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Hyaluronan networking via Ugi's condensation using lysine as cross-linker diamine

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Abstract

A new type of hyaluronan based polymeric network has been prepared applying the well known cross-linking processes based on aqueous Ugi condensation reactions. In this study lysine has been used as a cross-linking agent.

The structural and physico-chemical properties of the resulting hydrogels have been studied using solid state NMR spectroscopy and measurements of swelling in water and in aqueous NaCl solution.

Keywords: Hyaluronan; Hydrogels; Ugi's reaction; Solid state NMR

1. Introduction

Hydrophilic polymeric networks (hydrogels) (Rosiak & Yoshii, 1999) can fulfill the necessary requirements for their application in the biomedical sector such as controlled drug delivery (Peppas, Bures, Leobandung, & Ichikawa, 2000), efficient cell scaffolding (Kuo & Ma, 2001) and tissue engineering (Lee & Mooney, 2001).

To this end hydrogels composed of biocompatible polymers are conveniently selected; in addition the synthesis of the polymeric networks must be performed using procedures which do not alter the basic properties of the original material. Moreover, reaction with good yields and fast rates are desirable. Hyaluronan (HA, Fig. 1) (Meyer & Palmer, 1934; Laurent, 1998) is a versatile biopolymer which can be cross-linked in a variety of ways (Crescenzi, Francescangeli, Renier, & Bellini, 2002; Lapcik & Lapcik, 1998) taking advantage of the carboxylic functionalities present in the structure (Bulpitt & Aeschlimann, 1999). In previous papers, we reported on the use of the Ugi multicomponent condensation (Ugi, Lohberger, & Karl, 1991) for the synthesis of polymeric networks: this four-components aqueos condensation leads essentially to a single product with yields up to 85–90%.

In this paper, we report on the synthesis and on the preliminary characterization of hydrogels prepared using HA and lysine as a cross-linking reagent. This synthesis allows us to obtain HA hydrogels with suitable physical properties. These hydrogels have been characterized using solid state NMR spectroscopy and studying their swelling properties. The cross-linking degree has been evaluated by changing the molar ratio of the cross-linker diamine (lysine).

2. Materials and methods

2.1. Materials

A hyaluronic Acid (HA) sample, from Fidia Advanced Biopolymers (FAB Srl, Abano Terme, Padua, Italy) with $M_\eta = 65$ kDa, has been used throughout. Lysine ethyl ester dihydrochloride salt was supplied by Fluka (Milan, Italy);

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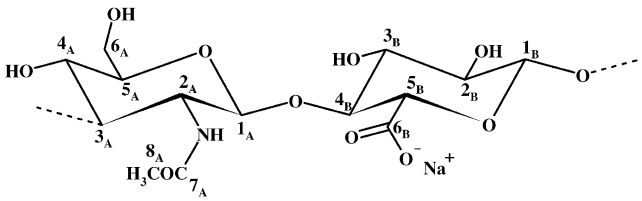


Fig. 1. Structure of HA repeating unit.

all other chemicals were reagent grade and have been used without further purification.

2.2. Hydrogel synthesis

In a typical experiment, 200 mg of HA are dissolved in 2.5 ml of distilled water (polymer concentration 8% w/V) to obtain a viscous solution. The solution is slightly acidified with two drops of HCl 2 M to obtain a pH of 4. The necessary amount (x) of lysine ethyl ester is then added to eventually obtain a given theoretical cross-linking degree, TCD, defined as the ratio between the number of moles of diamine and the number of moles of carboxylic groups of HA, i.e.:

$$\mathrm{TCD} = \frac{x/247}{200/400}\cdot 100$$

where 247 is the molecular weight of the lysine ethyl ester dihydrochloride salt and where 400 is the molecular weight of hyaluronic acid (sodium salt) repeating unit.

Finally, 100 μl of formaldehyde (40% w/V aqueous solution) and 100 μl of pure cyclohexylisocyanide are added to the solution after a few minutes. The solution is kept under stirring at room temperature for about 5 min, until it becomes very viscous. Then the solution is allowed to cure overnight. The gels obtained are dialyzed against 0.1 M Na_2CO_3 aqueous solution (pH ≅ 10) for 24 h, and afterwards against distilled water for 10 days.

Six different hydrogel samples have been synthesized with a TCD ranging from 6 to 20%.

2.3. NMR data

2.3.1. NMR in solution

Hyaluronic acid was solubilized in D_2O. 1D and 2D NMR spectra were performed at 300 K on a Bruker Avance-600 NMR spectrometer operating at 600.13 MHz with a Bruker z-gradient probehead. In the ^1H spectrum, the residual HOD signal was suppressed using a soft signal presaturation. ^1H and ^{13}C assignments were reported in ppm with respect to 2,2 dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as internal standard and were obtained using (Braun, Kalinowski, & Berger, 1998) COSY, TOCSY and HMQC experiments. All these 2D experiments have been acquired using a time domain of 1024 data points in F2 and 512 data points in F1, a relaxation delay of 2 s. The number of scans has been optimized in order to have a good signal/noise ratio. TOCSYgs experiment has been acquired with a mixing time of 80 ms. The HMQCgs experiment has been acquired using a coupling constant $^1J_{C-H} = 150$ Hz.

2.3.2. ^{13}C CP-MAS NMR

Solid state NMR measurements were performed both on a hyaluronic acid sample and on a lysine-based-HA dry network with a TCD equal to 12%. Samples were finely cut and packed into 4 mm zirconia rotors and sealed with Kel-F caps.

Solid state ^{13}C CP-MAS NMR spectra were performed at 50.13 MHz on a Bruker AMX-200 spectrometer. The spin rate was 8.5 KHz, the π/2 pulse width was 4 μs and the recycling time was 3 s.

Spectra were obtained using 512 data points in the time domain, zero filled and Fourier transformed to a size of 1024 data points.

The chemical shift was externally referred to tetramethylsilane.

The Cross-Polarization was performed applying the variable spin-lock sequence (Cook, Langford, Yamdagni, & Preston, 1996; Metz & Smith, 1994) known as Ramp-CP-MAS. The ramp can be applied on the X or on the ^1H channel. The ramp causes one of the channels to be spin-locked slightly off the Hartmann–Hahn condition, except in the middle of the ramp where the spin lock is exactly matched in the Hartmann–Hahn condition. This method allows us to overcome the motional modulation of the carbon and proton coupling caused by spinning the sample at high rate.

The ramp was applied on the ^1H channel and, during the contact time τ, the amplitude of the spin lock increases from 50 up to 100% of its maximum value.

2.4. Swelling measurements

The equilibrium water swelling (S_W) of the hydrogels is defined as the ratio between the weight of the networks in the swollen state (W_S), dialyzed for 10 days against distilled water, and the weight of the freeze-dried network (W_D):

$$S_W = \frac{W_S}{W_D}$$

We have also measured the re-swelling (RS_W) of the hydrogels, defined as the ratio between the weight of the network swollen in water (W_{RS}) following a freeze-drying treatment and after two days of water swelling, and the network weight in the freeze-dried state (W_D):

$$RS_W = \frac{W_{RS}}{W_D}$$

Hydrogel swelling in solutions of different ionic strengths (from 0.005 to 0.5 M NaCl), has been determined knowing the hydrogel weight in water (W_S) and measuring its weight

(W_I) after two days of dialysis against a solution of ionic strength I :

$$S_I = \frac{W_I}{W_S} \cdot S_W$$

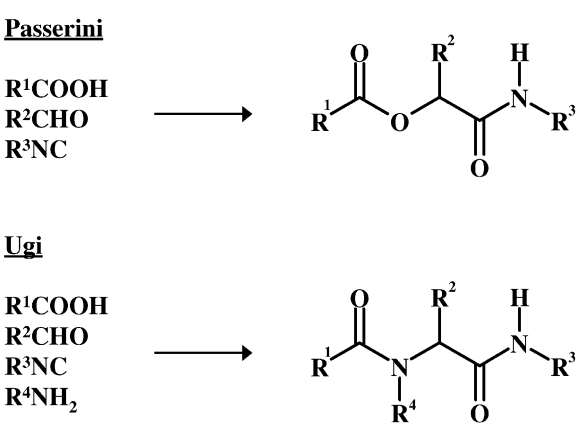
3. Results and discussion

3.1. Cross-linking process

In previous papers (de Nooy, Capitani, Masci, & Crescenzi, 2000; de Nooy, Masci, & Crescenzi, 1999), we have reported on the synthesis of new hydrogels starting from a few carboxylated polysaccharides, e.g. carboxymethylcellulose (CMC), cross-linked with 1,5-diaminopentane by means of the four component Ugi's condensation reaction (Scheme 1). The NMR analysis showed that the yield of the Ugi's reaction for this system was about 80%.

In order to obtain new HA-based networks, different cross-linking agents such as 1,5-diaminopentane, 1,4-diaminobutane and lysine were used. However, the hydrogels obtained from 1,5-diaminopentane and 1,4-diaminobutane were opaque, fragile and difficult to handle.

On the contrary, using lysine, HA-based hydrogels with suitable physical characteristic and good swelling properties were obtained. In the experiment, we did not use lysine but lysine ethyl ester to avoid interference of lysine carboxylic group in Ugi's reactions between amine groups and carboxylic groups of HA. After hydrolysis of the ester moieties, networks of univocal structure were obtained, as confirmed by NMR measurements (see Section 3.2). To achieve this end, a dialysis of the hydrogel samples against an alkaline solution was performed. This dialysis also allows us to hydrolyze the ester bonds formed along the hyaluronic acid backbone as a result of the concomitant Passerini's reaction (Ugi, Lohberger & Karl, 1991) (Scheme 1). This reaction involves only three components instead of four in the Ugi reaction, is kinetically favoured. In this way, lysine-HA based networks with TCD ranging from 6 to 20% were obtained. The hydrogel with a TCD equal to 12% has been characterized by ^{13}C solid state NMR spectroscopy.



Scheme 1. Representative Passerini and Ugi's reaction products.

3.2. ^{13}C CP-MAS NMR analysis

The ^{13}C CP MAS NMR spectrum of the HA sample is shown in Fig. 2(a), along with the resonance assignment. The assignment in the solid state has been obtained by comparing it with the assignment in aqueous solution reported in Table 1 and it is in agreement with the assignment reported in the literature (Poujani, Kuo, Harbison, Prestwitch 1992).

The methyl carbon resonance is at 24.7 ppm; the resonance due to carbon 2_A bearing the $NHCOCH_3$ group is at 56.4 ppm whereas the resonance due to the methylene carbon 6_A is at 62.8 ppm. Signals due to carbon 4_A and 3_A appear as shoulders at about 70 and 84 ppm, respectively. The resonance centered at 103 ppm is due to the anomeric carbons 1_A and 1_B whereas the resonance due to 7_A is observed at 178 ppm. The very intense resonance observed at about 76 ppm is due to all other methine carbons.

The ^{13}C CP-MAS spectrum of the lysine-based networks (TCD = 12%) is shown in Fig. 2(b).

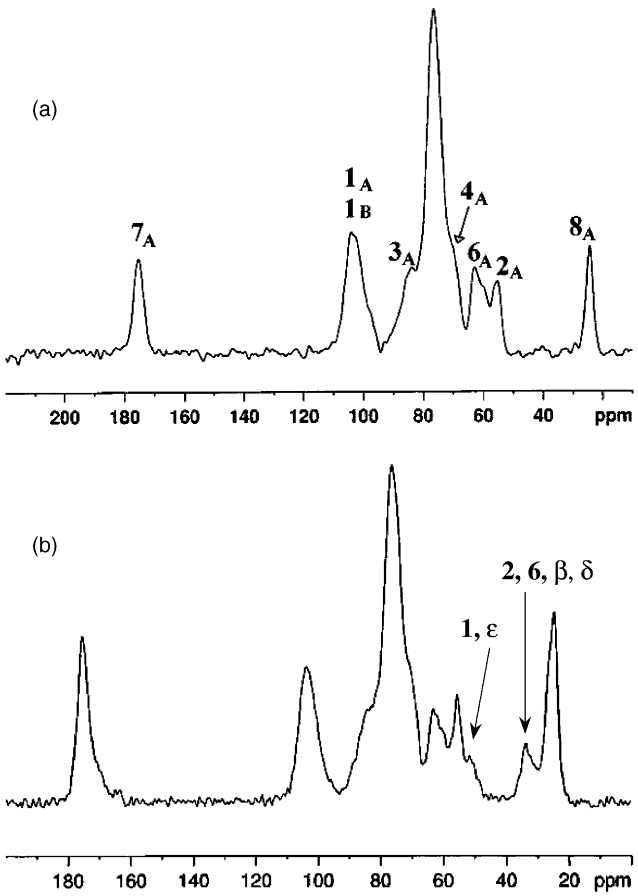


Fig. 2. (a) ^{13}C CP-MAS NMR spectrum of hyaluronic acid, along with the resonances assignment. (b) ^{13}C CP-MAS spectrum of a lysine-HA based networks with TCD equal to 12%.

Table 1
^{1}H and ^{13}C assignments of HA in D_2O at 300 K. Chemical shifts are reported in ppm with respect to DSS used as internal standard

	^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)
1_A	4.54	102.78	1_B	4.46	105.34
2_A	3.83	56.44	2_B	3.34	74.67
3_A	3.70	84.88	3_B	3.57	75.74
4_A	3.51	70.66	4_B	3.73	82.31
5_A	3.48	77.65	5_B	3.74	78.29
6_A	3.75	62.81			
	3.91				
8_A	2.01	24.72			

Besides the resonances due to HA, few other resonances are observed due to carbon atoms belonging to the chemical bridges between hyaluronic acid chains (Fig. 3).

The broad signal resonating at 33.6 ppm is due (de Nooy, Capitani, Masci & Crescenzi, 2000; Crescenzi, Francescangeli, Segre, Capitani, Mannina & Renier, 2002) to methylene carbons 2 and 6 of the cyclohexyl rings and also to the methylene carbons β and δ.

The broad signal at about 50 ppm is due to methine carbon 1 of the cyclohexyl rings (de Nooy et al., 2000; Crescenzi et al., 2002) and to methylene carbon ϵ. Resonances due to carbonyl carbons appear as a shoulder centered at about 170 ppm.

The area of the resonance due to methylene carbons 2, 6, β and δ and the area of the resonance due to the anomeric carbons, can be used to evaluate the degree of cross-linking.

However, since ^{13}C CP-MAS NMR technique is not quantitative, caution has to be used even for a semi-quantitative evaluation of the intensity and/or area of resonances in a ^{13}CP-MAS spectrum.

Since the Cross-Polarization process depends on the dipolar interaction between protons and carbons, the rate of the process is strongly dependent on the number of abundant spins I close to dilute spin S and on their distance from S. Thus, the Cross-Polarization technique is not quantitative.

In order to obtain semi-quantitative information, the cross-polarization dynamic must be carefully investigated (Stejskal & Memory, 1994).

In homogenous systems, the study of the cross-polarization dynamic allows to obtain the 'true' intensity and/or area of carbon resonances. For simple cases the kinetics of the cross-polarization dynamic can be described by the equation (Harris, 1990):

$$S(\tau) = S_0\left(\frac{1}{1-\frac{T_{IS}}{T_{1\rho}^{1H}}+\frac{T_{IS}}{T_{1\rho}^{13C}}}\right)\times\left[1-\exp\frac{-\left(1-\frac{T_{IS}}{T_{1\rho}^{1H}}+\frac{T_{IS}}{T_{1\rho}^{13C}}\right)\tau}{T_{IS}}\right]\times\exp\left(\frac{-\tau}{T_{1\rho}^{1H}}\right) \quad (1)$$

where S_0 is the area of the resonance at $\tau = 0$; $T_{1\rho}^{1H}$ and $T_{1\rho}^{13C}$ are the proton and the carbon spin–lattice relaxation times in the rotating frame; T_{IS} is the cross-relaxation time between protons and carbons.

CP-MAS spectra were run at different contact times τ_i with τ_i ranging from 0.05 to 8 ms.

The area of the resonances centred at 103 ppm (S1A) and 33.6 ppm (S1M), respectively, was reported against the contact time (Fig. 4).

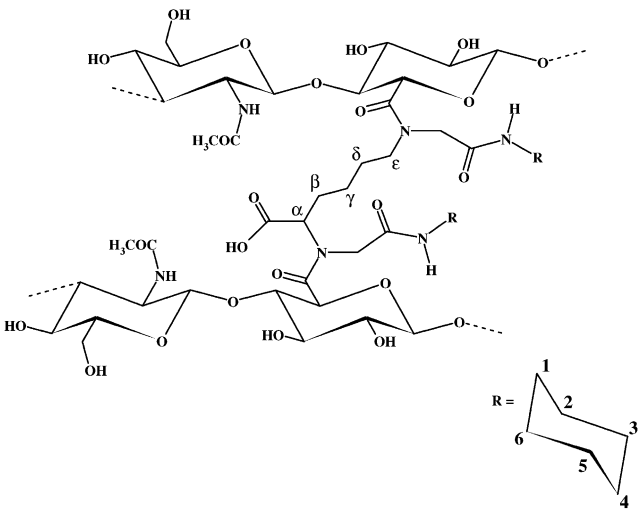


Fig. 3. Proposed structure of the network formed by HA via a Ugi's condensation with aqueous formaldehyde, cyclohexylisocyanide and lysine with carbon atoms assignment.

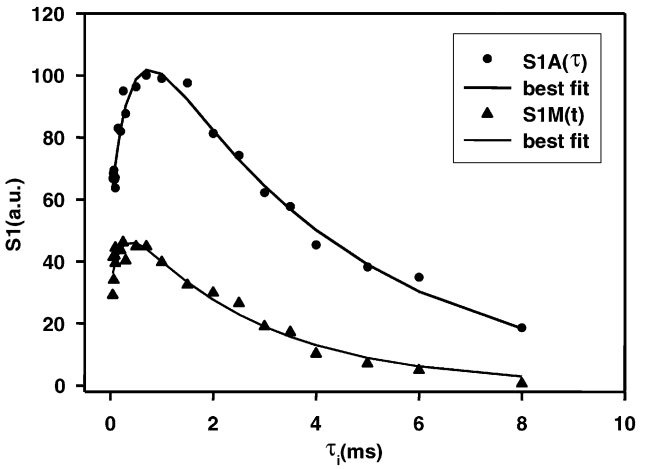


Fig. 4. Correlation between the area of the resonance due to the anomeric carbons (●) and the area of the resonance due to the methylene carbons 2, 6, β and δ (▲) and the contact time (networks with TCD equal to 12%). Line through experimental points are obtained applying a best fit procedure to Eq. (1).

Fitting these experimental data to Eq. (1) $T_{1\rho}^{1H}$ and S_0 are obtained:

$S_0(\text{S1A}) = 137 \pm 8 \qquad S_0(\text{S1M}) = 59 \pm 5$

$T_{1\rho}^{1H}(\text{S1A}) = 4.0 \pm 0.3 \text{ ms} \qquad T_{1\rho}^{1H}(\text{S1M}) = 3.2 \pm 0.5 \text{ ms}$

It is worth to note that the selected resonances show the same $T_{1\rho}^{1H}$ values within experimental errors. Thus the proton spin-diffusion is active to average the $T_{1\rho}^{1H}$ values, i.e. the sample can be considered an homogeneous system.

S_0 values can be used to obtain a quantitative evaluation of the real cross-linking degree (RCD):

$$\text{RCD(S1)} = [(\text{S1M/nc})/(\text{S1A})] \times 100 = (8 \pm 2)\%$$

where nc = 6 is the number of carbon atoms which contribute to the intensity and/or the area of the S1M resonance.

Ugi's condensation performed with the system HA/lysine/formaldehyde/cyclohexylisocyanide exhibits a yield, considering the experimental errors, around 75%, very close to the value obtained with other polysaccharides (de Nooy et al., 2000).

3.3. Swelling data

In Fig. 5 the swelling data (water, 25 °C) as a function of TCD for lysine-based HA hydrogels are reported. As expected an exponential trend is observed; note that in a polyelectrolyte-based hydrogel the increase of the concentration of the crosslinker makes the net's mesh closer and furthermore subtracts ionized groups. Moreover, a high TCD causes a high concentration of highly hydrophobic groups (cyclohexylisocyanide). As a consequence a decrease of the total amount of water absorbed by the gel occurs.

In Fig. 6 the *log* S_w vs. log TCD plot is shown. According to the equations:

$$\log S_W = A + B \cdot \log \text{TCD} \quad (2)$$

$$S_{\text{lysine}} \cdot \text{TCD}^{\alpha} = k \quad (3)$$

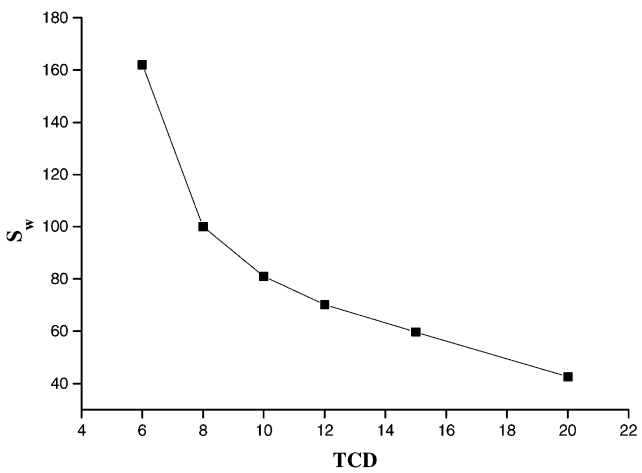


Fig. 5. Correlation between the swelling in water S_W at 25 °C, and TCD.

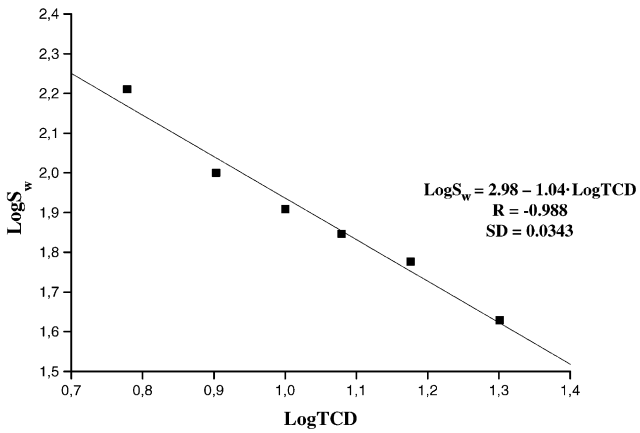


Fig. 6. log S_W, vs. log *TCD* in water at 25 °C for lysine based hydrogels.

the parameters α and k are obtained:

$\alpha = 1.04 \qquad k = 957$

It is worth to note that Eq. (3) can be interpreted as a kind of 'state function' which relates the 'volume' (S) of the hydrogel to its 'pressure' (TCD) in water at 25 °C.

We have also determined the 're-swelling' capacity of the networks, i.e. their ability to absorb water after a freeze-drying treatment. In Fig. 7, we compare the swelling S_w and the re-swelling RS_w data. Note that in all cases the RS_w value is slightly lower than the corresponding S_w value, hence the freeze-drying process slightly alterates the network structure. Due to the high air content, the ensuing samples are opaque, filamentous and float.

3.4. Influence of ionic strength

We have studied the influence of ionic strength, I, on the swelling of the lysine-based gel (TCD = 8%). As expected for ionic hydrogels, the increase of the ionic strength causes a partial screening of the fixed network charges lessening electrostatic repulsions and promoting a network shrinkage, i.e. a swelling decrease. Plotting log S_I vs. *log I* (Fig. 8) and

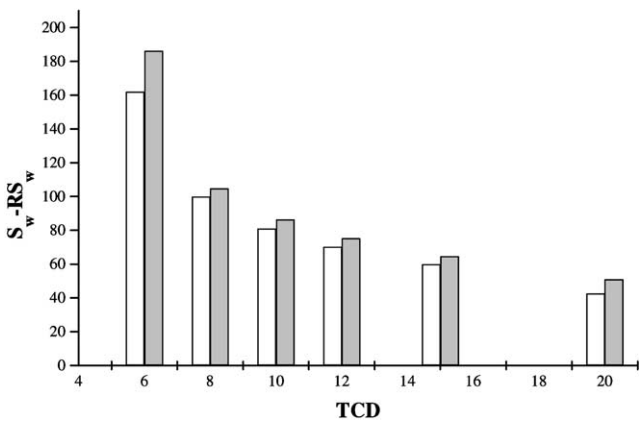


Fig. 7. Comparison between swelling (S_W) (■) and re-swelling (RS_W) (□) in water, at 25 °C, for lysine based hydrogels.

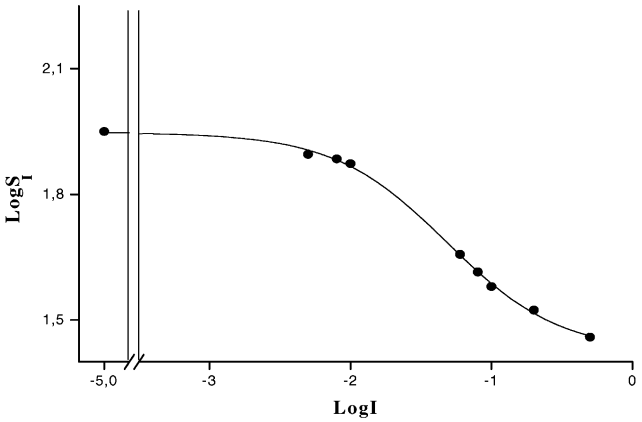


Fig. 8. $\log S_I$ vs. $\log I$ at 25 °C, for a lysine based hydrogel, with TCD = 8%.

imposing $I = 0.00005$ M for the water, we obtain a sigmoidal trend, typical of many hydrogel categories, foreseable in terms of non-gaussian statistics of the chains (Schröder & Oppermann, 1996).

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