# The Molecular Weight Cut-Off of Microcapsules Is Determined by the Reaction Between Alginate and Polylysine

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Mammalian cells encapsulated in alginate-polylysine microcapsules are used as artificial organs in cancer research and in biotechnology. These applications require microcapsules with a reproducible mol. wt. cut-off. The high cost of the polycation, polylysine, requires an efficient preparation procedure. This article shows that the overall reported contact time of 5 minutes at ambient conditions should be increased several times in order to reach a maximal binding between the calcium alginate beads and 0.1% (w/v) polylysine solutions. An increase of the polylysine concentration from 0.0125% to 0.8% (w/v) resulted in a faster maximal binding, but the amount of polylysine bound increased also. Immersion of calcium alginate beads with a diameter of 750 $\mu$m, prepared from 1 mL alginate, in 30 mL of a 0.8% (w/v) polylysine solution, resulted in a polylysine spill of more than 89%. The time required to reach a maximal binding was related to the reaction temperature. The interaction zone between calcium alginate beads and fluorescein isothiocyanate-labeled polylysine solutions was visualized with a confocal laser scanning microscope as a function of time. Microcapsules, prepared at 40°C with 0.1% (w/v) polylysine solutions with mol. wts. between 12 and 249.2 kD, were permeable for fluorescein isothiocyanate-labeled dextran, mol. wt. 4.7, but not for 40.5 kD. Higher polylysine concentrations resulted in a membrane with a mol. wt. cut-off lower than 4.7 kD. 

Key words: alginate • polylysine • microcapsules • optimization • cut-off • FITC-dextran • FITC-polylysine

## INTRODUCTION

Microencapsulation of mammalian cells prevents immune rejection, which allows liver[4] or pancreas[2,3,11,25,31–33,35,40,42,43,48] transplantation, the evaluation in vivo of antineoplastic[6] and antiviral drugs.[29,34] Prokop[36] and Glacken et al.[15] reviewed the use of microencapsulated hybridoma cells in bioreactors allowing higher cells densities (up to $10^7$ cells/mL) as compared with suspension cell cultures ($10^6$ cells/mL),[9,14,15,17,20,22,37,38] resulting in a higher purity of the monoclonal antibodies[9,10,14,17,19,20,24,27,36–38] or biomolecules as IFN[22] and lymphokines,[36] and facilitating the separation of the cells from the medium by means of gravity settling. The biomolecules can be partitioned into either the microcapsule or into the medium. One of the disadvantages of the encapsulation technique is the cost of the process, mainly of the polylysine.[15]

The above-mentioned applications required microcapsules with a known and reproducible mol. wt. cut-off. Several attempts to modify the mol. wt. cut-off were undertaken.[1,16,17,24,41] Some authors used polylysine concentrations between 0.005% and 1% (w/v) and reaction times between 3 and 10 minutes (Table I). Nevertheless, it is still reported that the "quality varies"[11] or that "variations . . . are difficult to eliminate."[42]

In this study, the influence of the reaction time, the reaction temperature, and the polylysine concentration on the amount of polylysine reacted and its influence on the mol. wt. cut-off were determined.

## EVALUATION

### Preparation of Calcium Alginate Beads

Microcapsules were prepared according to the method described by Lim and Moss[32] and modified by Vandenbossche et al.[44,45,47] Membrane filtration was selected for the sterilization of the alginate[46] (Pronova MVG, Protan, Drammen, Norway). The 1.0% (w/v) alginate dispersion in saline was filtered through a 0.22-$\mu$m membrane filter (SM16534, Sartorius, Göttingen, Germany) and dropped, via a droplet-forming device,[30] in a glass vessel containing 30 mL of a 1.3% (w/v) $CaCl_2$ solution. The droplets jellified and formed calcium alginate beads with a diameter of about 750 $\mu$m. Calcium alginate beads, prepared from 1.0 mL sodium, alginate, were kept for another 5 minutes in the $CaCl_2$ solution. The calcium alginate beads and all the solutions added were kept at 40°C unless otherwise indicated.

#### *Reaction Kinetics*

The calcium alginate beads were washed twice with saline. Next, 30 mL of a poly-L-lysine · HCl solution

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**Table I.** Experimental conditions used during the calcium alginate-polylysine reaction.

| Mol. wt. polylysine (kD) | Concentration polylysine (%) | Contact time (min) | Reference |
|---|---|---|---|
| 16–22 | 0.05 | 10 | 11, 35 |
| 17–22 | 0.05 | 5 | 25 |
| 17 | 0.005 | 6 | 18 |
| 18 | 0.05 | 6 | 48, 49 |
| 20 | 0.05 | 6 | 7 |
| 22 | 0.05 | 6 | 12, 25, 27 |
| 22 | 0.05 | 10 | 5 |
| 25 | 0.02 | 6 | 43 |
| 35 | 0.1 | 6 | 28 |
| 35 | 0.02 | 5 | 3 |
| 35 | 0.02 | 3–5 | 33 |
| 39 | 0.08 | 6 | 44, 45 |
| 41 | 0.1 | 5–10 | 10 |
| 42 | 0.05 | 3 | 22 |
| 42 | 0.05 | 6 | 21 |
| 50 | 0.05 | 6 | 8 |
| 170 | 0.03 | 6 | 2 |
| 249 | 0.1 | 15 | 47 |
| 270 | 0.05 | 6 | 25 |
| Not specified | 0.005 | 6 | 40 |
| | 0.02 | 3–5 | 32 |
| | 0.02 | 10 | 6 |
| | 0.035 | 6 | 26 |
| | 0.05 | 6 | 4, 14, 42 |
| | 0.05 | 8 | 39 |
| | 1.0 | 5–7 | 31 |

(Sigma, St. Louis, MO) in saline were poured over the beads. Before and 1, 2, 5, 10, 20, 40, 60, 180, 240, and 300 minutes after the addition of the polylysine solution, 100-$\mu$L samples were taken, assessed for polylysine concentration, and, if necessary, diluted in order to fit between the linear interval of the calibration curve. In order to exclude sorption, a polylysine solution was added to an empty glass vessel. The 100-$\mu$L sample was added to 0.75 mL of the red pyrogallol-molybdate complex (cat. no. 003-0309–02, Sopa Chem®, Brussels, Belgium) and 1.9 mL saline. The change in absorbency (UV-140-02 spectrophotometer, Shimadzu, Kyoto, Japan) was measured at 598 nm after an incubation of 20 min at 25°C. A linear interval ($r^2 = 0.99$) was found between the absorbency and polylysine concentrations from 50 to 250 $\mu$g/mL.

## Preparation of Microcapsules

The calcium alginate beads were washed twice with saline. Next 30 mL of a polylysine solution in saline were poured over the beads. The microcapsules were washed twice with saline and incubated during 5 minutes with a 0.05% (w/v) alginate dispersion in saline. After a wash with saline, the mol. wt. cut-off was determined.

### *Determination of the Mol. Wt. Cut-Off*

A 1-mL sample of the microcapsules was mixed with 1 mL of a 0.1% (w/v) fluorescein isothiocyanate-labeled dextran (FD) solution. The mol. wts. of the FD-4 (Sigma) and the FD-40 (Sigma) were 4.7 kD and 40.5 kD, respectively. The polydispersity factor was 1.25 for both FDs. The microcapsules were incubated for 24 h at 37°C and observed with a confocal laser scanning microscope (CLSM, Lasersharp Ltd., Bio-Rad, Abingdon, UK). The permeability for the FDs of 10 individual microcapsules was determined as described previously.[46] Briefly, the fluorescence was measured at an equatorial, optical, section of a microcapsules. Diffusion was concluded if the light intensity inside and outside the microcapsules did not differ significantly ($P = 0.05$).

## Modifications of the Preparation Procedure

### *Mol. Wt. of Polylysine and Reaction Time*

The mol. wt. cut-off of microcapsules prepared from calcium alginate beads left in contact with 0.1% (w/v) polylysines with a mol. wt. of 21.7 and 126.2 kD (Sigma) during 1, 5, 15, and 30 min was determined. Whereas only a contact time of 30 min, was tested for polylysines with a mol. wt. of 12, 62.5, and 249.2 kD (Sigma).

### *Polylysine Concentration*

The reaction kinetics between calcium alginate beads and polylysine (mol. wt. 38.5 kD) concentrations of 0.0125, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.3, 0.4, 0.6, and 0.8%

(w/v) were calculated. The mol. wt. cut-off of microcapsules, prepared from calcium alginate beads left during 30 minutes in contact with polylysine of a mol. wt. of 21.7 at concentrations of 0.1, 0.2, 0.3, and 0.4% (w/v), was determined.

### *Reaction Temperature*

The reaction kinetics for polylysine (mol. wt. 38.5 kD) concentrations of 0.1% (w/v) at 4°, 20°, 35°, and 40°C were determined. The influence of the reaction temperatures at 4°, 20°, and 40°C on the mol. wt. cut-off was determined for a 0.1% (w/v) polylysine concentration (mol. wt. 21.7 kD).

### *Release of Polylysine From the Microcapsules*

After a contact time of 30 min with the calcium alginate beads, the solutions containing 0.1%, 0.2%, and 0.4% (w/v) polylysine (mol. wt. 38.5 kD) were removed and replaced by saline. Samples were taken during up to 3 h at regular time intervals and the amount of polylysine released from the microcapsules was determined. In order to exclude sorption and desorption to the glass vessel, saline was also added to an empty glass vessel that had been in contact with the polylysine solutions during 30 min.

### *Increased Amount of Polylysine*

The reaction kinetics of 120 mL, instead of 30 mL, for a 0.025% (w/v) polylysine (mol. wt. 38.5 kD) concentration were investigated.

### *Visualization of the Polylysine Layer*

Microcapsules were prepared using 0.1% and 0.4% (w/v) fluorescein isothiocyanate-labeled polylysine (FITC-polylysine, mol. wt. 18.3 kD; Sigma) left in contact during 1, 5, 15, and 30 min at 40°C with the calcium alginate beads. The microcapsules were washed three times with saline and kept at 37°C. After an overnight and 1-week incubation, the thickness of the polylysine layer was measured on optical sections, made with the CLSM. The influence on the membrane thickness was statistically evaluated using the two-tailed Mann-Whitney $U$ test ($n$ = 5, $P$ = 0.01).

## RESULTS

### Mol. Wt. of Polylysine and Reaction Time

The microcapsules prepared at 40°C with 0.1% (w/v) polylysine solutions of a mol. wt. between 12 and 249.2 kD, and a reaction time between 1 and 30 minutes, were all permeable for FD-4, but not for FD-40. The thickness of the fluorescent 18.3-kD polylysine layer increased as a function of the contact time with the polylysine solutions (Figs. 1 and 2).

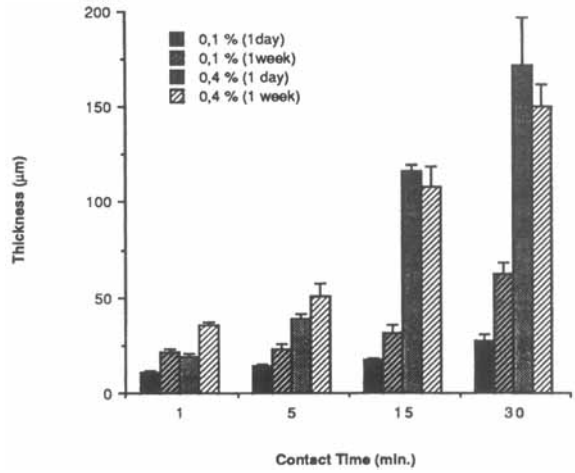


**Figure 1.** Thickness of the FiTC-polylysine layer after 1, 5, 15, and 30 min contact time between the calcium alginate beads and the polylysine solutions of 0.1% and 0.4%, measured after 1 day or 1 week incubation.

### Polylysine Concentration

The time to reach the maximal binding between the calcium alginate beads and the polylysine solutions from 0.0125% to 0.8% (w/v) decreased from 5 h or more to 10 min (Fig. 3). The maximal amount of polylysine bound to the calcium alginate beads, increased linearly ($y = 5.1x + 6762$; $r^2 = 0.85$) from 9.9 mg to 27.0 mg for concentrations from

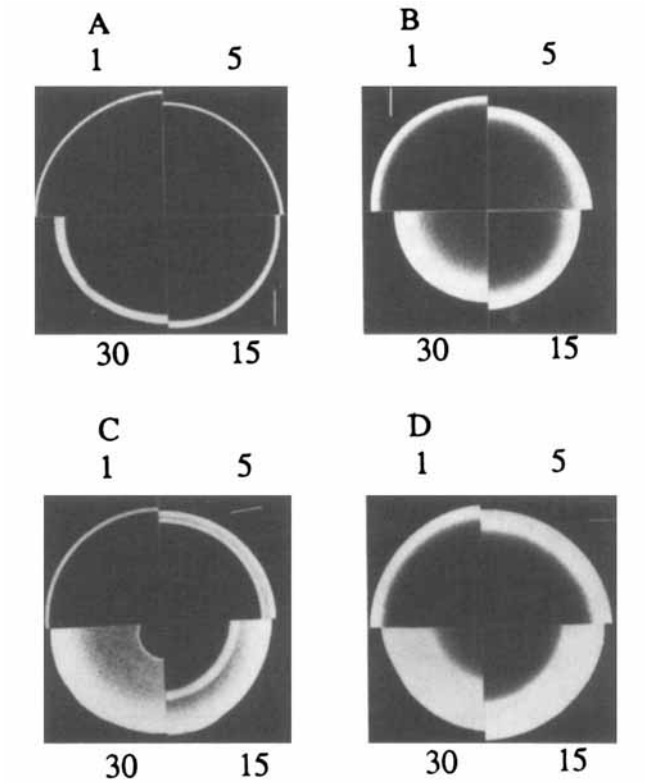


**Figure 2.** Confocal images of calcium alginate beads after 1, 5, 15, and 30 min reaction time with a 0.1% (w/v) FITC-polylysine solutions [after 1 day (A) and 1 week (B) incubation] and a 0.4% (w/v) solution [after 1 day (C) and 1 week (D) incubation]. Scale bar = 100 $\mu$m.

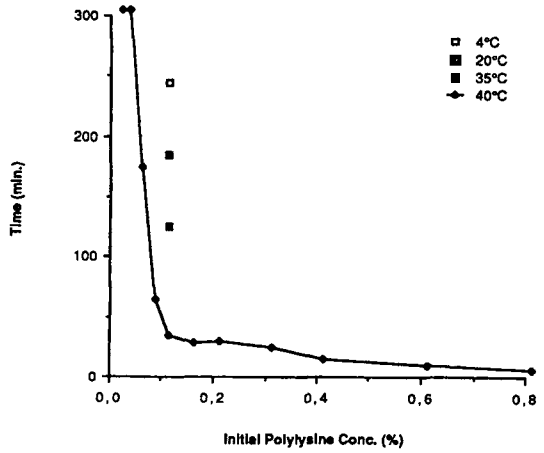


**Figure 3.** Time required to reach a maximal binding between calcium alginate beads and polylysine (mol. wt. 38.5 kD) as a function of the polylysine concentration and the reaction temperature.

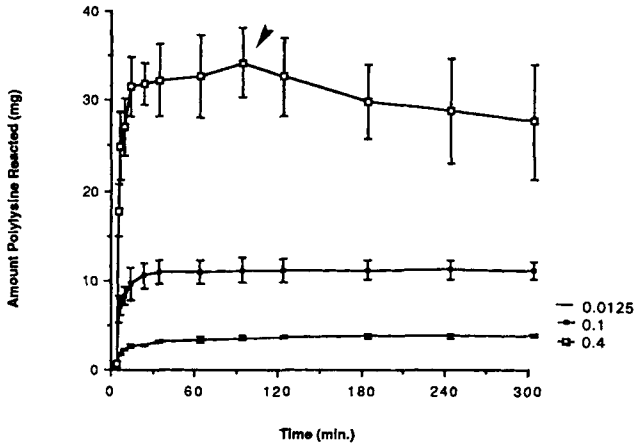


**Figure 5.** Mean (±SD) amount of polylysine (mol. wt. 38.5 kD) bound to calcium alginate beads as a function of time for three different polylysine concentrations. The arrow indicates the maximal binding for a 0.4% polylysine concentration, followed by a decrease in the amount of polylysine bound.

0.05% to 0.4% (w/v). As shown in Figure 4, this increase leveled off at concentrations higher than 0.4% (w/v). When concentrations of 0.0125% and 0.025% (w/v) were used, nearly all the polylysine added, 3.6 and 7.2 mg, respectively, bound to the calcium alginate beads. The sorption of the polylysine to the empty glass vessel was always lower than 1%. Microcapsules prepared with polylysine solutions of 0.2% (w/v) and higher showed, as a function of time, a maximum (arrow on curve of 0.4%; Fig. 5) in the amount of polylysine bound to the calcium alginate beads. There was a direct correlation between the amount of polylysine added and the decrease in amount polylysine bound after reaching the maximum value.

Microcapsules prepared at 40°C with 0.1% polylysine were permeable for FD-4, whereas microcapsules prepared with polylysine concentrations of 0.2%, 0.3%, and 0.4% were not.

The fluorescent polylysine layer was always significantly thinner for polylysine concentrations of 0.1% as compared with 0.4%. After 30-min contact with polylysine concentrations of 0.1% or 0.4% (Fig. 2), the polylysine penetrated 35 ± 11% and 85 ± 10% of the intracapsular space, respectively.

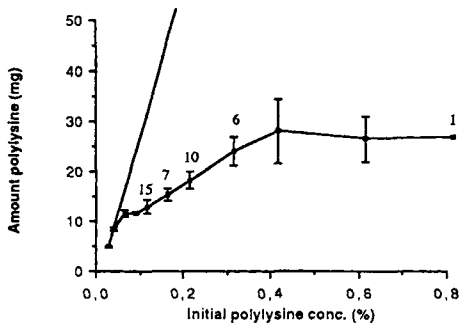


**Figure 4.** Mean (±SD) amount of polylysine (mol. wt. 38.5 kD) bound after a contact time of 5 h (■) and the amount polylysine added (−), as a function of the polylysine concentration ($n$ = 3, unless otherwise indicated).

## Release of Polylysine From the Microcapsules

After 30-min contact, 0.8%, 8.4%, and 23.5% of the amount of polylysine that previously bound to the calcium alginate beads was released from microcapsules prepared with polylysine concentrations of 0.1%, 0.2%, and 0.4% (w/v), respectively. No release of polylysine from the control vessel was detected.

## Reaction Temperature

As shown in Figure 3, the time to reach a maximal binding between alginate and a polylysine concentration of 0.1% (w/v) decreased from 240 minutes to 30 minutes when the temperature was increased from 4° to 40°C. The microcapsules, prepared at 40°C, always excluded the FD-40, whereas 20% and 10% of the microcapsules prepared at 4° and 20°C, respectively, were permeable for FD-40.

## Increased Amount of Polylysine

When 120 mL of a 0.025% (w/v) polylysine solution (30 mg polylysine) were added, 8.7 mg of the polylysine bound to the calcium alginate beads, whereas 7.2 mg of the polylysine bound if only 30 mL (7.5 mg polylysine) were added. When 120 mL were used, a maximal binding was observed after 2 h, whereas no maximal binding was reached before 5 h reaction time when 30 mL were used.

## DISCUSSION

The time required to reach a maximal binding decreased six times by doubling the polylysine concentration from 0.05% to 0.1% (w/v). A further increase of the concentration had a less pronounced effect on the time to reach maximal binding. Not only the reaction rate, but also the amount of polylysine bound increased. The increase in

amount of polylysine bound to the calcium alginate beads can be explained by the thicker capsule membrane as shown in Figures 1 and 2. The membrane thickness was also determined by the contact time with the fluorescent polylysine. Once the polylysine was replaced by saline, only a limited diffusion of polylysine toward the core of the microcapsule was observed during 1 week incubation. This indicates that only a high concentration gradient allows deep penetration of the polylysine. When polylysine concentrations of 0.2% (w/v) and higher were used, a fraction of the bound polylysine released after reaching maximal binding. This release could be caused by a weaker binding of the polylysine molecules to the microcapsules. The release of polylysine from the beads was confirmed by a second set of experiments in which the polylysine solution was replaced by saline after 30-min contact. There was a direct correlation between the amount of polylysine added and the amount of polylysine released. This diffusion of polylysine might explain the failure of shielding the polylysine layer by an additional alginate layer in order to improve tissue biocompatibility after implantation.[45] A preliminary experiment did not show a difference in brittleness or cell viability between microcapsules with an interaction time between calcium alginate and polylysine of 5 min, as compared with 30 min.

In this experimental setup the total surface of the calcium alginate beads was about 85 $cm^2$. When the polylysine/surface of calcium alginate beads ratio was lower than 0.088 ($mg/cm^2$), nearly all the available polylysine was used. As shown in Figure 4, the amount of polylysine spilled increased from 32% to more than 89% when polylysine concentrations of 0.05% and 0.8% were used, respectively. As postulated above, the concentration of polylysine influenced the way in which polylysine interacted with the calcium alginate beads, therefore, the reaction circumstances should be well standardized, especially for low polylysine/surface ratios.

A reaction time shorter than the time required to reach a maximal binding will be sensitive to small fluctuations, having a large influence on the amount of polylysine bound. Interruption of the reaction as suggested by Goosen et al.,[16,17] King et al.,[24] and Shimi et al.,[41] seems therefore not the best tool to modify the mol. wt. cut-off of the microcapsules. On the other hand, increasing the reaction time above the time required to reach maximal binding did not influence the amount of polylysine bound. Because of the minimal rearrangement, the limited spill of the polylysine and a reasonable reaction time of 30 min at 40°C, the authors selected a polylysine concentration of 0.1% (w/v) for their application.

Confocal microscopy was useful for the evaluation of the membrane thickness, but could not be applied for the evaluation of the membrane density, because of the low resolution. An electron microscopical evaluation.[41,45] could not be used for the visualization of the membrane density, because the dehydration of the hydrogel microcapsules led to artifacts. Apparently, the polylysine concentration and the reaction temperature influenced the density of the capsule membrane. This is shown by the exclusion of FD-4 when increasing the polylysine concentration from 0.1% to 0.2%, 0.3%, and 0.4%, and by the permeability for FD-40 at decreased reaction temperatures. However, at 40°C, reaction times below 30 min did not result in microcapsules permeable for FD-40. This might, in contrast with the data provided by the kinetic experiments, indicate a fast establishment of the capsule membrane. But, short reaction times might lead to inconsequent results. The permeability of the microcapsules prepared with a contact time of 6 minutes by Goosen et al.[16,17] and King et al.[24] increased when using polylysines of a higher mol. wt., whereas Shimi et al.[41] did find an inverse correlation between the microcapsule permeability and mol. wt. of polylysine. This contradiction might also be related to the technical limitations of the measuring techniques. Goosen et al.[16,17] and King et al.[24] measured a decrease of mol. wt. markers in the medium surrounding the microcapsules, whereas Shimi et al.[41] measured an increase in the medium caused by the release of encapsulated mol. wt. markers. As discussed previously,[46] The method used in this study measured the penetration of a fluorescent marker in individual microcapsules.

In conclusion, investigators will have to choose an optimal polylysine/surface ratio for their application, taking into account the polylysine spill, the influence of decreasing polylysine concentrations during the reaction, the sensitivity to variations in the number or dimensions of the calcium alginate beads, and the corresponding time required to reach a maximal binding.

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