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A fluorescence method for the determination of the molecular weight cut-off of alginate-polylysine microcapsules

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Abstract—Several applications of microcapsules for the encapsulation of living cells or macromolecules require well defined pore sizes. The molecular weight cut-off of alginate-polylysine microcapsules has been determined using a range of fluorescent labelled dextran molecules. The diffusion of the fluorescein isothiocyanate labelled (FITC)-dextrans into the microcapsules was followed by fluorescence and confocal laser scanning microscopy. The permeability of microcapsules for FITC-dextrans with a molecular weight of 4 700 daltons and the impermeability for FITC-dextrans with a molecular weight of 40 500 daltons was confirmed with both techniques. Determination of the molecular weight cut-off, using confocal laser scanning microscopy was more reliable and required a smaller sample than fluorescence measurements.

Several applications of microcapsules for containing living cells or macromolecules, require characterization of the molecular weight cut-off of the capsule wall. Microcapsules, with a molecular weight cut-off of about 100 000 daltons prepared with alginate and polylysine have been used in artificial organs, including pancreas (Lim & Sun 1980; Leung et al 1983; Lim 1984; O'Shea et al 1984; Sun & O'Shea 1985; O'Shea & Sun 1986), liver (Sun et al 1986) and parathyroid (Darquy & Sun 1987; Fu & Sun 1989). This microencapsulation procedure used the natural polysaccharide, alginate, and the synthetic homopolymer, polylysine, to form a polyelectrolyte membrane. The concentration, the molecular weight and contact time of those polymers determine the molecular weight cut-off of the membrane. Goosen et al (1985) and King et al (1987) used two different approaches to determine the molecular weight cut-off. The leaking of encapsulated proteins from microcapsules into the medium was measured or the penetration of proteins added to the medium containing microcapsules was followed with time. A theoretical advantage of the encapsulation of proteins to determine the molecular weight cut-off is the possiblity of detecting a small number of leaking capsules. However, during the preparation procedure the proteins diffuse out of the alginate beads. A disadvantage of addition of proteins to the medium containing capsules, is that inhomogeneity within one batch cannot be detected, as small numbers of permeable or non-permeable capsules will not be detected.

In this study the comparison was made between a new approach, microscopic visualization of fluorescent markers inside and outside the microcapsules, and measurements in the supernatant after addition of the fluorescent markers to the medium containing capsules.

Materials and methods

Preparation of microcapsules. A production protocol, suggested by Lim & Sun (1980), was modified according to literature data (Lim 1982, 1984, 1985; Goosen et al 1985). Sodium alginate (1·0%, w/v) (Pronova MVG, Protan, Drammen, Norway) in Moscona's solution (950 mL distilled water, 8 g NaCl, 0·3 g KCl, 0·05 g NaH_2PO_4, 0·025 g $KH_2PO_4.H_2O$, 1·0 g $NaHCO_3$, 2·0 g dextrose, HCl 1 M at pH 7·2) was dropped into 30 mL of a $CaCl_2$ solution (1·3%, w/v) by means of a double needle system (Lim 1984). The droplets with a diameter of about 700 μm gelified and formed Ca-alginate beads. When a volume of 1·0 mL Na-alginate was extruded, the beads were kept for an additional 5 min in the $CaCl_2$ solution. The $CaCl_2$ solution was removed. The beads were washed twice with 30 mL saline (0·9% NaCl). Thirty mL (0·1%, w/v) poly-L-lysine HBr (mol. wt 249 200, Sigma, St. Louis, MO) in saline, preincubated at 40°C, was poured over the beads. After a contact time of 15 min at 40°C, two washes with 30 mL saline were performed. A solution of 0·05% Na-alginate in saline was added and, after 5 min, replaced by saline pending the determination of molecular weight cut-off.

Determination of the molecular weight cut-off. The molecular weight cut-off was defined as the molecular weight of the fluorescein isothiocyanate labelled dextran (FITC-dextran) that did not diffuse into the microcapsules. FITC-dextran solutions with molecular weights of 4700 daltons (FD-4) and 40 500 daltons (FD-40) (Sigma, St. Louis, MO) were separately added to the batch of microcapsules. The diffusion of the FITC-dextrans from the medium (i) into the capsules (ii) were determined as a function of time, for three separate batches of microcapsules.

(i). FITC-dextran concentration in the medium. Two mL of a concentrated FITC-dextran solution (0·1%, w/v) was added to a sample of microcapsules, corresponding to 0·9 mL extruded alginate, in 2 mL saline. The control solution consisted of 2 mL saline and 2 mL concentrated FITC-dextran solution. The microcapsules were incubated at 37°C; 50 μL samples were taken from the medium surrounding the capsules at 2, 10, 20, 30, 60, 120, 180 and 300 min, diluted with 1·0 mL saline and the fluorescence measured with a fluorescence meter (SFM 25, Kontron, Zurich, Switzerland) (ex = 465, em = 517 nm).

The fluorescence was expressed as the percentage of the fluorescence of a control solution, containing the same volume of saline instead of capsules. The fluorescence of the medium was plotted as a function of time.

(ii). FITC-dextran concentration in the microcapsules. An FITC-dextran solution (0·5 mL, 0·0015%, w/v) was added to a sample of microcapsules, corresponding to 0·1 mL extruded alginate, in 0·2 mL saline. The microcapsules were incubated for 24 h at 37°C and observed with the MRC-500 confocal scanning laser microscope (Lasersharp Ltd, Bio-Rad, Abingdon, UK). The principle and advantage of confocal microscopy versus conventional imaging are described by Shotton (1989) and White et al (1987). Confocal microscopy allows the observation of objects in the absence of out-of-focus blur. It generates digital images of optical sections with the possibility for extensive image processing. Medium (100 μL) containing microcapsules was placed on a slide with a central spherical well (15 mm diameter, 3 mm maximal depth, Vel, Leuven, Belgium).

The Bio-Rad MRC-500 scanning laser system is equipped with an Ar^+-ion laser, mounted on a Leitz Orthoplan fluorescence microscope (objective 10×, numerical aperture 0·3) (Wetzlar, Germany) and controlled by an RM-Nimbus IBM-

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compatible PC-AT. The focus plane was set, by the motor driven micrometer screw, at the equator of the capsule after determination of the object diameter. The mean of 50 consecutive, Kalman filtered, scans of a single field was accumulated in the image memory. Pixel grey values (between 0 and 255) outside and inside the microcapsules were evaluated on a single image with the software command "STAT", which calculated and displayed mean and standard deviation of the grey value in a selected rectangle of variable dimensions. The distribution of pixels with a particular light intensity showed a normal distribution inside and outside the capsules. A significant difference in grey value was accepted with a confidence interval of 95%, if no overlap in mean light intensity ± twice s.d. between two rectangles with a surface of 0·010 mm^2 inside or outside of eight capsules per batch, was detected.

Results

FITC-dextran concentration in the medium. The fluorescence in the medium decreased towards the level of the control solution after addition of FD-4 to the medium containing capsules (Fig. 1a). This indicated that the FD-4 diffused homogeneously in the medium and the capsules. The concentration of the FD-40 in the medium remained higher than in the control experiment (Fig. 1b), indicating a smaller distribution volume because of the non-homogeneous diffusion in the medium and in the capsules.

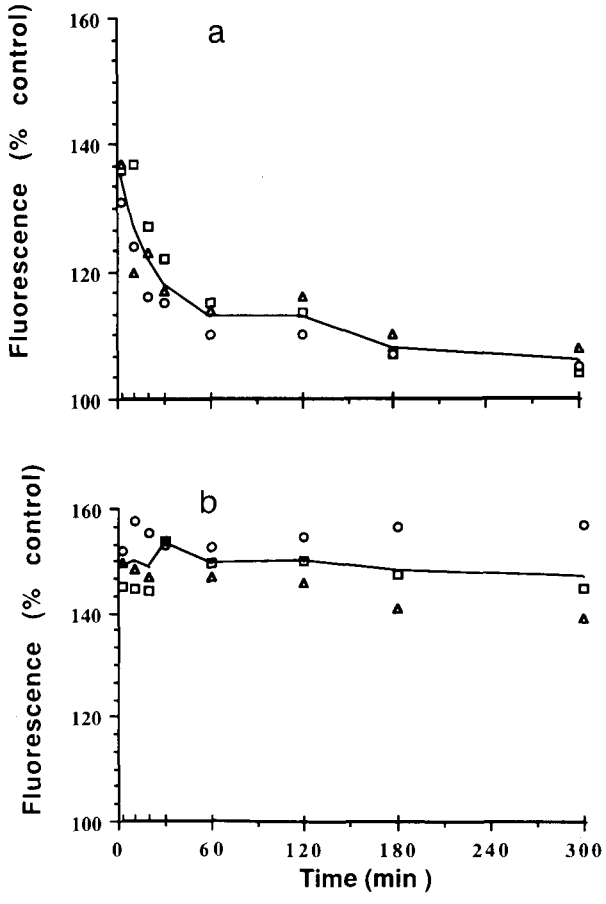

FIG. 1. Mean relative (—) fluorescence (% of the control) (n = 3) of the medium as a function of time after addition of FITC-dextran with a molecular weight of 4700 daltons (a) or 40 500 daltons (b) to the medium with capsules. Individual values are indicated by ○, □ and △.

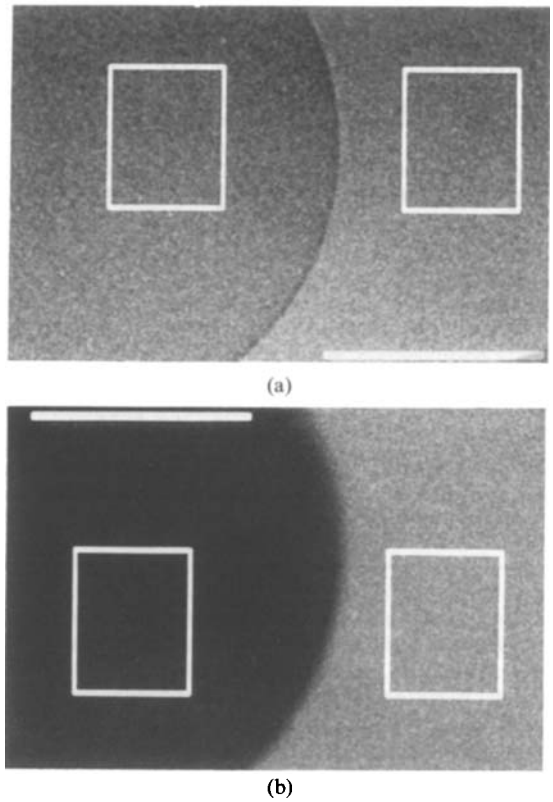

FIG. 2. Confocal image of a capsule 24 h after addition of FITC-dextran with a molecular weight of 4 700 daltons (a) or 40 500 daltons (b). (Scale bar = 250 μm).

FITC-dextran concentration in the microcapsules. Images of microcapsules, 24 h after addition of FD-4 and FD-40, are shown in Fig. 2. Analysis of the 8 images per batch showed no significant difference in light intensity between the inside and the outside of the microcapsules after addition of FD-4. This indicated a homogeneous distribution of FD-4 in the capsule and the medium (Fig. 3). A significantly lower light intensity inside the capsules was observed after addition of FD-40. This indicated that the FD-40 did not diffuse through the capsule wall.

Discussion

Fluorescence measurements and visualization of the diffusion into microcapsules prepared with alginate and polylysine indicated a molecular weight cut-off of the microcapsules lower than 40 500 daltons. The molecular weight cut-off of the microcapsules under investigation differs from the cut-off obtained by Goosen et al (1985), whose capsules were permeable to bovine albumin (mol. wt 66 000) and impermeable to γ-immunoglobulins (mol. wt 150 000). Differences in preparation technique and the origin of raw materials for the manufacturing of the microcapsules influence the molecular weight cut-off (Goosen et al 1985; King et al 1987). The molecular shape and charge of the molecular weight marker might also influence the observed value.

The influence of modifications of the production protocol or change in raw material batch should be investigated for capsules with a critical molecular weight cut-off. Application of microencapsulated cells for organ transplantation (O'Shea et al 1984; Sun & O'Shea 1985; O'Shea & Sun 1986) or use of microcapsules to study cell interactions (Vandenbossche et al 1990) require a

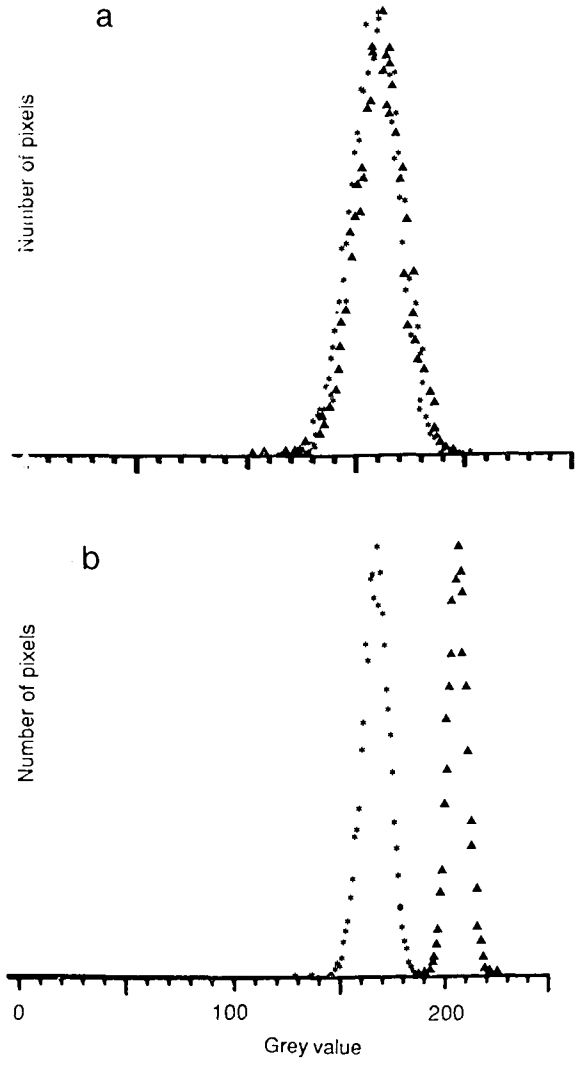

FIG. 3. Grey value in 0·010 mm^2 rectangles inside (▲) and outside (*) the capsule after addition of FITC-dextran with a molecular weight of 4 700 daltons (a) or 40 500 daltons (b). (0 = white, 255 = black).

careful validation of the preparation method to assure a reproducible molecular weight cut-off within and between batches.

Major difficulties in interpretation of the concentrations in the supernatant arise when the molecular weight cut-off of the capsules is not homogeneous in one batch. When the membrane of a minority of capsules is permeable, the concentration in the supernatant will decrease during the diffusion experiments, whereas a few non-permeable capsules will not be detected in a batch of permeable capsules. The sensibility or detection limit of measurements in the supernatant depends on the ratio of supernatant volume to capsule volume, and is limited by the volume of the capsule suspension and the volume of supernatant needed for sampling.

The ability to evaluate the molecular weight cut-off of individual microcapsules allows the evaluation of reproducibility within one batch, several molecular weight markers can be used to characterize the same batch and larger amounts of capsules are available after characterization. Leaking or impermeable capsules can be detected and withdrawn during or after use.

In conclusion, two evaluation techniques showed the permeability of microcapsules prepared with alginate and polylysine for FITC-dextrans with a molecular weight of 4 700 daltons and the impermeability for FITC-dextrans of 40 500 daltons.

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