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Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system

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Abstract

The mechanisms of internal and external gelation and their impact on alginate in its use as a coat and as a delivery system for drugs and living cells were investigated. Surface topography, thickness, mechanical and permeation properties, drug encapsulation efficiency and drug release profiles of alginate matrices were evaluated. Comparable extent of cross-linkages was produced by the two gelation methods as indicated by comparable Ca^{2+} contents between externally and internally cross-linked films but the distributions of cross-linkages were different. External cross-linking produced thinner films with smoother surface, greater matrix strength, stiffness and permeability than internally cross-linked films. Externally cross-linked micropellets were also capable of greater drug encapsulation efficiency and slower drug release rate. The differences in the properties observed were due to the different gelation mechanisms involved and the physical form of the matrix produced. External gelation is the preferred method in producing cross-linked alginate for coating and encapsulation purposes.

Keywords: Mechanism of cross-linking; Internal gelation; External gelation; Alginate

1. Introduction

Alginate, a naturally occurring polysaccharide in brown algae, is a biocompatible polymer that is commonly employed as cross-linked matrices or coats in drug delivery systems (Abletshauser, Schneider, & Rupprecht, 1993; Badwan, Abumalooh, Sallam, Abukalaf, & Jawan, 1985; Iannuccelli, Coppi, Vandelli, Leo, & Bernabei, 1995; Tomida, Mizuo, Nakamura, & Kiruya, 1993). It is also extensively used to encapsulate living cells, such as yeasts for fermentation and pancreatic islets for clinical applications (Najafpour, Younesi, & Ismail, 2004; Tze, Cheung, Tai, & Ye, 1998). Alginates can be cross-linked by external or internal gelation method using polyvalent cations, such as Ca^{2+}. In the production of micropellets by external gelation, the alginate-drug solution is extruded as droplets into a solution of calcium salt. For internal gelation, an insoluble calcium salt is added to the alginate-drug solution and the mixture extruded into oil (Liu et al., 2002). The latter is acidified to bring about the release of Ca^{2+} from the insoluble salt for cross-linking with the alginate. A number of studies have reported that the micropellets produced by the internal gelation methods have more homogeneous but less dense matrices with larger pore sizes (Choi, Park, Hwang, & Park, 2002; Liu et al., 2002; Quong, Neufeld, SkjåK-BræK, & Poncelet, 1997; Vanderberg & De La Noüe, 2001). Liu et al. (2002) attributed this to the displacement of Ca^{2+} by H^+ due to the acid added. While the acid in the oil phase liberated Ca^{2+} from the insoluble salt, it also competed with Ca^{2+} for interaction with the alginate. Despite their homogeneity, the internal gelated matrices were more permeable, resulting in lower encapsulation efficiencies and faster release rates (Quong et al., 1997; Vanderberg & De La Noüe, 2001). If the permeability of a matrix is primarily affected by competition between Ca^{2+} and H^+, the limitations of the internal gelation method may be overcome by manipulating the pH of the medium and the amount of calcium salt employed.

In our laboratory, alginate micropellets were prepared by internal and external gelation methods. The pH of the medium required to bring about almost complete release of Ca^{2+} from the insoluble salt, calcium carbonate, was determined. The pH of the medium employed by

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Table 1
Median particle size of calcium carbonate batches

Type of calcium carbonate used	Code	Median diameter (μm)
Unmilled calcium carbonate[a]	$CaCO_3$-S	5.663 ± 0.18
Milled calcium carbonate[a,c]	$CaCO_3$-M	4.123 ± 0.061
Nanoparticulate calcium carbonate[b]	$CaCO_3$-N	0.071 ± 0.013

[a] Particles sized by laser diffraction technique (Coulter, Particle Sizer LS 230, Coulter, USA) (replicates, $n \geq 9$).

[b] Particles were coated with Osmium and sized by scanning electron microscope (Shimadzu, SS-550, Japan) (particles, $n \geq 20$).

[c] Calcium carbonate was comminuted using an air jet mill (Hosokawa Alpine AG, 100 AFG, Germany) with classifier wheel rotational speed of 18,000 rpm at a milling pressure of 0.5 MPa.

the 2 methods was kept at a constant pre-determined value to avoid the effect of the actual pH of the medium. Although the effect of pH was eliminated, the micropellets produced by the 2 methods were obviously different in morphology. These preliminary findings suggested that the gelation mechanism played an important role. A good understanding of the mechanisms and their effects is critical to the successful application of alginate in the design of delivery systems.

This study attempted to investigate the mechanisms of internal and external gelation and their impact on alginate in its use as a coat and as a delivery system for drugs and living cells. In all the studies reported in the literature, the medium employed in the internal and external gelation methods was not standardized and the pH was not reported, resulting in varying observations (Choi et al., 2002; Liu et al., 2002; Quong et al., 1997; Vanderberg & De La Noüe, 2001). Therefore, in this study, an aqueous medium was used and its pH was standardized so that the effect of internal and external gelation mechanisms on the properties of the matrix could be compared. Properties that affect the function of the matrix as a coat, such as thickness, extent of cross-linking, tensile strength, elastic modulus, solubility, degree of hydration and drug permeability, were evaluated. In addition, the surface topography of the matrix was determined as this parameter was reported to affect the success rate of encapsulated cells in immunoisolation devices (Lanza et al., 1991). Films were employed as models to avoid the effects of size and shape of the test sample on the properties of the matrix, as well as ease of measurement. Alginate micropellets were also prepared to assess the influence of gelation mechanisms on the morphology, encapsulation efficiency and release properties of alginate particulate delivery systems.

2. Experimental section

2.1. Materials

Sodium alginate (Manucol® DH, Kelco, ISP-Alginates Industries, USA) was used in this study. Glacial acetic acid, nitric acid (70%), perchloric acid (60%) [Merck, Germany] and sodium acetate anhydrous (AnalaR®, BDH Laboratory Supplies, England) were used as supplied. Calcium carbonate batches of different median particle sizes were used (Table 1): unmilled calcium carbonate ($CaCO_3$-S, Merck, Germany), milled calcium carbonate ($CaCO_3$-M) and nanoparticulate calcium carbonate ($CaCO_3$-N, Nano-Materials Technology, Singapore). Acetaminophen (BP grade) was used as the model drug.

2.2. Methods

2.2.1. Preparation of alginate films

2.2.1.1. Sodium alginate films. A 2%w/w solution of sodium alginate was prepared by dissolving an appropriate amount of sodium alginate in deionised water. The solution was left to stand overnight to remove air bubbles. The films were produced by a solvent evaporation technique. Thirty grams of the sodium alginate solution was transferred into respective glass petri dishes of 10 cm diameter and oven-dried on a leveled surface at 40 °C to constant weight. The dried films were then removed from the petri dishes and stored in a desiccator at 24 ± 1 °C until use.

2.2.1.2. Calcium carbonate–sodium alginate films. A specific amount of calcium carbonate ($CaCO_3$-S, $CaCO_3$-M or $CaCO_3$-N) was suspended in an appropriate amount of deionised water, ultrasonicated for 30 min and further mixed using a magnetic stirrer. The required amount of suspension was added to sodium alginate solution which was thoroughly mixed to give a final alginate concentration of 2%w/w. Films were prepared and stored as previously described.

2.2.1.3. Preparation of calcium alginate film matrices. Externally cross-linked films were prepared by immersing each sodium alginate film for about 24 h in 50 ml of 1% sodium acetate with an appropriate amount of glacial acetic acid (Solution A), to which the required amount of calcium carbonate was added (0.15, 0.25 or 0.35 g of $CaCO_3$-M). Glacial acetic acid was added to liberate Ca^{2+} from the insoluble salt. Internally cross-linked films were produced by immersing each calcium carbonate–sodium alginate film for about 24 h in 50 ml of Solution A. The cross-linked films were washed with 50 ml of deionised water thrice and oven-dried at 40 °C to constant weight. The dried films were stored in a desiccator at 24 ± 1 °C for at least 72 h prior to

Table 2
Types of alginate films studied

Type of Film	Code
Sodium alginate	SA
Calcium alginate externally cross-linked with different amounts of $CaCO_3$-M	
0.15 g	CAE 0.15
0.25 g	CAE 0.25
0.35 g	CAE 0.35
Calcium alginate internally cross-linked with different amounts $CaCO_3$-M	
0.15 g	CAI 0.15
0.25 g	CAI 0.25
0.35 g	CAI 0.35
Calcium alginate internally cross-linked with 0.25 g of calcium carbonate batches of different particle sizes	
$CaCO_3$-M	CAI 0.25
$CaCO_3$-S	CAI 0.25S
$CaCO_3$-N	CAI 0.25N

further test. The different types of alginate films prepared are shown in Table 2.

2.2.2. Characterization of alginate films

2.2.2.1. Morphology and surface topography. The morphology of the films was examined under a light microscope (Olympus, BX16TRF, Japan). Quantitative surface measurement was carried out by scanning probe microscopy (Shimadzu, SPM 9500, Japan) in the dynamic mode, at a scan rate of 1 Hz. A scan area of 25 μm ×25 μm with Z range (depth of scan) set at 1.5 μm was employed in the acquisition of surface images. At least 30 scans were carried out for each type of film and the mean roughness values (R_a) calculated (Heng, Chan, & Lim, 2000).

2.2.2.2. Film thickness. The thickness of the films was measured by using a micrometer (Mitutoyo, 293-721-30 CE, Japan). At least 5 thickness readings from different regions of each film were taken and the results averaged.

2.2.2.3. Weight of alginate film before and after cross-linking. The weights of alginate film before (W_b) and after (W_c) cross-linking were determined. For films containing $CaCO_3$, the corresponding weight of the calcium salt was deducted from W_b. For each type of film, the individual weights of 5 film samples were determined and results averaged.

2.2.2.4. Apparent solubility and hydration index. Rectangular pieces of films, 1 cm×7 cm, were cut and stored in a desiccator for at least 72 h before testing. Each piece of film was weighed (W_1), immersed in 40 ml of deionised water in a boiling tube, which was then placed in a shaker water bath (Lauda, M20S, Germany) at 37 °C. At the end of 6 h, the film was removed, placed between two pieces of filter paper to remove surface water and weighed (W_0). It was then spread out on a clean petri dish and oven-dried at 40 °C to constant weight. The dried film was kept in a desiccator for at least 72 h before reweighing (W_2). The apparent solubility (W_A) was calculated as follows:

$$W_A = \frac{W_1 - W_2}{W_1} \times 100\% \tag{1}$$

The hydration index (W_H) was expressed as a percentage of the total hydrated film weight as shown:

$$W_H = \frac{W_0}{W_1} \times 100\% \tag{2}$$

For each type of film, 5 determinations were made and the results averaged.

2.2.2.5. Tensile properties. The thickness of films, 1 cm× 7 cm, was measured at 5 random points (Mitutoyo, 293-721-30 CE, Japan) and mean thickness calculated. Only film samples with deviation of thickness within 10% from the mean were used. The tensile strength was determined (Shimadzu, EZ tester, Japan) with an initial grip separation of 5 cm and a test speed of 10 mm/min. Tensile strength was calculated by dividing the maximum load with the original cross-sectional area of the test film. Elastic modulus, which describes the rigidity or stiffness of the film, was obtained from the gradient of the initial linear portion of the load-deformation curve. For each type of film, at least 5 determinations were made and the results averaged.

2.2.2.6. Permeation profile. A two-compartment horizontal diffusion cell was used. The film was securely mounted between the compartments. The donor compartment contained 250 ml of acetaminophen solution (0.48 mg/ml) while the receptor compartment was filled with an equal volume of deionised water. The film area available for permeation was 8.042 cm^2. The diffusion cell was placed in a shaker water bath (Lauda, M20S, Germany) at 37 °C. At predetermined time intervals over 6 h, 5 ml samples were withdrawn from the receptor compartment with replacement of deionised water to maintain constant volume. Sink conditions were maintained throughout the permeation study as no more than 5% of the initial drug quantity in the donor compartment entered the receptor compartment (Aslani & Kennedy, 1996). The samples were analyzed spectrophotometrically (Shimadzu, UV-1201, Japan) at 242 nm. All experiments were repeated at least 5 times and the results averaged.

The total amount of drug, Q_t, which penetrated through a membrane in time, t, from the donor compartment at constant concentration (C) to the receptor compartment at sink condition is given by:

$$Q_t = \mathrm{AKhC}\left[D\frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(\frac{-Dn^2\pi^2 t}{h^2}\right)\right] \tag{3}$$

where A is the actual surface diffusion area, K is the partition coefficient of the drug between the membrane and the donor solution, h is the membrane thickness, and D is the drug diffusion coefficient through the membrane. The product of Kh and D/h^2, which is equal to the permeability coefficient (P), was calculated by curve fitting the permeation data to the above equation (Crank, 1975) using a statistical program (GraphPad Prism 3). At least 5 determinations were carried out for each type of film and the mean permeability coefficient calculated.

2.2.2.7. Calcium ion content. Approximately 50 mg of dried calcium alginate film was accurately weighed. Twenty millilitres of concentrated HNO_3 was added and heated for 1 h, keeping the solution slightly boiling throughout. On cooling, 10 ml of perchloric acid was added and heated at a slightly boiling state for 1.5 h, cooled and made up to 100 ml with deionised water. After appropriate dilutions, the calcium ion contents were determined by atomic absorption spectrometry (Shimadzu, AA6800, Japan) at 422.7 nm. Three determinations were carried out for each type of film and the results averaged.

2.2.2.8. Fourier transform infra-red spectroscopy (FTIR). Absorption spectra were obtained using a FTIR spectrometer (Jasco, FTIR-430, Japan) equipped with a TGS detector. The alginic acid employed was obtained by precipitation of sodium alginate using 0.1 N HCl. Potassium bromide discs containing 1%w/w of film material were scanned at 4 mm s^{-1} with a resolution of 4 cm^{-1} over 400–4000 cm^{-1}, averaging over 128 scans for each type of film.

2.2.3. Preparation of calcium alginate micropellets

Acetaminophen was added to 30 g of 2% w/w alginate solution to give a drug to polymer ratio of 1:2. Externally cross-linked micropellets were produced by drop-wise extrusion of the drug-polymer solution into 50 ml of Solution A, to which 0.25 g of calcium carbonate ($CaCO_3$-M) was added. In the preparation of internally cross-linked micropellets, $CaCO_3$ was added to the drug–polymer solution instead and the mixture extruded into Solution A. Extrusion was carried out at a flow rate of 0.7 g min^{-1} using a peristaltic pump (Gilson, Minipuls 3 Model M312, France) through either a syringe needle tip (20 gauge) or a silicon tubing with an internal diameter of 2 mm. The alginate micropellets were allowed to cure overnight in the cross-linking solution before collection. The micropellets were washed thrice with 50 ml of deionised water and oven-dried at 40 °C to constant weight.

2.2.4. Characterization of calcium alginate micropellets

2.2.4.1. Drug content. An accurately weighed amount of alginate micropellets was diluted to 50 ml with 0.1 N HCl. The sample was placed in an ultrasonic water bath for three consecutive periods of 40 min each, with a resting period of 40 min in between. It was then allowed to stand overnight at room temperature. Aliquot samples were removed through a 0.45 μm filter and appropriately diluted with 0.1 N HCl. The acetaminophen content was assayed spectrophotometrically (Shimadzu UV-1201, Japan) at 245 nm. For each sample, at least three determinations were carried out and the drug content of the micropellets was reported as the mean amount of drug in 50 mg of alginate micropellets.

2.2.4.2. Drug release. An accurately weighed amount of alginate micropellets was placed in a cylindrical basket, which was then immersed in 900 ml of deionised water at 37 ± 0.5 °C (USP Apparatus 1, Optimal DT-1, USA). The basket was rotated at 50 rpm. Five millilitres of samples were collected at specified time intervals. The samples were passed through a 0.45 μm filter and then assayed spectrophotometrically (Shimadzu, UV-1201, Japan) at 242 nm. Three dissolution runs were carried out for each batch of alginate micropellets and the results averaged.

2.3. Statistical analysis

Data were reported as mean ± standard error of mean (SE). Differences were assessed for significance using the one-way ANOVA or unpaired t-tests as appropriate. The level of significance was set at $\alpha = 0.05$.

3. Results and discussion

Sodium alginate could be converted to alginic acid at pH below the p*K*a values of β-D-mannuronic acid (3.38) and α-L-guluronic acid (3.65) (Haug, 1964). Addition of glacial acetic acid to liberate Ca^{2+} from the insoluble calcium salt would reduce the pH of the medium drastically. Hence, sodium acetate was employed to keep the pH relatively constant, at about 4. Calcium carbonate was used in both external and internal gelation to keep the source of Ca^{2+} constant. Different amounts of calcium carbonate were used to compare their influence with that of gelation mechanisms.

3.1. FTIR of alginate films

The presence of a very strong peak at around 1737 cm^{-1} for alginic acid has been reported by Dupuy, Arien, and Perrot Minnot (1994). The peak was suggested to be due to a C=O stretch, attributed to the free carboxyl (–COOH) group present in the alginic acid. In this study, strong peaks around 1737 cm^{-1} were absent in the FTIR spectra of the cross-linked alginate films, indicating interaction involving C=O groups (Fig. 1). It could therefore be inferred that the pH of the medium employed promoted interaction between Ca^{2+} and the alginate molecule. This supported the earlier proposal that the displacement of Ca^{2+} by H^+ could be controlled by careful manipulation of the pH of the medium. The FTIR spectra of the cross-linked alginate films appeared similar and thus it was unable to detect changes in the matrix due to different gelation mechanisms.

3.2. Morphology, surface topography and calcium content of alginate matrices

Films were produced by both external and internal gelation methods using varying amounts of $CaCO_3$ (Table 2). The dried cross-linked alginate films were clear. Examination of internally cross-linked films under 40× magnification showed the presence of abundant minute cavities, which were absent in the externally cross-linked films.

Cross-linking by either method was found to increase the surface roughness of the films (Table 3 and Fig. 2). It was postulated that calcium ions in the external gelation method would first cross-link the film surface extensively as it was the first point of contact. This would draw the polymer chains closer to form a less permeable surface to

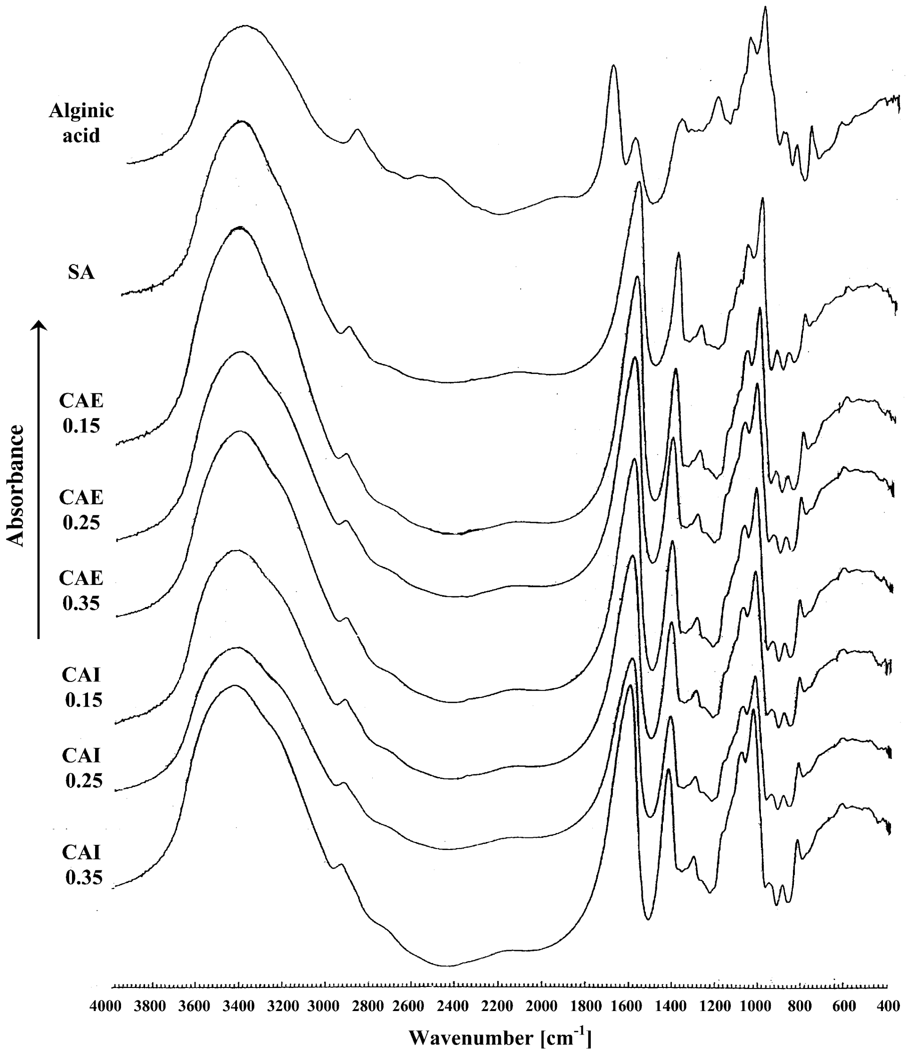


Fig. 1. FTIR spectra of alginic acid and alginate matrices.

Table 3
Surface roughness, mechanical properties and calcium content of dried calcium alginate films

Film Code	R_a value (nm)	Tensile strength (N/mm^2)	Elastic modulus (N/mm^2)	Amount of Ca^{2+}per g of cross-linked film (mg/g)
SA	44.7±3.00	78.8±2.13	1674.7±81.4	–
CAE 0.15	45.1±0.77	89.3±3.55	3142.0±91.3	67.01±0.885
CAE 0.25	53.5±1.16	95.9±2.78	3017.9±127.7	76.42±1.719
CAE 0.35	52.6±1.26	99.9±4.92	2533.2±163.8	79.48±1.841
CAI 0.15	51.0±1.23	88.2±3.16	2974.4±112.2	65.71±0.392
CAI 0.25	59.0±1.62	86.6±4.67	2646.3±79.9	73.55±0.480
CAI 0.35	66.2±2.21	87.2±2.13	2210.7±220.2	76.24±0.716
CAI 0.25S	–	84.5±3.26	3230.3±199.0	–
CAI 0.25N	–	90.2±1.61	3270.2±287.6	–

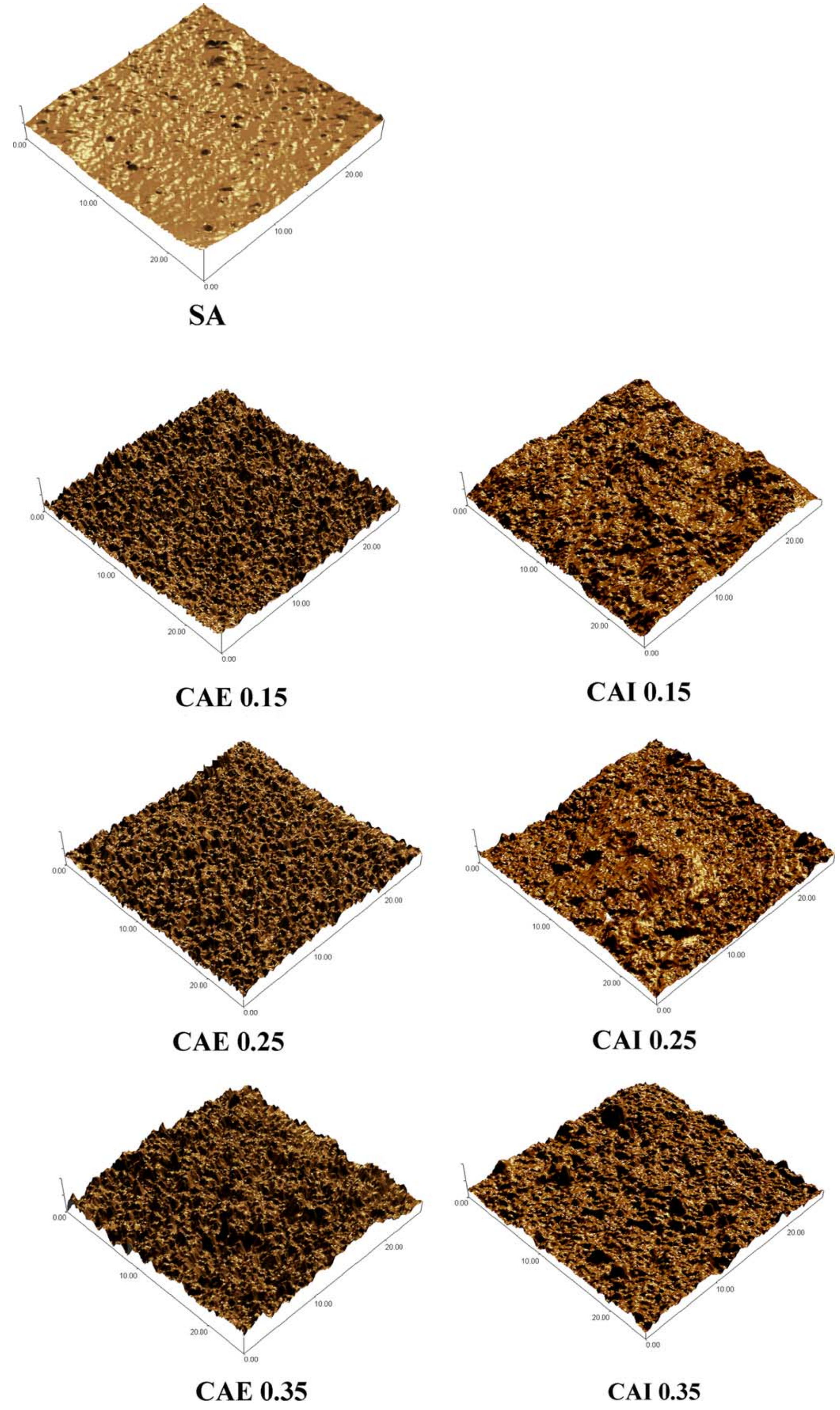
the diffusion of Ca^{2+} into the interior. Consequently, a less homogeneous alginate matrix resulted due to a relatively less cross-linked interior and a highly cross-linked surface. A homogeneous matrix is essential for uniform contraction of the matrix during drying, which, in turn, is critical to the formation of a smooth surface. The surface roughness and Ca^{2+} content of externally cross-linked alginate films increased significantly ($p<0.05$) when the amount of $CaCO_3$ used was increased from 0.15 to 0.25 g (Table 3). Further increase to 0.35 g of $CaCO_3$ produced insignificant change in surface roughness, suggesting that 0.25 g of $CaCO_3$ was sufficient to produce a highly cross-linked surface that strongly impeded diffusion of Ca^{2+} into the interior. However, in earlier studies, alginate microspheres were found to release drug rapidly (Chan, Heng, & Wan, 1997; Wan, Heng & Chan, 1994). Since some of the drug molecules studied were larger than Ca^{2+}, the reduced permeability of the cross-linked surface to Ca^{2+} could not be attributed to the size of Ca^{2+}. It was more likely that the sodium ions displaced by the calcium ions led to the gradual development of a positively charged microenvironment, which showed a repelling effect on the calcium ions.

In the internal gelation method, the insoluble $CaCO_3$ was uniformly distributed in the sodium alginate matrix. The liberation of Ca^{2+} for cross-linking was accompanied by the evolution of CO_2, which created abundant minute cavities throughout the cross-linked matrix. The evolution of carbon dioxide near the surface aptly accounted for the rougher surface of internally cross-linked alginate films, compared to externally cross-linked or non-cross-linked sodium alginate films. A linear relationship ($r^2=0.999$) between surface roughness of internally cross-linked films and amount of calcium carbonate used was observed. It is clearly seen that the surface topography was significantly affected by the gelation mechanism and the amount of calcium salt used. Internal gelation and larger amounts of $CaCO_3$ are therefore not recommended for cross-linking of alginate matrix used in providing immunoprotection to cells in immunoisolation devices as the success rate of transplantation has been reported to be adversely affected by surface roughness of the matrix membrane (Lanza et al., 1991).

3.3. Thickness and weight of the alginate films

The thickness of alginate films is shown in Table 4. With the exception of CAE 0.35 films, the rest were markedly thinner than the corresponding films before cross-linking. Although a smaller amount of $CaCO_3$ was used, CAE 0.15 films were significantly thinner than CAE 0.25 films ($p<0.05$). These findings were not in agreement with those reported in the literature, which seemed to indicate that a larger amount of calcium salt produced greater cross-linkage and consequently thinner films. The change in film weight was found to be minimal compared to the change in film thickness after cross-linking. Stochiometrically, one mole of calcium ion (atomic weight ≈40 g) displaces two moles of sodium ions (total atomic weight ≈46 g). As there is little difference between the corresponding weights of the cations exchanged, the weights of the film before and after cross-linking were expected to be comparable. Any significant change in weight would imply a loss in polymer content through dissolution during the cross-linking process. The percent reduction in thickness of films externally cross-linked with 0.15 g of $CaCO_3$ (45.9%) was markedly higher than the corresponding reduction in weight (6.5%). Hence, the loss in polymer content could only account for the reduction in film thickness to a small extent. CAE 0.25 films were significantly thinner ($p<0.05$) than CAE 0.35 film but both films were of comparable weights and Ca^{2+} contents. These findings supported earlier postulation of greater surface cross-linkage and lower Ca^{2+} permeation at high calcium salt concentration in the external gelation method. In contrast, the greater Ca^{2+} permeation at lower calcium salt concentration promoted cross-linkage across the matrix resulting in greater shrinkage and thinner films.

The amount of glacial acetic acid used in this study was predetermined to bring about complete liberation of Ca^{2+} from the insoluble $CaCO_3$. Hence, all the $CaCO_3$ employed for internal gelation was available for cross-linkage. Insignificant difference ($p>0.05$) between the Ca^{2+} content of the CAI 0.25 and CAI 0.35 films showed that 0.25 g of $CaCO_3$ was sufficient for maximum cross-linkage (Table 3). Interestingly, the Ca^{2+} contents between the corresponding



SA

CAE 0.15

CAI 0.15

CAE 0.25

CAI 0.25

CAE 0.35

CAI 0.35

Fig. 2. Scanning probe microscope images of alginate films.

Table 4
Thickness and weight of dried alginate films

Film code	Thickness (mm)			Weight (g)		
	Before cross-linking	After cross-linking	% change	Before cross-linking	After cross-linking	% change
CAE 0.15	0.0495 ± 0.0005	0.0268 ± 0.0011	−45.86	0.552 ± 0.006	0.516 ± 0.005	−6.52
CAE 0.25	0.0495 ± 0.0005	0.0352 ± 0.0009	−28.89	0.549 ± 0.005	0.551 ± 0.004	+0.36
CAE 0.35	0.0495 ± 0.0005	0.0510 ± 0.0013	+3.03	0.545 ± 0.008	0.563 ± 0.008	+3.30
CAI 0.15	0.0659 ± 0.0019	0.0383 ± 0.0009	−41.88	0.570 ± 0.003	0.526 ± 0.005	−7.72
CAI 0.25	0.0719 ± 0.002	0.0513 ± 0.0001	−28.65	0.582 ± 0.002	0.565 ± 0.003	−2.92
CAI 0.35	0.082 ± 0.0008	0.0574 ± 0.0012	−30.00	0.591 ± 0.0009	0.574 ± 0.003	−2.88

films externally and internally cross-linked with different amounts of $CaCO_3$ were comparable ($p > 0.05$) but CAI 0.15 and CAI 0.25 films were thicker than the corresponding externally cross-linked films. This showed that the different gelation mechanisms resulted in comparable extent but different distribution of cross-linkage, giving rise to structurally different matrices. The thickness of internally cross-linked matrices was dependent on the balance between opposing effects of cross-linkage and carbon dioxide produced, whereas the thickness of externally cross-linked films was controlled by the distribution pattern of cross-linkage. Cross-linking of alginate by Ca^{2+} would bring the polymer chains closer together leading to film contraction and decreased film thickness. On the other hand, evolution of CO_2 from the reaction between H^+ and $CaCO_3$ resulted in the formation of abundant minute cavities, hence contributing to volumetric increase of the internally cross-linked matrix. Both effects were expected to increase with greater amounts of calcium salt used. The results clearly showed that CO_2 released had a greater contribution to film thickness than matrix contraction due to cross-linking.

3.4. Mechanical properties of alginate films

The tensile properties of the different types of films are given in Table 3. Cross-linked alginate films were significantly stronger than sodium alginate films ($p < 0.05$) indicating that the extent of cross-linkage increased the mechanical strength of the matrix. Statistical analysis showed minimal differences in the tensile strength between film matrices cross-linked externally with different amounts of $CaCO_3$ ($p > 0.05$). However, CAE 0.25 and CAE 0.35 had greater extent of cross-linkage as reflected by their significantly higher calcium contents than CAE 0.15 ($p < 0.05$) (Table 3). This implied that the distribution of cross-linkage had a strong influence on the mechanical strength of films, with a more uniformly cross-linked matrix being stronger. The tensile strength and calcium contents of internally cross-linked films showed similar trends. Increased availability of Ca^{2+} was accompanied by higher level of CO_2 liberation. Hence, formation of films of comparable strengths resulted as the increased extent of cross-linking was compromised by some extent of integrity impairment due to CO_2 generated on the mechanical strength of films. The latter was further illustrated by the formation of stronger externally cross-linked films compared to the corresponding internally cross-linked ones ($p < 0.05$).

The presence of cavities, due to liberation of CO_2, caused disruption of the alginate matrix, thereby compromising its tensile strength. The effect of cavities was further investigated by employing $CaCO_3$ batches of different particle size (Table 1). Smaller particles of $CaCO_3$ were expected to produce smaller and more uniformly distributed cavities. However, no difference in tensile strength between CAI 0.25, CAI 0.25S and CAI 0.25 N was observed ($p > 0.05$), indicating stronger influence of cross-linkage than cavities on mechanical strength of the film. The two gelation mechanisms gave rise to similar extent of binding between Ca^{2+} and alginate as illustrated by insignificant difference in both Ca^{2+} content between CAE 0.15 and CAI 0.15 and between CAE 0.25 and CAI 0.25 (Table 3). The cross-linked films had higher elastic modulus values than sodium alginate films, indicating lower flexibility of the cross-linked matrix (Table 3). Despite insignificant differences in Ca^{2+} content, the externally cross-linked films had higher elastic modulus values than the corresponding internally cross-linked films. This further supported the postulation that the two gelation mechanisms produced films with comparable extent of cross-linkage but with different matrix structure. The greater flexibility of internally cross-linked films could be attributed to the microscopic cavities dispersed in the matrix. Increasing amount of $CaCO_3$ used increased the flexibility of both externally and internally cross-linked films though it had insignificant effect on their tensile strength. This confirmed the contributory effect of cavities on film flexibility. It also showed that a film with poorly cross-linked interior, albeit a highly cross-linked surface, was more flexible. More importantly, the flexibility of the films could be modified, without affecting their mechanical strength, by varying the amount of $CaCO_3$ employed. In addition, the flexibility of internally cross-linked films could be increased by employing $CaCO_3$ of smaller particle size ($p < 0.05$).

The mechanical properties of alginate matrices were significantly affected by the gelation mechanisms and the amounts of $CaCO_3$ employed. External gelation produced stronger films while internal gelation produced more flexible films. Each method has its merits and is potentially useful for cross-linking alginate for use as a coat or delivery system.

3.5. Apparent solubility of alginate films

Sodium alginate films dissolved within minutes after immersing in water at 37 °C. On the contrary, calcium alginate films were practically insoluble as less than 8%w/w of the film was found to dissolve in water after 6 h (Fig. 3). Statistical analysis showed insignificant change in dry film weight before and after immersion in water ($p > 0.05$). This implied that the calcium alginate films employed in the permeability studies would remain intact throughout the experiment. Nonetheless, the apparent solubility of cross-linked films was found to increase with decreasing amount of $CaCO_3$ used. This was attributed to the higher proportion of unreacted sodium alginate, which was more soluble than calcium alginate.

3.6. Permeation study of alginate films

After an initial lag time, the amount of acetaminophen diffusing across the calcium alginate matrix as a function of time became constant (Fig. 4). The diffusion of water-soluble drug, acetaminophen was expected to occur through aqueous pores or channels in the film matrix. Increased cross-linking diminished the pores or channels, as reflected by lower permeation coefficients and hydration indices of CAE 0.15 and CAI 0.15 compared to those of CAE 0.25 and CAI 0.25, respectively (Table 5). Earlier results showed that CAE 0.25 and CAE 0.35 were cross-linked to comparable extents, with the latter having a more highly cross-linked surface. The difference in permeation coefficients between CAE 0.25 and CAE 0.35 showed that the distribution of cross-linkages in the matrix had a significant effect on drug permeation. A more highly cross-linked surface was more effectively than a more uniformly cross-linked matrix to impede drug permeation, as reflected by a lower permeation coefficient for CAE 0.35 (Table 5). More rapid release of drugs from homogeneous alginate gel beads than inhomogeneous alginate gel beads had been observed by Martinsen, Storrø, and SkjåK-BræK (1992). Earlier results also showed comparable extents of cross-linkage between CAE 0.15 and CAI 0.15 and between CAE 0.25 and CAI 0.25 (Table 3), with the internal gelation method producing a more uniformly cross-linked matrix. However, the internally cross-linked films had lower permeation coefficients than the corresponding externally cross-linked films ($p < 0.05$) (Table 5). This suggested that the microscopic cavities formed by the liberation of CO_2 in internally cross-linked films impeded drug diffusion. Matrices, which were internally cross-linked with a larger amount of $CaCO_3$, were expected to have numerically more or large cavities. All these matrices were found to have lower hydration indices (Table 5). It could be inferred that the cavities were air vesicles, which disrupted liquid continuity in the matrix.

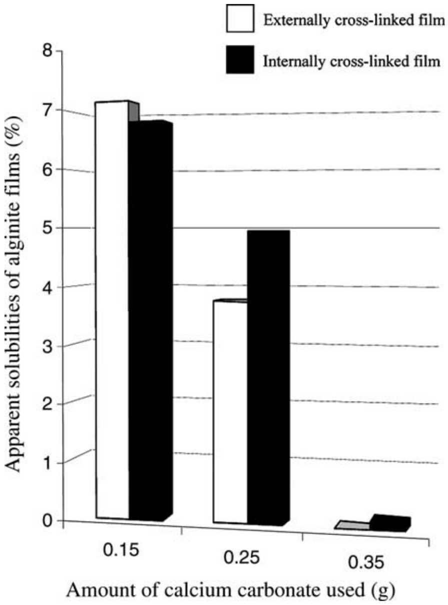


Fig. 3. Apparent solubilities of externally cross-linked and internally cross-linked alginate films at 37 °C.

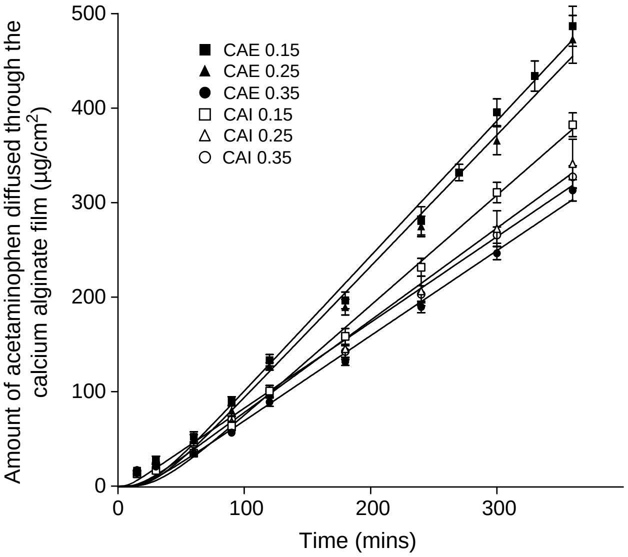


Fig. 4. Drug permeation profiles of calcium alginate films.

Table 5
The hydration index (W_H) and permeability coefficient (P) of calcium alginate films

Film code	W_H (%)	P (mm/min)
CAE 0.15	1010.83±35.40	$3.03\times10^{-2}\pm1.32\times10^{-3}$
CAE 0.25	511.85±8.78	$2.92\times10^{-2}\pm1.79\times10^{-3}$
CAE 0.35	321.37±4.13	$1.88\times10^{-2}\pm6.70\times10^{-4}$
CAI 0.15	562.31±14.40	$2.44\times10^{-2}\pm9.87\times10^{-4}$
CAI 0.25	406.46±8.94	$1.88\times10^{-2}\pm5.08\times10^{-4}$
CAI 0.35	327.24±11.32	$1.89\times10^{-2}\pm1.35\times10^{-3}$

As water is an important medium for drug diffusion, this aptly accounted for the lower drug permeation coefficient of such matrices. Other workers have observed that internally cross-linked beads were buoyant in water (Choi et al., 2002), which could also be explained by the above postulation. The internal gelation method is useful in producing cross-linked alginate coats for retarding or controlling drug release.

3.7. Formation and morphology of calcium alginate micropellets

Calcium alginate micropellets were produced to examine the effects of different gelation mechanisms on the properties of calcium alginate particles for the encapsulation of drugs and cells. Sufficiently strong calcium alginate micropellets were formed by extrusion through a needle tip or silicon tube, using $CaCO_3$: sodium alginate ratio employed for CAE 0.25 and CAI 0.25 films. The silicon tube, which had a larger orifice than the needle tip, resulted in larger micropellets (Fig. 5). Irrespective of the droplet size, the external gelation method produced generally spherical alginate micropellets while the internal gelation method produced particles of irregular shape (Fig. 5). This clearly showed that rapid cross-linking by Ca^{2+} was crucial for the formation of spherical alginate particles by the extrusion technique. It was interesting to note that the shape of the alginate particles became more regular as the production by internal gelation method progressed. This was probably due to the accumulation of Ca^{2+} that was gradually released from the cross-linked droplets in Solution A, which enabled more rapid and extensive cross-linkage on the surface of subsequent extruded droplets. Further studies showed that spherical beads could be obtained by internal gelation method if a small amount of calcium chloride was added to Solution A.

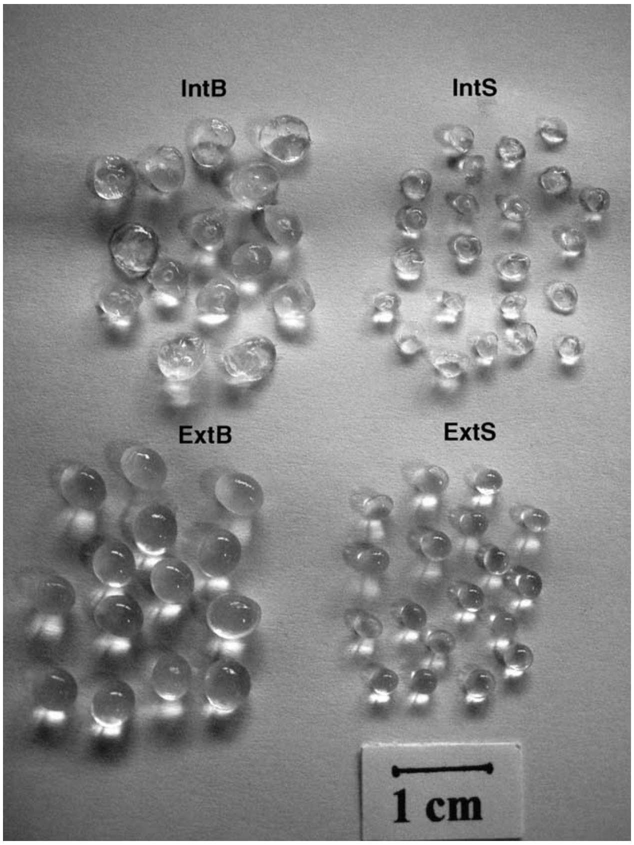


Fig. 5. Calcium alginate micropellets produced by extrusion through a 20 gauge needle tip (S) or silicon tube with a diameter of 2 mm (B) and cross-linked by external (Ext) and internal (Int) gelation methods.

Table 6
Drug content of calcium alginate micropellets

Type of calcium alginate micropellets	Code	Drug content (mg)[a]	Diameter of micropellets (mm)[b]
Produced by extrusion through a 20 gauge needle tip			
External gelation method	ExtS	3.439 ± 0.051	2.778 ± 0.248
Internal gelation method	IntS	2.768 ± 0.012	–
Produced by extrusion through a 2 mm diameter silicone tube			
External gelation method	ExtB	3.723 ± 0.021	4.583 ± 0.432
Internal gelation method	IntB	3.325 ± 0.026	–

[a] Drug content per 50 mg dried micropellets.

[b] Mean of diameters from at least 10 micropellets. The diameter of internally cross-linked micropellets was not determined due to difficulties to accurately measure the diameters of the irregularly shaped micropellets.

3.8. Drug content and drug release profiles of calcium alginate micropellets

The drug encapsulation efficiency was affected by the size of the micropellets. Calcium alginate micropellets of larger sizes had a higher content of acetaminophen (Fig. 5, Table 6). The alginate gel structure is governed by the kinetics of its formation which in turn affects the encapsulation efficiency of the micropellets. During the formation of externally cross-linked micropellets, diffusion of Ca^{2+} into the alginate droplet and the self diffusion of alginate molecules towards an inward moving calcium-induced gelling zone resulted in a dense 'membrane' of high alginate concentration at the Ca^{2+}-alginate interface (Skjåk-Bræk, Grasdalen, & Smidsrød, 1989), retarding the diffusion of acetaminophen out of the micropellets. On the other hand, release of CO_2 from $CaCO_3$ within the alginate matrix gave rise to a more porous matrix network in the internal gelation method resulting in greater loss of acetaminophen during the production process. The difference in drug encapsulation efficiencies of the micropellets, regardless of the size of micropellets, indicated differences in the gelation mechanisms of the

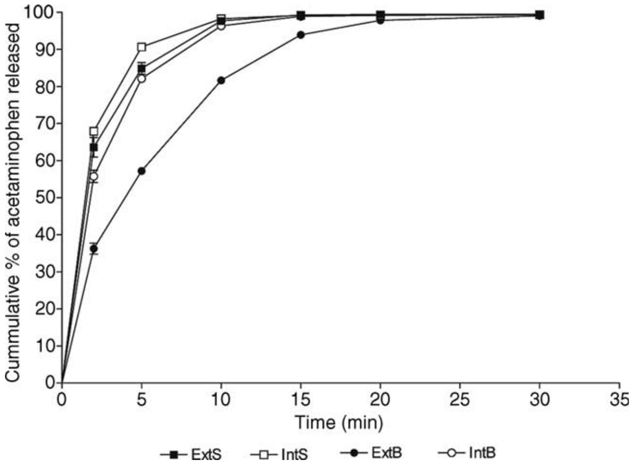


Fig. 6. Release profiles of acetaminophen from calcium alginate micropellets.

cross-linking methods used and the associated differences in the resultant alginate gel matrices.

Drug release from calcium alginate micropellets was rapid and complete drug release achieved within 30 min (Fig. 6). Nevertheless, the cross-linked alginate micropellets remained discrete, intact and formation of a gelatinous mass was not observed at the end of drug release studies. Faster rate of drug release was observed from micropellets of smaller size due to a larger surface area available for drug release. Internally cross-linked micropellets generally exhibited a faster rate of drug release than the externally cross-linked ones (Fig. 6). This observation showed an opposite trend to the results obtained from the film studies where a less permeable film was produced by the internal gelation method. This difference in the trends observed highlighted the significant effect of size and shape of the micropellets on the release profile of acetaminophen. Externally cross-linked micropellets being almost spherical would have a relatively smaller surface area to volume ratio as compared to the more irregularly shaped internally cross-linked micropellets. Film studies had showed that internally cross-linked films were thicker than the externally cross-linked films due to increased matrix volume as a result of CO_2 release. The volume of an equivalent internally cross-linked matrix was higher than that of the externally cross-linked matrix, thus giving rise to a comparatively greater surface area for drug release.

During drying, water was removed from the micropellets. The water-soluble acetaminophen would be drawn out and deposited on the surface of the micropellets together with the water during the drying process. The internally cross-linked micropellet with abundant microscopic cavities had a larger surface area for drug deposition, resulting in faster drug release from internally cross-linked micropellets. Thus, more rapid drug release was observed for internally cross-linked micropellets.

The properties of alginate matrix are influenced by the different gelation methods could have a pronounced effect on the drug content and release profile. Externally cross-linked calcium alginate appeared to be more suitable for drug and cell encapsulation capable of greater drug encapsulation efficiency and slower rate of drug release.

4. Conclusion

Cross-linked alginate matrices were produced by the external and internal gelation methods. In the external gelation method, the Ca^{2+} in the cross-linking solution would first cross-link the film surface drawing the polymer chains closer to form a less permeable surface to the diffusion of Ca^{2+}. This resulted in a matrix with a highly cross-linked surface and a less well cross-linked interior. Thus, in external gelation method, there was an optimal amount of cross-linker that could be used to produce matrix with the desired characteristics and further increase in amount of cross-linker employed did not exert significant changes in the matrix properties. In internal gelation method, the $CaCO_3$ was dispersed throughout the alginate matrix and release of CO_2 from the reaction between the acid and carbonate led to the formation of abundant cavities within the film. The difference in the matrix structure and properties observed in this study was due to the different gelation mechanisms involved in the cross-linking process as well as the amount of cross-linker employed. Comparable extent of cross-linkage was formed by the two gelation methods, as indicated by the insignificant difference in the Ca^{2+} contents between the externally and internally cross-linked films, but gave rise to different distribution of the cross-linkages throughout the matrix. This difference in structural aspect of the matrix also contributed to the differences in matrix properties observed.

Alginate matrix cross-linked by internal gelation exhibited greater surface roughness than externally cross-linked films with increased surface roughness observed when more calcium salt was used. Internal gelation method and larger amounts of $CaCO_3$ are not recommended for cross-linking of alginate matrix used in providing immunoprotection to cells in immunoisolation devices as the success rate of transplantation has been reported to be adversely affected by surface roughness of the matrix membrane. Externally cross-linked alginate matrix generally exhibited greater matrix strength than internally cross-linked matrix although the amount of cross-linker used could be adjusted to obtain films of comparable matrix strength for the two-gelation methods. Matrix flexibility could be modified by controlling the amount and size of $CaCO_3$ employed in internal gelation method, with little impact on the matrix strength. Both methods are potentially useful for use as a coat or delivery system. Internally cross-linked alginate films were less permeable to acetaminophen making it attractive for use as a polymer coat for retarding or controlling drug release. External gelation method is the preferred method for use in drugs and cell encapsulation, producing cross-linked alginate matrix capable of higher drug encapsulation efficiency and slower release of drugs.

The difference in gelation mechanisms between the two cross-linking methods had given rise to different matrix structures of the cross-linked alginate. The differences in structural aspects of the cross-linked matrix were responsible for the distinction in the properties of the matrices produced and hence, their suitability for use in coating applications as well as encapsulation of drugs and cells.

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