved 0.1% w/v in 310 mOsm Ca^{2+}-free KRH (135.0 m*M* NaCl, 4.7 m*M* KCl, 25.0 m*M* Hepes, 1.2 m*M* KH_2PO_4, and 1.2 m*M* $MgSO_4$). The microcapsules were rinsed again and then immersed in a 10× diluted solution of alginate (i.e. 0.19%, 0.34% or 0.35% w/v in Ca^{2+}-free KRH) for 5 min, with the aim of binding any unbound PLL at the surface. In all cases, the same type of alginate was used for both the microcapsule gel core and the coating step. After final rinsing, the microcapsules were stored in KRH until analysis. Final APA microcapsules measured 600–750 μm in diameter. All solutions used for microcapsule fabrication were sterilized by filtration (0.22 μm).

Protein adsorption to microcapsules

Samples of 30 microcapsules were transferred to a polypropylene test tube. The supernatant (i.e., KRH) was removed by aspiration. Serum was diluted 1:1 in KRH and 300 μL of the diluted serum was added to each test tube. Samples were incubated in a warm water bath at 37°C with gentle agitation for 1 h. Afterwards, the serum was removed and the microcapsules were rinsed five times with KRH. For certain experiments (see the results

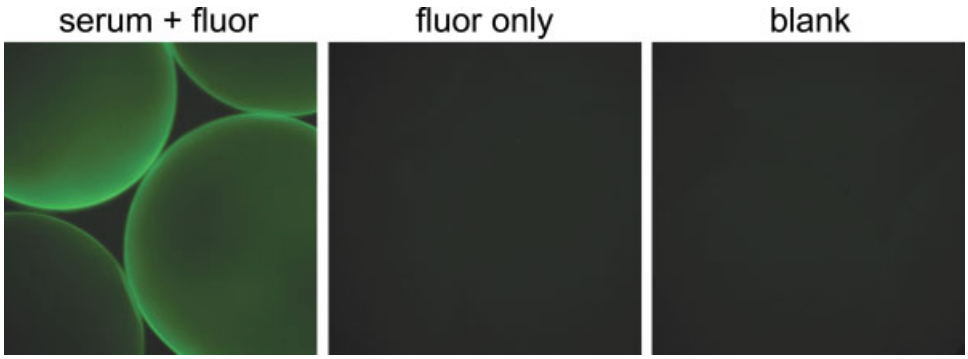

Figure 1. Fluorescent signal emitted from alginate-poly-L-lysine-alginate (APA) microcapsules upon excitation by a light having a wavelength (λ) of 485 nm, as observed by microscopy. Microcapsules were either pre-incubated in serum then incubated with a FITC-labeled antibody to human immunoglobulins IgG/IgM/IgA ("serum + fluor"), incubated with only the FITC-labeled antibody ("fluor only"), or incubated with neither ("blank"). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

section), the serum was replaced with human PF or a 1:1 mixture of serum and PF.

Measurement of Ig adsorption

The amounts of Ig that adsorbed to the microcapsule surfaces were measured using direct immunofluorescence. Seventy-five microliters of dilute FITC-conjugated rabbit antibody to human IgG/IgM/IgA (diluted 1:600 in KRH) was added to each sample of microcapsules. Immediately afterwards, the samples were kept in the darkness at room temperature for 1 h. The microcapsules were then rinsed five times with KRH and subsequently transferred to a 96-well plate (Corning® flat bottom). The fluorescence intensity emitted from the samples was quantitatively measured using a Bio-Tek FL600™ fluorescence microplate reader (Bio-Tek Instruments, USA). An excitation wavelength of 485 nm and emission wavelength of 530 nm was used in order to detect the FITC label. A top-reading measurement was taken at a sensitivity setting of 200. Readings were always taken in triplicate. The labeling efficiency of the antibody was also confirmed by fluorescence microscopy using an Olympus IMT-2 inverted microscope (Olympus, Japan).

Statistical analysis

Measured values were compared using the Wilcoxon Signed Ranks Test with the aid of SPSS® statistical analysis software (SPSS, USA). A difference for which $p < 0.05$ was considered to be statistically significant. In general, quantitative results (represented as bar graphs) are expressed as the mean fluorescence intensity over an n value of 6 ± the standard error of the mean (SEM).

RESULTS

Direct immunofluorescence was applied in order to quantify the extent of immunoglobulin (Ig) adsorption to microcapsules preincubated in either serum or PF. A microscopic examination of APA microcapsules prepared of purified intermediate-G alginate and preincubated in serum (Fig. 1), confirmed that the fluorescence staining was specific for the protein-covered microcapsule surface. These results not only confirm that Ig adsorbs to APA

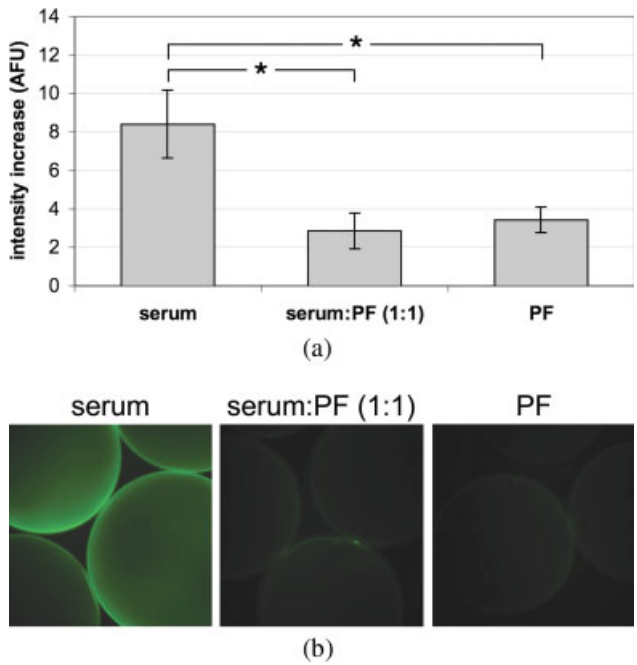

Figure 2. Extent of immunoglobulin (Ig) adsorption to APA microcapsules that were incubated in serum, peritoneal fluid (PF) or a 1:1 mixture of serum and PF. Ig adsorption is proportional to the intensity of fluorescence emitted from the samples upon excitation. (a) Quantification of Ig adsorption, which is represented by an "intensity increase" (i.e. the average increase in fluorescence intensity vs. microcapsules that were incubated with only the FITC-labelled antibody) ± standard error of the mean, SEM ($n = 6$). AFU = arbitrary fluorescent units, $*p < 0.05$. (b) Microscope observation of the fluorescent light emitted by the microcapsules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microcapsules, but also demonstrate the adequacy of our experimental approach.

In the case of *in vivo* studies, the microcapsules are in contact with both blood and PF upon their implantation in the peritoneal cavity (i.e., the conventional transplantation site). Therefore, we quantified and compared the adsorption of Ig onto APA microcapsules after incubation in serum, PF, and a 1:1 mixture of PF and serum. As shown in Figure 2, the Ig adsorbed in significantly greater amounts ($p < 0.05$) to the microcapsules when they were incubated in serum (8.4 ± 1.8 arbitrary fluorescent units, AFU) compared to those that were incubated in either PF

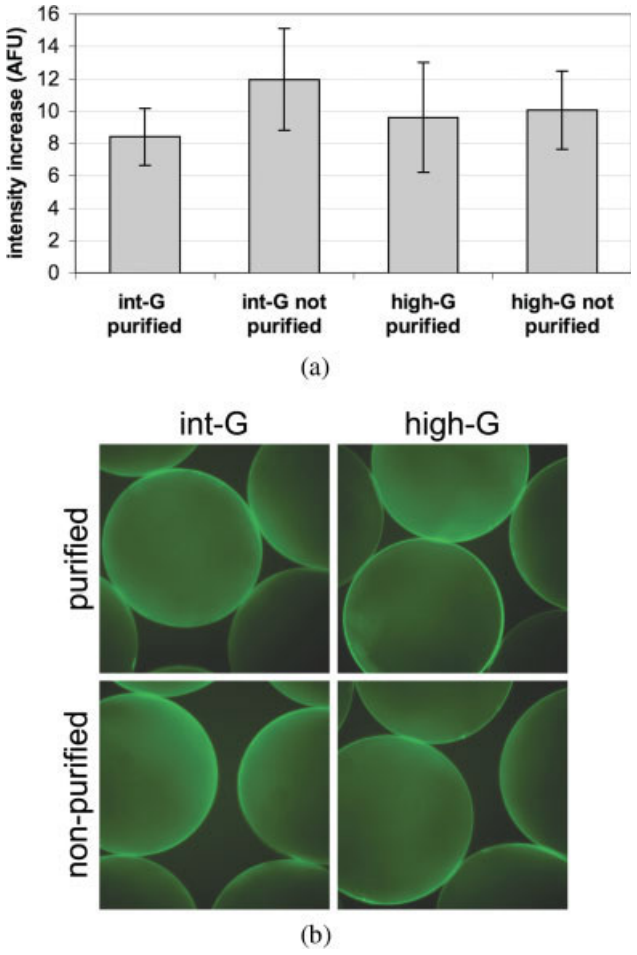

Figure 3. Extent of Ig adsorption to APA microcapsules that were incubated in serum. The microcapsules were prepared of alginates varying in chemical composition (intermediate-G vs. high-G) and in purity (purified vs. nonpurified). Ig adsorption is proportional to the intensity of fluorescence emitted from the samples. (a) Quantification of Ig adsorption, which is represented by an "intensity increase" (i.e. the average increase in fluorescence intensity vs. microcapsules that were incubated with only the FITC-labeled antibody) ± SEM ($n = 6$). AFU = arbitrary fluorescent units, *$p < 0.05$. (b) Microscope observation of the fluorescent light emitted by the microcapsules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

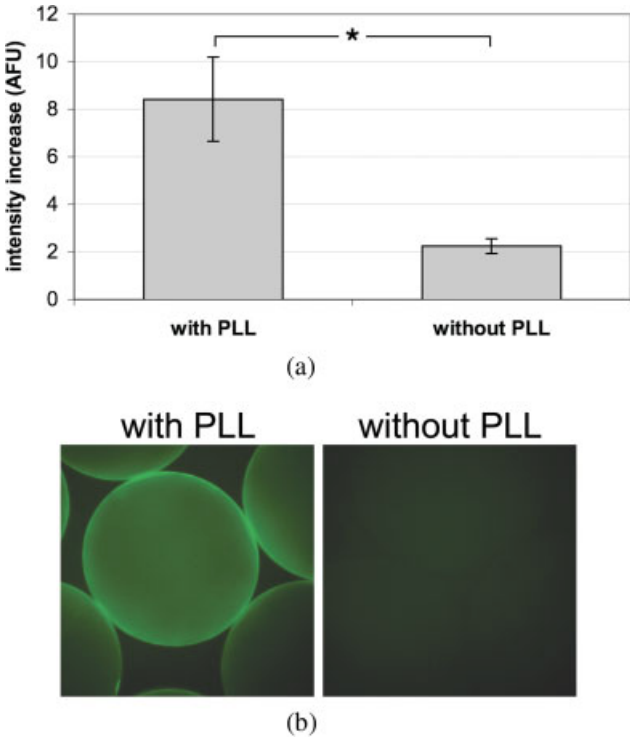

Figure 4. Extent of Ig adsorption to APA microcapsules ("with PLL") or to calcium alginate beads ("without PLL") that were incubated in serum. The samples were prepared of purified intermediate-G alginate. Ig adsorption is proportional to the intensity of fluorescence emitted from the samples. (a) Quantification of Ig adsorption, which is represented by an "intensity increase" (i.e. the average increase in fluorescence intensity vs. samples that were incubated with only the FITC-labeled antibody) ± SEM ($n = 6$). AFU = arbitrary fluorescent units, *$p < 0.05$. (b) Microscope observation of the fluorescent light emitted by the samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(2.9 ± 0.9 AFU) or the serum/PF mix (3.4 ± 0.7 AFU). Since incubation in serum was associated with the highest adsorption levels, we used serum as the Ig source for subsequent studies.

To investigate the influence of the microcapsule characteristics on the extent of Ig adsorption, we quantified and compared the adsorption onto APA microcapsules prepared of purified versus nonpurified alginates, and of intermediate-G versus high-G alginates. There were no statistically significant differences between the amounts of Ig adsorbed onto each of the samples tested (Fig. 3).

Next, we investigated whether it is the PLL-based membrane or the alginate matrix that is responsible for the observed adsorption of Ig to the capsules. To do this, we compared the extent of Ig adsorption onto calcium alginate beads to the adsorption onto APA microcapsules after incubating each in serum. As shown in Figure 4(a), in the case of samples prepared of purified intermediate-G alginate, the

measured fluorescence intensity was fourfold higher when PLL was included in the membrane. The experiments were repeated for samples prepared of high-G alginates and nonpurified alginates; the results (not shown) consistently confirmed that the PLL membrane is a principal mediator of Ig adsorption to APA microcapsules.

DISCUSSION

Generally speaking, following the implantation of a medical device such as microcapsules, the typical sequence of events is as follows: Immediately after injury, there is acute inflammation that, if persists, leads to chronic inflammation, granulation tissue, foreign body reaction, and finally fibrosis.[5,6,14–17] Acute inflammation, the first phase upon injury, is characterized by the exudation of fluid and plasma proteins, as well as the attraction of leukocytes to the site of injury. Subsequently, neutrophils and macrophages become activated. In general, these immune cells will recognize the implant as being "foreign" only if it is coated by opsonins, i.e. serum proteins for which these cells have specific membrane receptors.[17–19] Several immunoglobulins, particularly IgG but also IgA and IgM, are considered to be opsonins. In the case of cell-containing microcapsules, opsonization of the implant during acute inflammation plausibly leads to events that can be disastrous for graft viability, particularly the further release of cytotoxic cytokines associated with chronic inflammation and frustrated phagocytosis, as well as the formation of fibrotic tissue during the final stage of the healing process.

It is well recognized that the biological response to APA microcapsules is significantly influenced by the chemical properties and the quality of the alginate. We have observed that microcapsules prepared of purified intermediate-G alginate remain free of any significant foreign body response for prolonged periods of time after implantation, while microcapsules prepared of high-G alginate are consistently associated with low recovery rates and extensive overgrowth.[20,21] It is also well known that microcapsules composed of alginates that are not properly purified induce a severe inflammatory response.[12,22–24] Yet, the mechanisms that explain the influence of the alginate properties on the overall biocompatibility of the microcapsule are still not well understood. Therefore, we investigated the extent of immunoglobulin (Ig) adsorption onto the surface of APA microcapsules composed of alginates varying in chemistry and purity level as these microcapsules consistently demonstrate various degrees of biocompatibility when implanted. This approach was based on the hypothesis that opsonization by Ig is the principal event that mediates or leads to the immune response to implanted microcapsules. However, it was observed that altering the alginate properties had no statistically significant effect on the quantity of Ig that adsorbed to the corresponding APA microcapsules. Taking into account our previously observed *in vivo* response to microcapsules of the same chemical composition, these results suggest that Ig adsorption plays a negligible or a minor role in mediating an immune response to the implanted microcapsules. At the same time, it is worth noting that the microcapsules prepared of purified intermediate-G alginate adsorbed the lowest amount of Ig. This finding corroborates our *in vivo* observation that microcapsules prepared of this particular alginate provoke a minor inflammatory response compared to microcapsules prepared of high-G or impure alginates.[20,21] Thus, it remains plausible that Ig adsorption has a certain degree of influence on the inflammatory response to implanted APA microcapsules.

We observed that the extent of Ig adsorption to the microcapsules was significantly lower in the absence of the PLL-based membrane. Since we have recently confirmed, using zeta potential measurements, that adding the PLL membrane creates a net charge at the surface of APA microcapsules,[25] the results of this study indicate that the positive charges of the polycation are attracting the immunoglobulin. This is in alignment with the general observation that, as opposed to highly charged surfaces, neutral (and hydrophilic) polymers have minimal or weak interactions with most aqueous proteins.[26] The biological implications of these results can be interpreted in two ways. On one hand, if it is to be assumed that Ig adsorption inevitably leads to inflammation, then the observation that the PLL attracts Ig to the microcapsule surface supports the numerous published studies which imply that exposed PLL is mainly responsible for the inflammatory response to APA microcapsules.[15,21,27,28] On the other hand, these results do not necessarily reflect our own *in vivo* observations. We have seen that, in the case of APA microcapsules prepared of purified intermediate-G alginate, complete microcapsules that include the PLL membrane are just as biocompatible as the corresponding alginate gel beads. From this point of view, our results suggest that Ig adsorption to the microcapsule surface does not always lead to a severe inflammatory response or fibrosis.

In this study, we observed that Ig adsorption to the microcapsules was greater from serum than from PF or from a serum/PF mix. This is explained by the differences in protein concentrations between the two fluids. PF is a transudate of plasma, thus they share similar protein compositions. However, due to the fact that the peritoneal membrane limits the dif-

fusion of larger molecules (>20 kDa), PF contains similar concentrations of smaller solutes, lower concentrations of high molecular-weight molecules, and an overall protein concentration of ~50% that of plasma protein concentrations.[29–32] The molecular weight of human immunoglobulin ranges from about 150 to 900 kDa,[11] thus their concentration is expected to be lower in PF than in serum. In turn, protein adsorption patterns from mixed solutions are influenced by (among other factors) the relative bulk concentrations and molecular weights of each protein type. For instance, smaller proteins at higher concentrations tend to adsorb before larger proteins.[26,33]

The results of this study provide indications that Ig adsorption may influence the inflammatory response to APA microcapsules, yet it is clear that the combined effect of IgG, IgM, and IgA adsorption is not the only determining factor for microcapsule biocompatibility. While IgG is known to be a highly effective opsonizer, there are a number of other serum proteins that have also been seen to influence implant biocompatibility, for example the complement factor C3b, whose potential role in these experiments must not be ignored.[17] A growing number of researchers recognize that proteins of all types are very important to biomaterials science because of their tendency to deposit on surfaces as a tightly bound adsorbate, and more importantly due to the strong influence that these deposits have on subsequent cellular interactions with an implant surface.[34] Proteins have been measured on biomaterial surfaces within 1 s of implantation, thus protein adsorption occurs well before cells arrive at the surface.[35] On another note, in the particular case of microcapsules for cell therapy, protein adsorption should be considered not only in terms of its influence on the cellular response to the implants, but also in its influence on the capsule bioperformance by its potential to alter the selective permeability of the microcapsule's immuno-protective membrane. While this aspect has not been directly studied in the case of alginate microcapsules, protein adsorption has been seen to reduce the hydraulic permeability and solute sieving of polysulphone membranes.[36,37]

CONCLUSIONS

It was clearly proven that human immunoglobulin (IgG/IgM/IgA) adsorbs to the surface of APA microcapsules *in vitro*. This was demonstrated using the relatively simple method of direct immunofluorescence. The extent of immunoglobulin (Ig) adsorption is not significantly influenced by the chemical composition and purity of the alginate used for microcapsule fabrication, yet it is highly dependent on the presence of the polylysine membrane, indicating that the positive charges of the polycation are mainly responsible for binding the Ig. Since IgG, IgM, and IgA are known to be opsonizing proteins that lead to complement activation upon their adsorption to foreign surfaces, these findings are useful for explaining the mechanisms of the immune response to APA microcapsules.

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