Zeta-potentials of alginate-PLL capsules: a predictive measure for biocompatibility?

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Abstract: Alginate-poly-L-lysine (PLL) microencapsulation of cells is a promising approach to prevent rejection in the absence of immunosuppression. Clinical application, however, is hampered by insufficient insight in factors influencing biocompatibility of the capsules. By now, it has been accepted that not only the chemical composition of the materials applied but also other factors contribute to bioincompatibility. The zeta-potential serves as a measure for the electrical charge of the surface and has been shown to be a predictive value for the interfacial reactions between the biomaterial and the surrounding tissue in other applications. In the present study, we have assessed the streaming potential of alginate-PLL capsules composed of either low-, intermediate-, or high-guluronic (G) alginate to calculate the zeta-potential. The zeta-potentials of the capsules were compared to the biological response against the capsules at 4 weeks after implantation in the rat. We show that high-G and low-G alginates provoke a more severe response in the rat than capsules prepared of intermediate-G alginate. This correlates with a higher zeta-potential of the high-G and low-G alginates and by a change in zeta-potential at lower pH. These lower pH-levels are common directly after implantation as the consequence of a host-response associated with mandatory surgery. Our results suggest that we should not only consider the capsule properties under physiological circumstances to explain bioincompatibility but also the capsule features during common pathophysiological situations. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 80A: 813–819, 2007

Key words: transplantation; alginate; microencapsulation; immunoisolation

INTRODUCTION

Transplantation of cells for treatment of human disorders such as hormone or protein deficiencies is not applied on a large scale due to the necessity to use life-long immunosuppression for preventing rejection of the graft. To overcome the necessity to apply immunosuppression, many have proposed technologies to immunoisolate hormone- or protein-secreting cells in semipermeable membranes to protect donor-cells against antibodies and cytotoxic cells of the host immune system. This immunoisolation by encapsulation not only allows for successful transplantation of cells in the absence of immunosuppression[1–3] but also for transplantation of cells from nonhuman origin, that is xenografts, which overcomes the obstacle of limited supply of donor tissue. Because of these benefits, the feasibility of transplanting cells in immunoprotective membranes is under study for the treatment of a wide variety of endocrine disorders.[4–8]

Microencapsulation of tissues in alginate-poly-L-lysine (PLL) based capsules, as originally described by Lim and Sun[3] is the most commonly applied procedure for immunoisolation. During recent years, important advances have been made with this technology. The first allotransplantations in humans with encapsulated parathyroid cells and islets have been successfully performed.[9,10] Although this illustrates the principle applicability of the alginate-encapsulation technique, a fundamental barrier has to be overcome since graft survival varies considerably from several days to months.[1,11–13]

Recently, it became more accepted that the host-responses against immunoisolating cells is far more complicated than originally assumed. Until a few years ago, the variations in success rate was usually

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attributed to differences in the chemical composition.[14–17] By now it has been accepted that many more factors can contribute to bioincompatibility. The response already starts with the mandatory surgery to implant the "foreign material."[18–20] This induces an inflammatory response due to rupture of blood vessels which is associated with influx of inflammatory cells and release of bioactive factors such as cytokines and fibronectin. It is generally assumed that the adsorption of proteins to the capsules is the first step towards adherence of inflammatory cells and overgrowth of the capsules with necrosis of the enveloped cells as a consequence. Also, the cells in the capsules contribute to bioincompatibility by releasing soluble bioactive factors that influence the immunological response.[21,22]

An event that is insufficiently realized is that the direct environment of the capsules changes directly after implantation. A pertinent change is a drop in pH as the consequence of a temporary inflammation process due to the mandatory surgery. Such a drop in pH can for instance induce changes in the charge density of the capsules and make the capsule more vulnerable for adhesion of proteins and cells. Capsules should be able to withstand these kind of environmental changes.

The zeta-potential is a measure for the electrical charge of the surface and a predictive value for the interfacial reactions between the biomaterial and the surrounding tissue.[23–25] We designed a technology to measure the zeta-potential of alginate-based capsules and applied this technology to compare three commonly applied types of alginate with a different composition. This was done under physiological and low pH levels. The results were compared with the corresponding biocompatibility of the capsules in rats.

MATERIALS AND METHODS

Design of the study

Capsules were prepared of alginates with either a low, an intermediate, or a high guluronic acid (G) content. High viscosity alginate solutions were applied to produce capsules with an adequate mechanical strength. The capsules were inspected before and after implantation in order to confirm that the majority of the capsules were intact. Only highly purified alginates were applied in order to exclude that contaminating components were the cause of an inflammatory response.

Capsules were implanted in the peritoneal cavity of AO-rats, that is the usual transplantation site for an encapsulated islet graft and retrieved one month later. Capsules retrieved by peritoneal lavage were always processed for histological examination of the degree of overgrowth of the graft (i.e. a measure for the biological response against the capsules[1,2,26,27]). Portions of the capsules were subjected for analysis of the zeta-potentials of the capsules.

Graft recipients

Male inbred Albino Oxford (AO/G) rats served as recipients of alginate-poly-L-lysine (PLL) capsules and were obtained from the Central Animal Laboratory of Groningen. Their body weights ranged from 300 to 350 g. NIH guidelines for the care and use of laboratory animals have been observed.

Alginates

Alginates contain various amounts of guluronic acid (G)-chains and of mannuronic acid (M)-chains. Low-G alginate (Mannucol), intermediate-G (Keltone LV), and high-G (Manugel) sodium alginates were obtained from Kelco International, London, UK. Purification of alginate was performed as described in detail elsewhere.[1] Alginates were dissolved at 4°C in Krebs-Ringer-Hepes (KRH) with an appropriate osmolarity to a solution with a viscosity of 4 cps. This viscosity is necessary for the production of spherical droplets without any tails or other imperfections associated with bioincompatibility. The viscosity of an alginate solution is determined by the concentration of alginate but different alginates have different viscosities. This implies for the low-G solution a concentration of 4.3%, for intermediate-G solution a concentration of 3%, and for the high-G a 2% solution to obtain a viscosity of 4 cps. The solutions were sterilized by 0.2 μm filtration.

Encapsulation

Capsules were produced according to a three step procedure. First, we produced rigid Ca-alginate beads, by converting an alginate solution into droplets using an air-driven droplet generator as previously described.[28] The droplets were collected in a Ca-rich solution (100 m*M* $CaCl_2$) to gelify into rigid Ca-beads. Secondly, the Ca-beads were subjected to a procedure to form a semipermeable PLL-membrane. Finally, to cover incompletely bound PLL, the alginate-PLL beads were coated with alginate again.[29] Capsules had a diameter of 600–700 μm. All procedures were performed under sterile conditions.

Capsules were injected into the peritoneal cavity. Upon peritoneal lavage, microcapsules were either freely floating and non-adherent, or adherent to the surface of abdominal organs. First, non-adherent microcapsules were retrieved by peritoneal lavage, and brought into a syringe with appropriate measures for quantification of the retrieval rate.[2] Subsequently, the microcapsules adherent to the surface of abdominal organs, were excised and processed for histology.

All surgical procedures were performed under isoflurane anesthesia.

Electrophoretic mobility

The zeta potential was measured using the Electro Kinetic Analyzer (EKA, Anton Paar GmbH, Austria). The EKA operates according to the principles of streaming potential and includes a powder measuring cell, the electrolyte circuit, and a pair of Ag/AgCl electrodes. The electrolyte ($10^{-3}M$ KCl solution) is forced through the measuring cell containing the sample. A pressure drop (ΔP) depending upon the flow resistance of the sample is detected across the measuring cell. The circulation of electrolyte through the cell results in a flow of ions (streaming current). The resulting potential difference (streaming potential, U_p) is detected by electrodes placed at each end of the cell. During a measurement, ΔP and U_p are recorded.

The zeta potential (ζ) was calculated using the equation as follows:

$$\zeta = \frac{U_p \eta æ}{\Delta P \varepsilon \varepsilon_0}$$

In this equation η is the dynamic viscosity of the electrolyte solution, æ is its electrical conductivity, ε is the liquid permittivity, and ε_0 is the permittivity of free space.

If not otherwise mentioned, the pH of the electrolyte solution was kept at 7.0 since this is the physiological pH to which capsules are exposed *in vivo*. During the assessment of the streaming potential, the temperature was kept at 25°C.

Microscopy

The biological response against capsules was assessed by quantifying the number of capsules overgrown by macrophages and fibroblasts.[26,27] Therefore, samples of adherent capsules recovered by excision and non-adherent, freely floating capsules were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05*M* phosphate in saline (pH 7.4), and processed for glycol methacrylate (GMA) embedding.[30] Sections were prepared at 2 μm and stained with Romanovsky-Giemsa stain and applied for detecting imperfections in the capsule membrane and for determining the number of capsules with and without overgrowth. The degree of capsular overgrowth was quantified by expressing the number of recovered capsules with overgrowth as the percentage of the total number of recovered capsules for each individual animal.[1,2,26,27]

Statistical analysis

Results are expressed as mean ± SEM. Statistical comparisons were made with the Mann Whitney U test. A p-value < 0.05 was considered statistically significant.

RESULTS

Biological responses and the integrity of capsules

Before and after implantation, we observed all batches of microcapsules to contain some imperfect capsules such as capsules with strains or capsules with a broken membrane. This number of imperfect capsules was higher with low-G alginate than with intermediate-G and high-G alginates since it was (14 ± 7)% with low-G and always less than 3% with the other types of alginate.

The biological responses against the capsules was determined by the type of alginate applied. In recipients of low-G capsules and high-G capsules, we found large numbers of capsules adherent to the surface of the abdominal organs and virtually all were overgrown (Fig. 1). These adherent capsules were mainly located on the omentum, low in the peritoneal cavity near the testis, and on the highest liver-lobes.

The overgrowth on capsules recovered by peritoneal lavage was composed of fibroblasts in case of low-G and intermediate-G alginate, but it was mainly composed of macrophages instead of fibroblasts when high-G alginate had been applied.

The number of freely floating capsules with overgrowth was larger with low-G and high-G alginate capsules than with intermediate-G alginate capsules (Table I). The high response against the low-G capsules can in part be explained by differences in physical integrity of individual capsules, since we observed many overgrown low-G capsules to contain a broken membrane. However, such a difference was not observed between intermediate-G and high-G alginates which implies that other, more systemical inadequacies such as chemical variations affecting all capsules than imperfections on individual capsules must also be considered as a cause for the difference in responses *in vivo*.

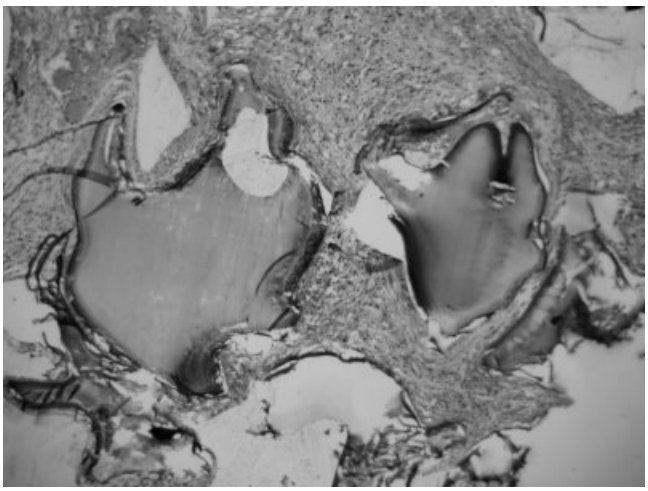

Figure 1. Alginate-PLL capsule prepared of high-G alginate, one month after implantation in the peritoneal cavity of AO-rats. The capsule is covered by macrophages and fibroblasts. GMA-embedded section, Romanowsky-Giemsa stain, original magnification ×800.

TABLE I
Recovery Rates and Percentage of Alginate-PLL Capsules with Overgrowth, 1 Month After Implantation in the Peritoneal Cavity of AO-Rats

Type of Alginate in Capsule	*n*	% Recovery	% Overgrowth
Low G-content	5	27 ± 8.2	63.2 ± 15.8
Intermediate G-content	7	89.2 ± 6.3	2.7 ± 0.4
High G-content	5	48.5 ± 8.6	52.0 ± 19.2

Capsules of alginates are prepared with low-G, intermediate-G, or high-G content.

Alginate-composition and zeta-potential of capsules

In a previous study, we have shown that high-G capsules bind more proinflammatory poly-L-lysine than intermediate-G alginate.[15,31] This has subsequently, been interpreted to be the causative factor for the difference in the biological response since more PLL may imply a higher positive charge density at the capsule surface and therefore a lower degree of biocompatibility.[15,31] Unfortunately, it was far from simple to assess the charge density of a capsule surface. We have applied gold labeling experiments, light and electron microscopy, and immunochemistry without success since the fragile features of capsules do not allow the required processing for these procedures. Therefore, we finally have assessed the streaming potential to calculate the zeta-potential which can be used as a measure for the charge density at the capsule surface. This was done for Ca-beads (i.e. capsules without PLL) and for capsules.

Figure 2 shows that with all three types of alginate the transition of Ca-beads to alginate-PLL capsule is associated with a statistical significant increase in zeta-potential ($p < 0.05$). This increase, however was more pronounced with high-G alginate than with alginates with a lower-G content.

Since during inflammation capsules may be exposed to variations in pH, we not only tested the zeta-potentials of the capsules at pH 7.0 but also at pH 5.4. As shown in Figure 3, the lowering of pH induces an increase of the zeta-potentials of the low-G and high-G capsules ($p < 0.05$) but not of the intermediate-G capsules. The increase in zeta-potential was more pronounced with low-G than with high-G alginates.

DISCUSSION

In the present study we attempted to apply capsules with a similar physical stability. This is mandatory to avoid imperfections on the capsules that can cause bioincompatibility. This could successfully be accomplished with intermediate and high-G alginates but not with low-G alginates. Capsules prepared of low-G alginate were sensitive for shear forces. This resulted in membrane imperfection on 14% of the capsules during the required convection during the encapsulation procedure and the subsequent gentle rinsing with a cannula. These imperfections cause inflammatory responses *in vivo*,[32,33] and should therefore be considered to be causative for the observed inflammatory response against the low-G capsules. However, differences in physical stability between capsules prepared of intermediate-G and

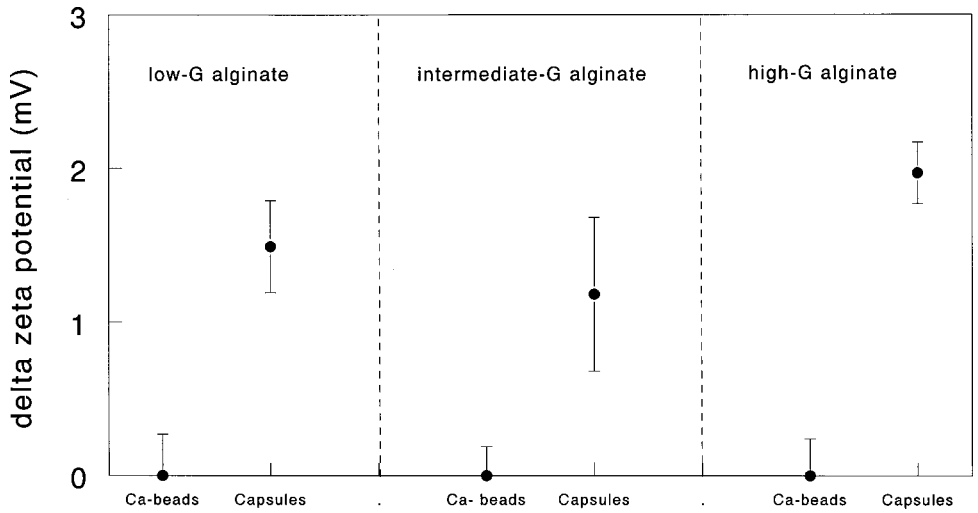

Figure 2. Increments in zeta-potential during transition of Ca-beads to alginate-PLL capsules for capsules prepared of low-G, intermediate-G, or high-G alginates. Values are presented as delta where the zeta-potential of Ca-beads was taken as the 0 value.

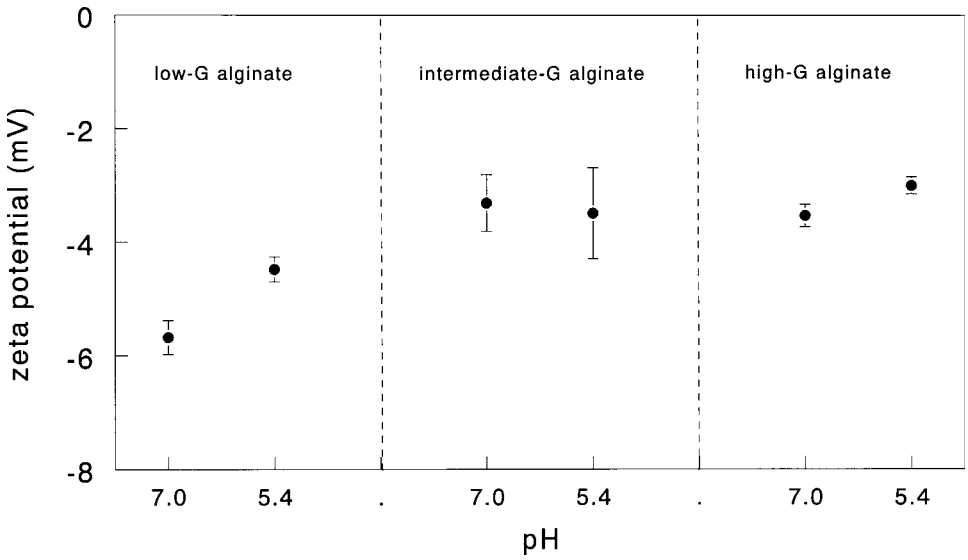

Figure 3. The effect of lowering the pH from 7.0 to 5.4 on the zeta-potentials of capsules prepared of low-G, intermediate-G, or high-G alginates.

high-G alginates can not explain the lower degree of biocompatibility of high-G capsules.

It has been shown that high-G alginates have some advantages over intermediate-G alginates for application in cell-encapsulation. It has been shown in *in vitro* studies that capsules prepared of high-G alginates have a higher mechanical stability than capsules prepared of intermediate-G alginate.[14,15,34,35] Also they contain much lower numbers of incompletely and therefore inadequately encapsulated cells.[32,33] Unfortunately, the majority of high-G capsules are overgrown by inflammatory cells and adherent to the abdominal organs whereas with intermediate-G alginate most capsules are freely floating in the peritoneal cavity and free of any adhesion of cells.[16] In a previous study we showed that a major difference between intermediate and high-G capsules is that high-G capsules bind more poly-L-lysine (PLL).[14,15] We postulated that this PLL binding was rather excessive due to limited amounts of binding sites for PLL on the high-G capsules.[14,15] This results in an increase in the amount of positive charges on the surface of the capsule which is well known to induce inflammatory reactions. Unfortunately, until now, we were not able to demonstrate this increase in positive charge at the capsule surface due to lack of appropriate technical means to measure this on the fragile capsules.

This is the first study, showing clearcut differences in the electric charge density of the capsule surface during transition from Ca-bead to alginate-PLL capsule. The increase in zeta-potential was more pronounced on the high-G capsules than on the intermediate-G capsules which confirms the hypothesis[14–16,36] that PLL-binding on high-G alginate is associated with a larger increase in charge density than with binding on intermediate-G alginate. It is plausible that this increase is causative for the observed biological effects against the high-G capsules.

Before clinical application of microcapsules, it is essential to have techniques available to assess the chemical characteristics of capsules in order to predict the biological response after transplantation. Our present results shows that zeta-potential measurement is such an approach. It is inexpensive, nonlaborous, and a rather fast method to test the adequacy of the capsules after the production procedure and before transplantation. It measures directly the charge distribution on the capsule surface which is a measure for the biological response *in vivo*.[23–25,37,38]

It has been shown that physiological pH changes during inflammation has a substantial effect on the zeta-potential and consequently the biological properties of some biomaterials such as hyaluronan acid.[24] This effect of pH on zeta-potentials is caused by a change of the average electric charge of ionized molecules in the biomaterials (e.g. proteins with dissociable chains, hydrogen ions, hydroxyl ions etc.), and through protein adsorption and conformational changes in and on the biomaterials.[25,37,39] Since inflammation and thus pH changes always occur in the first days after implantation of microcapsules, we tested the effect of a lowering of the pH on the zeta-potentials of capsules.

A pH dependent change of zeta-potential was observed on alginate-PLL capsules. This effect was most pronounced on low-G and high-G capsules but absent on intermediate-G capsules. This alginate-dependent effect of pH on zeta-potentials of capsules warrants some further consideration since it can explain why capsule grafts of low- and high-G cap-

sules are often found to be completely overgrown after implantation while with intermediate-G only a portion of the capsules are overgrown. Our observations suggest the following sequence of events. After implantation, a non-specific immune response is activated with influx of cells and pH changes in the implantation site as a consequence.[18,19,23] As previously shown with intermediate-G capsules, this normally induces cell adhesion on a small portion of the capsules with physical imperfection such as capsules with protruding cells or with broken membranes. However, this cell-adhesion is, on grafts composed of low- or high-G capsules, not only restricted to the imperfect capsules but can occur on all capsules since the pH-change in the immediate post-transplant period will increase the zeta-potential and facilitates the adherence of proteins and cells not on a portion of capsules but on all capsules.

The correlation between zeta-potential and biocompatibility was only studied after implantation of capsules in the peritoneal cavity, that is in peritoneal fluid. Other results may have been found when the capsules would have been implanted in other sites, or in direct contact with blood. We have chosen the peritoneal cavity as the site of interest since this is the conventional transplantation site for alginate-PLL capsules. For low-G materials we did not find a correlation in the peritoneal site since other, more pertinent biocompatibility issues did cause a biological response *in vivo*.

Our study not only shows that changes of capsule properties directly after implantation can explain bioincompatibility, it also shows that we should measure capsule properties under other circumstances than that of physiological homeostasis. After implantation many environmental changes may occur that influences the capsule properties. Ideally, capsules should be able to withstand these pathophysiological changes in order to provide optimal survival of the enveloped cells. Measurement of the zeta-potential may be a powerful tool to predict changes under pathophysiological circumstances such as during inflammation.

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