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Casein/gelatin beads: I. Cross-linker solution composition effect on cross-linking degree

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Abstract

The effect of the cross-linker solution composition (aqueous and organic ratio) on the cross-linking degree of hydrophilic casein/gelatin beads has been evaluated. Casein/gelatin beads with different radii have been prepared and treated over the same time with three different cross-linker solvent compositions containing *d,l*-glyceraldehyde at the same concentration. The cross-linking degree was studied not only comparing the results of swelling process and degradation rate, widely reported in literature as methods for the cross-linking degree evaluation, but also determining the solvent penetration rate and the *d,l*-glyceraldehyde reacting percentage. It has been observed, in fact, that the cross-linker solvent composition influences the penetration rate through the matrix of the cross-linker, thus controlling the homogeneity of the matrix cross-linking.

Keywords: Casein/gelatin beads; Cross-linking degree; *d,l*-Glyceraldehyde

1. Introduction

Protein microspheres have attracted considerable attention for several years as matrices for controlled drug delivery (Davis et al., 1984). In vivo studies show as microencapsulated drugs are more effective than parenteral solutions (Ratcliffe et al., 1984). Usually, microspheres are prepared by the emulsification solvent extraction method (Tanaka et al., 1963) and cross-linked either by adding an exact volume of the cross-linker solution in the preparative emulsion (Longo et al. 1982) or, after isolation, by suspending them in the cross-linker solution (Vandelli et al., 1995).

The rate and the kinetics of drug release depend on matrix characteristics, such as: (i) size and density of the microsphere; (ii) physicochemical properties of the drug; (iii) percentage loading and distribution of the drug; (iv) interactions between the drug and the matrix; (v) release environment; and (vi) extent and nature of cross-linking of the matrix (Tomlinson, 1983).

Glutaraldheyde is the aldehyde most frequently used for cross-linking proteins, such as albumin (Lee et al., 1981), gelatin (Tanaka et al., 1963; Hashida et al., 1979) and casein (Chien et al., 1987; Knepp et al., 1993; Latha and Jayakrishnan, 1994). Lysine is the amino acidic residue

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involved in the chemical cross-linking (Sokoloski and Royer, 1984) and the cross-linking extent is proportional to the concentration of glutaraldehyde (Hopwood et al., 1969).

Recently, in order to overcome the problem of cross-linker toxicity, *d*,*l*-glyceraldehyde has been proposed as a biocompatible cross-linker reagent (Vandelli et al., 1995).

The major parameters investigated affecting cross-linking are the cross-linking time (Vandelli et al., 1991) and the cross-linker percentage (Latha and Jayakrishnan, 1994), both if the cross-linker is introduced in the preparative emulsion or if the cross-linking is achieved post-synthesis. No studies, to our knowledge, have been reported on the cross-linker solvent aqueous/organic ratio. The importance of this factor is matched by the well-known matrix permeability correlation to both the diffusivity and the matrix/solution distribution coefficient of low molecular weight permeants (Baker and Lonsdale, 1974). Therefore it is reasonable to hypothesise that the permeability of the cross-linker can affect the cross-linking degree.

Up to now there is no absolute method to assess the cross-linking degree.

Many methods have been proposed and applied within several fields of research. For instance, solubility has been investigated to assess the cross-linking degree in such proteins as wool (Caldwell et al., 1966), turbidity measurement for albumin microspheres (Aguiar et al., 1967; Sheu et al., 1986; Rubino et al., 1993), and in vitro tests of swelling and degradation for albumin (Burger et al., 1985; Gupta et al., 1986) or casein (Jayakrishnan et al., 1994; Latha and Jayakrishnan, 1994) have also been widely used.

Table 1
Casein/gelatin ratios (p/p) in the preparative mixture; casein percentage in parentheses.

Batch name	Casein/gelatin ratio
Cagel (1:9)	1:9 (10% casein)
Cagel (1:3)	1:3 (25% casein)
Cagel (1:2)	1:2 (33% casein)
Cagel (1:1)	1:1 (50% casein)

In this paper besides swelling and degradation texts, the amount of *d*,*l*-glyceraldehyde reacting (Avigad, 1983) and the cross-linker solvent penetration rate are determined and applied to the cross-linking degree evaluation.

For this purpose, beads made of a mixture of casein and gelatin, have been prepared by the emulsification extraction method and cross-linked with *d*,*l*-glyceraldehyde after bead isolation.

2. Materials and methods

2.1. Materials

Soluble casein (light white; batch 9211260D, BDH Chemicals, Poole, England) and gelatin (225 Bloom) from calf skin (batch CW 05314MV, Aldrich, Milwaukee, WI, USA), *d*,*l*-glyceraldehyde (2,3-dihydroxypropionaldehyde, MW 90.08, Aldrich) mineral oil (Carlo Erba, Milan, Italy), methylene blue (Carlo Erba), trypsin (Sigma, St. Louis, MO, USA) and 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole reagent (AHMT, supplied under the label 'Purpald' was purchased from Aldrich) were used as received from the manufacturers.

2.2. Preparation of the uncross-linked beads

A 30% (w/v) solution in distilled water (20 ml) at 60°C containing mixtures of casein and gelatin (Table 1) was added dropwise to mineral oil (60 ml) preheated to 60°C.

The dispersion was stirred using a paddle stirrer holding constant dispersion time (10 min) and speed at 500 rpm.

As the emulsion was obtained, the temperature was lowered to 5°C by rapid cooling in an ice bath. Then, to solidify completely the droplets in the dispersed phase, 100 ml of previously cooled (5°C) acetone were added and the stirring was continued for 15 min.

The solidified systems were recovered by filtration and vacuum dried (10 mmHg) at room temperature.

2.3. Preparation of the cross-linked beads

The uncross-linked beads (1g) were cross-linked by continuous stirring (400 rpm) in solutions (20 ml) containing *d,l*-glyceraldehyde (0.5%, w/v).

The variables investigated are the cross-linking time (1, 6, 24 h at a fixed solution composition, mixture acetone–water, 3:1, v/v) and the cross-linker solution composition (either acetone–water mixtures, 3:1 or 1:1, v/v, or pure water solutions for a fixed cross-linking time interval 6 h).

The cross-linked samples were filtered, washed with 20 ml of cool acetone (5°C) and vacuum dried (10 mmHg) at room temperature.

After drying, the beads were sieved in order to obtain three different fractions: fraction A, diameter lower than 1 mm; fraction B, diameter between 1 and 2 mm, and fraction C, diameter between 2 and 3 mm.

2.4. Morphological characteristics

The morphological characteristics were determined by scanning electron microscopy (Model XL40, Philips, Eindhoven, The Netherlands).

The beads were put onto a double-sided tape on an aluminium stub, sputter coated with gold-palladium (CE-500, Polaron) in an argon atmosphere and examined at 15 kV.

2.5. Dynamic swelling process

The dynamic swelling process was carried out by allowing dry placebo uncross-linked and cross-linked systems to swell in deionized water at 25 ± 1°C, and examined using a microscopic procedure (Robert et al., 1987).

Briefly, six dry cross-linked and uncross-linked placebo systems were tested with an optical microscope (Carl Zeiss) equipped with an ocular micrometer (Galileo). Thus, the dry diameter (d_0) and the diameter changes (d_t) at fixed time intervals, after being suspended in deionized water, were measured until the systems achieved the equilibrium swollen value (d_∞).

Fraction A, for studying cross-linking time effect and fraction C for cross-linking solvent composition effect, were used.

2.6. Solvent penetration

The solvent penetration rate was determined with a method developed in our laboratory. The aim of this test is to indicate whether the solvent penetration is complete or a dry core remains, after the end of the cross-linking time. The test has been conducted only on beads with diameter $2 < D < 3$ mm, because the results of the penetration completion of smaller beads can be deducted comparing the measured penetration thickness and the bead diameter. Briefly, nine batches consisting of 20 uncross-linked beads each (average diameter, 2.6 mm) were suspended in the cross-linker solvent (three batches in acetone–water, 3:1, v/v, three batches in acetone–water, 1:1, v/v, and three batches in deionized water) containing methylene blue. After 1, 6 and 24 h the penetration was stopped by bead rapid filtering and freeze-drying (a batch for every cross-linker solvent system). The dried beads were cut by bistoury. In the cross-section the thickness of the blue layer at each penetration time was measured by the calliper (sensitivity 0.05 mm).

2.7. Quantitative determination of *d,l*-glyceraldehyde

The method originated from Avigad (1983) is a simple, sensitive spectrophotometric assay procedure for the determination of formaldehyde and short aliphatic hydroxyaldehyde at alkaline pH using an alkaline AHMT reagent.

The assay was performed by preparing the sample and the reagent blank solutions as follows: the sample solution is withdrawn from the cross-linker solution containing residual *d,l*-glyceraldehyde after cross-linking, filtered with a 0.45-μm Millipore filter and 1 ml diluted in a 250-ml flask with deionized water.

The reagent blank is obtained by diluting 750 μl of acetone (for samples cross-linked in the mixture water–acetone, 1:3) or 500 μl (for samples cross-linked in the mixture water–acetone, 1:1) in 250 ml of deionized water.

A total of 0.2 ml either of the sample or the blank was sampled into a 16×100-mm glass vial. After the addition of 0.3 ml of a 1% (w/v) AHMT

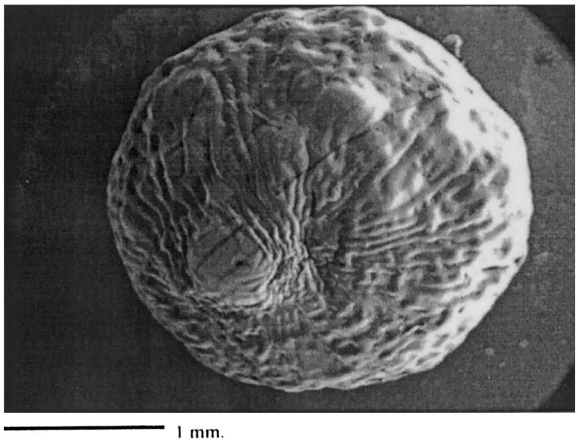

Fig. 1. Surface of Cagel bead.

Table 2
Solvent penetration rate (mm/h) through casein/gelatin beads (average diameter, 2.6 mm)[a]

Batch	A:W = 3:1 (v/v)		A:W = 1:1 (v/v)	
	Rate	*r*	Rate	*r*
Cagel (1:1)	0.026	0.995	0.182	0.984
Cagel (1:2)	0.012	0.999	0.042	0.999
Cagel (1:3)	0.009	0.985	0.035	0.966
Cagel (1:9)	0.003	0.997	0.042	0.999

[a] A:W, acetone–water; *r*, correlation coefficient.

in NaOH 1 N (freshly prepared for the day use) to each vial; the vials are incubated on a vibrating stirrer (15 Hz; Zx[3], Velp Scientifica, Cernate, Italy) at room temperature for 30 min. NaOH 1 N (0.5 ml) is then added to each tube. The thus developed chromogen is stable at room temperature for at least 30 min. This assay produces a linear response.

The *d*,*l*-glyceraldehyde is determined spectrophotometrically in the solution against the corresponding reagent blank at 540 nm.

The reacting *d*,*l*-glyceraldehyde was determined by the difference between the amount introduced for the cross-linking and the residual amount spectrophotometrically determined.

2.8. Biodegradation

The degradation study was conducted in a 0.01% (w/v) trypsin phosphate buffer solution (pH 7.4) at 37 ± 1°C. An exactly weighed amount of systems (20 mg) was suspended in the buffer solution and the time of complete degradation, determined by microscopic observation, was registered. The rate of complete degradation (mg/min) was mathematically calculated.

3. Results and discussion

3.1. Bead morphology

As pointed out by the SEM photomicrographs, the uncross-linked systems showed a nearly spherical geometry and a rough surface without any apparent pores, regardless of the casein/gelatin ratio (Fig. 1).

The bead cross-section presents a non-compact internal structure of the matrix in which randomly distributed large holes and cavities appear.

The number of cavities in the structure was affected by the casein percentage. This observation can be related to the incorporation of air bubbles during the aqueous solution preparation process because of the casein properties (Bulgarelli, 1998).

3.2. Cross-linking degree evaluation

3.2.1. Cross-linker solvent penetration rate

The thickness of the penetration layer and the corresponding penetration time expressed as penetration rates are presented in Table 2. The solvent penetration increases as the increase of both casein percentage in the matrix and water percentage in the cross-linker solution (Table 2).

The values presented show that the casein/gelatin matrix can be penetrated by methylene blue in all these solvents. As during the penetration uncross-linked beads swell and the swelling continues after its completion, the penetration thickness has to be compared with the swollen diameter referred to the same time interval. The beads with $D < 1$ mm (average dry bead diameter, 0.6 mm) can be considered completely penetrated after 1 h (swollen bead diameter, 1 mm), even in acetone–water (3:1, v/v); on the contrary, beads with $2 < D < 3$mm (average dry bead diameter, 2.6 mm) are completely penetrated only in water

Table 3
Thickness of the penetration layer (mm) of the aqueous solution containing methylene blue through casein/gelatin beads (average dry bead diameter, 2.6 mm; 1 h average swollen bead diameter, 2.8 mm; 6 h average swollen bead diameter, 3 mm; 24 h average swollen bead diameter, 4.2 mm); standard deviation in parentheses

Bach	Penetration layer		
	1 h	6 h[a]	24 h[a]
Cagel (1:1)	0.65 (± 0.22)	1.65 (± 0.14)	2.13 (± 0.26)
Cagel (1:2)	0.67 (± 0.09)	1.11 (± 0.28)	2.05 (± 0.20)
Cagel (1:3)	0.70 (± 0.20)	1.20 (± 0.14)	2.03 (± 0.26)
Cagel (1:9)	0.55 (± 0.11)	1.44 (± 0.14)	2.03 (± 0.23)

[a] Tests indicate that the penetration in the bead is complete; in fact, the measured thickness correspond to the average radium of the examined beads.

solution after 6 and 24 h (Table 3).

Then at a predetermined time (for example 1 h) the penetration thickness could be lower than the bead radius preventing a homogeneous cross-linking.

3.2.2. Dynamic swelling

The diameter of uncross-linked beads showed a maximum swollen value at $t = 1$ h and then approached gradually to a lower equilibrium value at $t = 4$ h (Table 4). This behaviour is probably due to the combination of both phenomena of matrix swelling and osmotic effect of uncross-linked protein diffusing through the matrix. Thus, the diameter first increases when water penetration is the predominant phenomenon, and then decreases, when diffusion predominates.

This behaviour disappears after cross-linking, in fact the diameter increases monotonically towards the equilibrium swollen value. The beads of fraction A cross-linked in acetone–water mixture (3:1, v/v) (Table 4) do not show remarkable differences between 6 and 24 h, but the equilibrium swollen value of 1 h cross-linked beads is significantly higher. These differences in swelling properties can be related to the cross-linking degree increase with cross-linking time.

The effect of casein percentage on matrix swelling seems to be the opposite: in fact, the equilibrium swollen values of 1 h cross-linked batches increase with the casein percentage (the swollen value of Cagel (1:2) do not appear to be inconsistent adding the standard deviation), whereas those of 6- and 24-h cross-linked batches decrease. Even if after 1 h the solvent penetration is complete in beads with $D < 1$ mm, we can suppose that the cross-linking is incomplete and that a partial osmotic effect of uncross-linked proteins diffusing through the matrix can drive the swelling behaviour, as hypothesised for uncross-linked systems.

The beads of fraction C, cross-linked for the same time (6 h) with the same cross-linker concentration, although with different cross-linker solvent compositions, showed an equilibrium swollen values decrease as the water percentage in the cross-linker solution increased in turn (Table 5). This strongly indicates that the cross-linking reaction was more complete in the solvent system containing 50% or more water.

Table 4
Parameters of the dynamic swelling process (D_∞/D_o) in water at 25 ± 1°C of uncross-linked and cross-linked beads with acetone–water mixture (3:1, v/v) containing 0.5% (w/v) *d,l*-glyceraldehyde, according to the cross-linking time; standard deviation in parentheses[a]

Batch	Uncross-linked		Cross-linked in A:W (3:1, v/v)		
	D_∞/D_o	D_{max}/D_o	1 h (D_∞/D_o)	6 h (D_∞/D_o)	24 h (D_∞/D_o)
Cagel (1:9)	3.00 (± 0.21)	/	2.10 (± 0.14)	1.76 (± 0.11)	1.68 (± 0.07)
Cagel (1:3)	3.15 (± 0.37)	2.75 (± 0.20)	2.17 (± 0.22)	1.75 (± 0.11)	1.68 (± 0.10)
Cagel (1:2)	3.30 (± 0.25)	3.50 (± 0.21)	1.90 (± 0.10)	1.76 (± 0.15)	1.60 (± 0.11)
Cagel (1:1)	3.60 (± 0.21)	3.75 (± 0.01)	2.30 (± 0.20)	1.56 (± 0.07)	1.55 (± 0.08)

[a] D_o, average dry bead diameter (fraction A = 0.6 mm); D_{max}, maximum swollen value (1 h); D_∞, equilibrium swollen value (4 h).

Table 5
Parameters of the dynamic swelling process (D_∞/D_o) in water at 25 ± 1°C of beads cross-linked with 0.5% (w/v) *d,l*-glyceraldehyde in the different cross-linker solvent system; standard deviation in parentheses[a]

Batch	Cross-linked (6 h)		
	A:W = 3:1 (v/v) (D_∞/D_o)	A:W = 1:1 (v/v) (D_∞/D_o)	Water (D_∞/D_o)
Cagel (1:9)	1.61 (± 0.03)	1.50 (± 0.04)	1.34 (± 0.01)
Cagel (1:3)	1.87 (± 0.20)	1.55 (± 0.07)	1.56 (± 0.05)
Cagel (1:2)	1.91 (± 0.18)	1.60 (± 0.03)	1.68 (± 0.02)
Cagel (1:1)	2.12 (± 0.10)	1.79 (± 0.06)	1.84 (± 0.08)

[a] D_o, average dry bead diameter (fraction C = 2.6 mm); D_∞, equilibrium swollen value (4 h).

3.2.3. Glyceraldehyde reacting with proteins

The amount of *d,l*-glyceraldehyde reacting with proteins increases with the cross-linking time; the reaction rate is particularly fast during the first hour of cross-linking, when most of the surface amino groups are free (Fig. 2). This may have caused a cross-linking degree increase with time in fraction A, because beads are completely penetrated after as short as 1 h by all the solvents used, as previously presented.

The bead surface area decrease makes the *d,l*-glyceraldehyde percentage decrease in turn (Fig. 3). This could be due to the lesser amount of reaction sites available, because while the same cross-linking time is maintained, the solvent penetration thickness remains unchanged, the solvent penetration rate being the same. In this way, the volume of matrix being in contact with the cross-linker solvent decreases as the batch diameter increases. Thus, in large size matrices the cross-linking may involve only a superficial layer and the inner core does not appear to be cross-linked.

As the penetration thickness increases along with both the casein percentage in the matrix and the water percentage in the cross-linker solvent (Table 3), so does the actual percentage of *d,l*-glyceraldehyde reacting with proteins.

Fig. 4 shows the *d,l*-glyceraldehyde increase with the aqueous percentage in the cross-linker solution.

Figs. 2–4 show how the reacting *d,l*-glyceraldehyde increases with the casein percentage in the matrix.

This is in accordance with the results of the study on the solvent penetration.

3.2.4. Biodegradation

The cross-linking time increase leads to a decreased degradation rate (Fig. 5); the same effect is observed for bead biodegradation when the water percentage in the cross-linker solution composition increases (Fig. 6). This behaviour shows how both the water percentage in the cross-linker solvent and the cross-linking time increase can modify the cross-linking degree. Both variables, in fact, improve the solvent penetration in the matrix and increase the reacting *d,l*-glyceraldehyde.

In both Figs. 5 and 6 the biodegradation rate and the casein percentage in the matrix show a downward trend, whatever cross-linking conditions are applied. Casein percentage improves the cross-linker penetration through the matrix and the reacting *d,l*-glyceraldehyde.

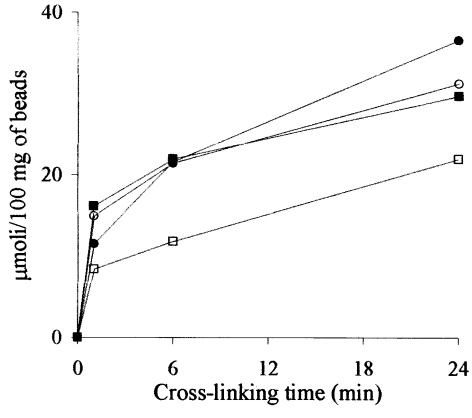

Fig. 2. Effect of the cross-linking time on reacting *d,l*-glyceraldehyde (µmol/100 mg) with casein/gelatin beads (fraction A). Lines are drawn for clarity, but do not indicate curve fitting. Key: (□) Cagel (1:9); (■) Cagel (1:3); (○) Cagel (1:2); (●) Cagel (1:1).

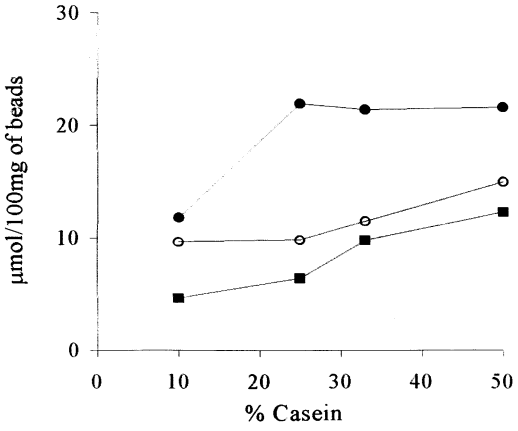

Fig. 3. Effect of the bead surface area on *d,l*-glyceraldehyde (µmol/100 mg), that reacts over 6 h. Lines are drawn for clarity, but do not indicate curve fitting. Key: (■) $D > 2$ mm; (○) 1 mm $< D <$ 2 mm; (●) D < 1 mm.

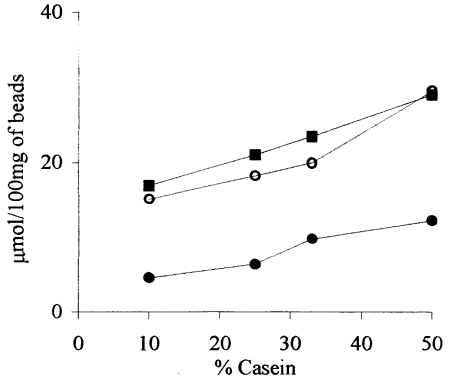

Fig. 4. Effect of the cross-linker solution composition on *d,l*-glyceraldehyde percentage that reacts with beads (fraction C) over 6 h. Lines are drawn for clarity, but do not indicate curve fitting. Key: (■) water; (○) A:W = 1:1; (●) A:W = 1:3.

Both the increase in water fraction in the cross-linker solution composition and the casein percentage in the matrix result in the increase of cross-linking degree.

4. Conclusion

It is confirmed that a hydrophilic matrix can also be penetrated by an organic solvent system; however, in order to obtain a homogeneously cross-linked matrix, the cross-linking parameters (i.e. the cross-linker solvent composition, the cross-linker concentration and the cross-linking time) should be carefully selected.

As a matter of fact, the same cross-linking time and cross-linker concentration may lead to differ-

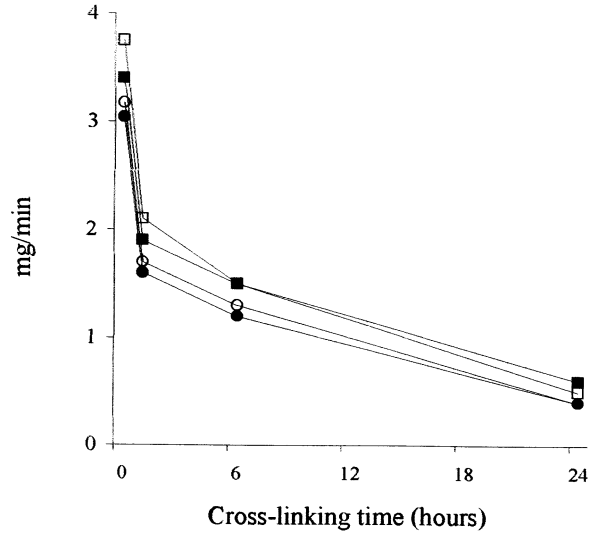

Fig. 5. Degradation rate (mg/min) of beads cross-linked in acetone:water mixture 3:1 (v/v) as a function of the cross-linking time. Lines are drawn for clarity, but do not indicate curve fitting. Key: (□) Cagel (1:9); (■)Cagel (1:3); (○) Cagel (1:2); (●) Cagel (1:1).

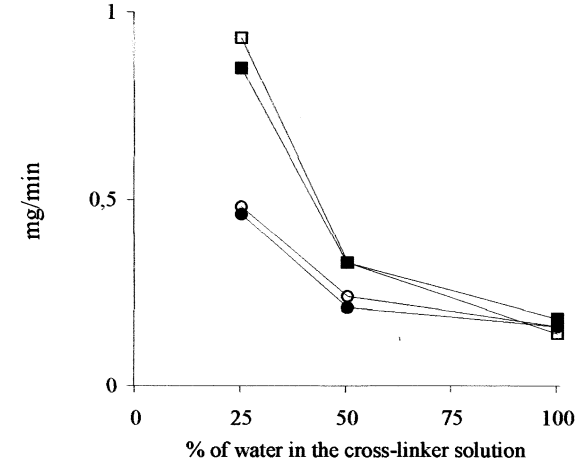

Fig. 6. Degradation rate (mg/min) of cross-linked beads (fraction C) as a function of the cross-linker solution composition. Lines are drawn for clarity, but do not indicate curve fitting. Key: (□) Cagel (1:9); (■) Cagel (1:3); (○) Cagel (1:2); (●) Cagel (1:1).

ent matrix characteristics. Large size beads, partially cross-linked, act as reservoir devices, containing an uncross-linked core in a cross-linked coat; on the contrary, small size beads, homogeneously cross-linked, act as monolithic devices.

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