Hydrogels of Enzymatically Modified Dextrin

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Dextrin is a glucose-containing saccharide polymer linked by α-(1→4) D-glucose units and is produced by partial hydrolysis of starch. Hydrolysis can be accomplished by the use of acid, enzymes, or by a combination of both. In this work, the *Bacillus subtilis* protease Proleather FG-F catalyzed the transesterification of the soluble polysaccharide with vinyl acrylate (VA), using anhydrous dimethylsulfoxide (DMSO) as reaction medium. Different degrees of substitution (DS, defined as the amount of acrylate groups per 100 glucopyranose residues) ranging from 11 to 19 % were achieved by controlling the molar ratio of VA to dextrin. Dextrin derivatized with VA (dextrin-VA) was characterized by gel permeation chromatography. Gels were obtained by free radical polymerization of aqueous solutions of dextrin-VA with different degrees of substitution and monomer concentration. Swelling experiments have been performed in order to determine the gel pore sizes. Specific physical properties, as degradation behaviour were also evaluated. These hydrogels are being developed as scaffold materials for bioactive molecule and cell delivery, tissue engineering and a variety of other biomedical applications

Keywords**:** Dextrin, Enzyme, Transesterification, Vinyl Acrylate (VA), Hydrogels, Scaffold Materials

Introduction

Hydrogels are a class of three-dimensional, highly hydrated polymeric networks (Peppas *et al.*, 2000). They are composed of hydrophilic polymer chains, which can be either synthetic or natural. The structural integrity of hydrogels depends on crosslinks formed between polymer chains by chemical bonds and physical interactions. Hydrogels are providing new opportunities for the development of a variety of medical applications (Peppas *et al*., 1996), including drug delivery systems (McCulloch and Shalaby, 1996), scaffold materials to organize cells into a three-dimensional architecture, tissue replacements (Kane *et al*., 1996), wound dressings (Ishihara *et al*., 2001; Chen *et al*., 2003) and immobilization of proteins, cells, among others.

Since the clinical use of hydrogels is increasing, considerable efforts have been made in order to develop new hydrogels from a variety of synthetic and natural materials (Drury and Mooney, 2003). The characterization of its structure and physical properties has also been achieved.

In this work we report a procedure to prepare dextrin hydrogels. Dextrin is a glucose-containing saccharide polymer linked by α-(1→4) D-glucose units, having the same general formula as starch, but smaller and less complex. This polysaccharide is produced by partial hydrolysis of starch, which can be accomplished by the use of acid, enzymes, or a combination of both. Dextrin is a widely used material with a variety of applications, from adhesives to food industry and textiles. Moreover, because of the biocompatibility and degradability (Wong and Mooney, 1997; Marques *et al*., 2002) of starch-based materials, a dextrin-containing hydrogel might be specially suitable for biomedical applications.

To prepare the hydrogels, dextrin was modified with vinyl acrylate (VA) with the enzymatic procedure reported by Ferreira *et al*. (2002). The *Bacillus subtilis* protease Proleather FG-F was used to catalyze the transesterification of dextrin with VA, using anhydrous dimethylsulfoxide (DMSO) as reaction medium. Gels were obtained by free radical polymerization of aqueous solutions of dextrin-VA with different degrees of substitution and monomer concentration.

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Experimental Section

Materials

Proleather FG-F, a protease from *Bacillus subtilis,* was obtained from Amano Enzyme Co. Dextrin - Koldex 60 starch (M_w= 45700 Da as determined by gel permeation chromatography (GPC) analysis) was a generous gift from Tate & Lyle. Vinyl acrylate (VA) was from Aldrich, *N,N,N',N'*-tetramethylenethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from BioRad, dimethylsulfoxide (DMSO) and acetone were from AppliChem and polyethyleneglicol (PEG 200 g/mol) was obtained from Riedel-d Haën. DMSO was dried with 0.4 nm molecular sieves at least overnight before use. Regenerated cellulose tubular membranes with 3500 MWCO were obtained from Membrane Filtration Products.

Gel Permeation Chromatography (GPC)

GPC analysis was performed with a FPLC LKB.UV-MII (Pharmacia) equipped with a column packed with Sephacryl HR 400. The eluent was 0.1 M NaOH at a flow rate of 0.35 mL/min. Calibration was made with dextran standards in the molecular weight ranging from 10 to 2000 kDa. The GPC chromatograms were obtained from samples dissolved in 0.1 M NaOH at a final concentration of 5 mg/mL.

Pretreatment of Proleather and determination of proteolytic activity in DMSO

Proleather FG-F was "pH adjusted" as described by Ferreira *et al.* (2001). The enzyme was resuspended in 20 mM phosphate buffer at pH 8. After freezing, the sample was lyophilized on a CHRIST ALPHA2-4 lyophization unit (B. Braun Biotech International) for 48 h. The percentage of active enzyme in commercial powder was 8.9 % (w/w) as obtained by BCA Protein Assay (Pierce) using BSA as protein standard. The proteolytic activity of Proleather FG-F in either 0.2 M phosphate buffer pH 8 or DMSO was determined with casein as substrate. Briefly, in the presence of a protease the casein is cleaved, exposing primary amines. The compound TNBSA (2,4,6-Trinitrobenzene sulfonic acid) reacts with these exposed amines thereby producing an orange-yellow colour that can be quantified as 450 nm absorbance. Control blanks for each sample were included in the assay to correct the effect of primary amines on proteins in the protease sample. The absorbance values were then converted to equivalent subtilisin (a *Bacillus subtilis* protease) concentration using a calibration curve. One unit of protease activity (U) is defined as the production of 1 mM of free amine per minute per milligram of enzyme.

Enzymatic synthesis of dextrin-VA

The enzyme-catalyzed modification of dextrin was as described by Ferreira *et al.* (2001) with few modifications. Briefly, dextrin (4 g) and an amount of VA ranging from 381.6 to 636 μL were dissolved in anhydrous DMSO (60 mL) and the reaction initiated by adding 600 mg of "pH adjusted" Proleather FG-F. The reaction mixtures were then incubated at 50 ºC with magnetic stirring, for 72 h. After this time, the mixtures

were dialyzed for 5 days against HCl aqueous solution, pH 3, at 4 ºC. The aqueous solutions of dextrin-VA were precipitated in a 4-fold excess of acetone and further centrifuged at 9000 rpm for 15 min. Finally, after freezing, the precipitates were lyophilized for 48 h. Blank reactions in the absence of enzyme, or in the absence of VA, were carried out at the same time and in the same conditions. The purification of the reaction mixtures was performed as described above.

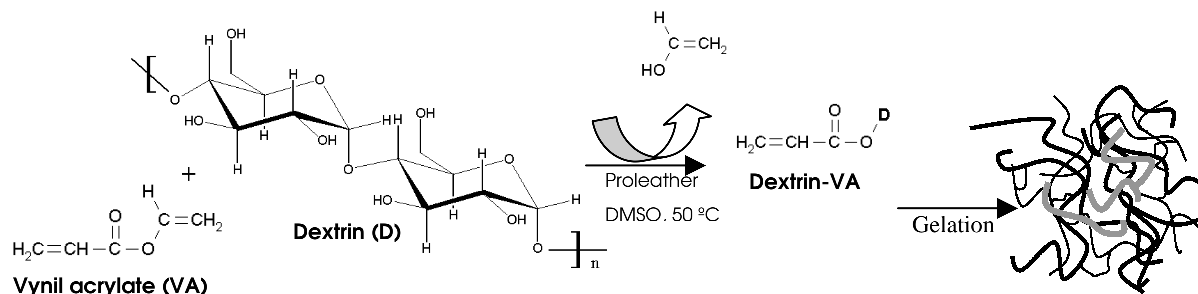

Figure 1. Schematic of the dextrin-VA synthesis and formation of the hydrogel networks.

The degree of substitution (DS, defined as the amount of acrylate groups per 100 glucopyranose residues) was determined by titration according to Vervoort *et al.* (1997). Dextrin-VA samples (100 mg) were dissolved in 2 mL of 0.1 M NaOH and stirred for 72 h at room temperature, to obtain alkaline hydrolysis of the ester. Molar consumption of NaOH was determined by back titration with 0.1 M HCl, using phenolphthalein as indicator.

Preparation of dextrin-VA hydrogels

Dextrin-VA hydrogels were obtained upon free radical polymerization of aqueous solution of dextrin-VA with different degrees of substitution and monomer concentration. Two different amounts of dextrin-VA (300 and 400 mg) were dissolved in 900 µL of 0.2 M phosphate buffer, pH 8.0, and bubbled with nitrogen for 2 min. The gelation reactions were initiated by adding 90 µL APS (80 mg/ml in 0.2 M phosphate buffer, pH 8.0) and 90 µL TEMED (13.6 % (v/v) in water, pH adjusted to 8.0 with HCl) and allowed to occur for 30 min at room temperature. All the recipients in which gelation took place were coated with a polyethyleneglicol solution (PEG 200 g/mol) to simplify the removal of the hydrogel following polymerization.

Swelling experiments

After the polymerization reaction, gels were removed from the casting recipients and immersed in 25 mL of 0.01M citrate-phosphate buffer, for 20 days at room temperature, to allow swelling. At time intervals, gels were removed, blotted with filter paper to remove water excess, weighted and returned to the buffer solution until weight stabilization (*Ws*) was observed (5-6 days). After this time gels were lyophilised and the dry weight was determined, *Wd*. The swelling ratio at equilibrium (SRE) was calculated according to equation 1.

$$\text{SRE} = \frac{Ws - Wd}{Wd} \qquad \text{(Equation 1)}$$

SEM Analysis

SEM micrographs were obtained, showing both internal and external structure of all the hydrogels. Samples were either flash-freeze with liquid nitrogen or freeze at -80 ºC. Freeze-dried samples were gold sputter coated (SC 502 Golden Sputter, Fison instruments) All micrographs were taken using an S 360 Scanning Electron Microscope (Leica, Cambridge).

Results and Discussion

Enzymatic synthesis of dextrin-VA

The use of enzymes has received great attention during the past several decades (Cooney and Hueter, 1974; Zacks and Klibanov, 1985; Klibanov, 1997; Klibanov, 2001). Early enzymatic approaches include the suspension of enzymes in nearly anhydrous organic solvents, although without high catalytic yields (Sergeeva *et al*., 1997, Akkara *et al*., 1999). However, in a previous study Ferreira and colleagues demonstrated a successful enzymatic modification of a polysaccharide solubilized in a DMSO solution. In this work we have modified dextrin with the same enzyme-catalyzed procedure used by Ferreira (2002). The proteolytic activity of the enzyme Proleather FG-F in either 0.2 M phosphate buffer pH 8 or DMSO was assessed (Figure 2). As can be seen, although with a smaller activity than that obtained with the buffer solution, the enzyme remains catalytically active when is solubilized in a DMSO solution. Additionally, a time-course reaction, carried out for six days, showed that the enzyme is very stable in DMSO, keeping nearly 80 % of the initial activity.

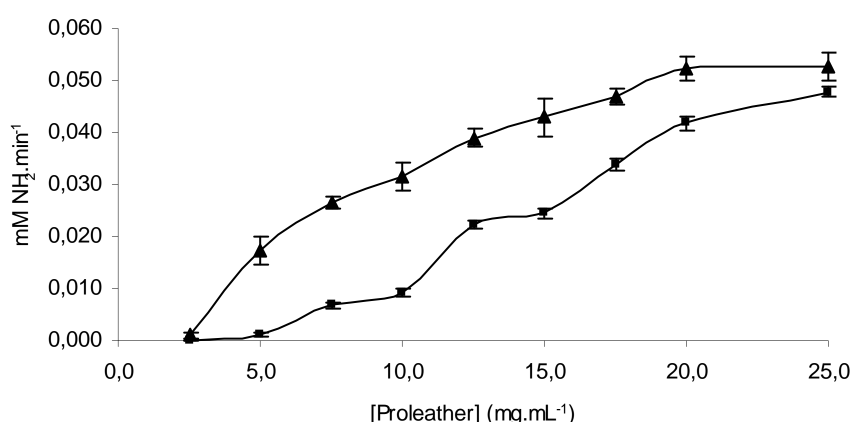

Figure 2. Production of NH_2 per minute, as function of Proleather concentration. The proteolytic activity was determined in 0.2 M phosphate buffer pH 8 (▲) or DMSO (■), by using casein as substrate.

The reactions for the synthesis of dextrin-VA were performed in the presence of 10 mg/mL Proleather FG-F for 72 h at 50 ºC. The results are presented in Table 1. These results show that dextrin-VA with different DS (ranging from 10 to 20 %) can be obtained by varying the concentration of the acyl donor. A percentage of spontaneous transesterification reaction, corresponding to a DS of 1.2 %, was obtained in the blank assay in the absence of the enzyme. This percentage corresponds to efficiency far lower than that obtained with the active enzyme, which reaches values over 70 %. These high percentages allow us to conclude that most of the VA was attached to dextrin molecules, since a very low amount of VA is retained in the blank assays.

The polymer purification procedure based on a dialysis against an HCl aqueous solution, followed by a precipitation with an excess of acetone, revealed to be efficient. The obtained yields were greater than 58 %, superior to values referred in previous works (Ferreira *et al*. 2002).

Table 1. Degree of substitution obtained with different VA concentrations and isolated yields of dextrin-VA monomer

Gel	Theorethical DS[a] (%)	DS (%)[b]	Efficiency[c]	Isolated yield[d] (%)
1	15	11.01	73.40	61.85
2	20	14.43	72.15	63.54
3	25	19.03	76.12	58.31

a Determined as molar ratio of VA to dextrin glucopyranose residues

b Determined by titration of the molar consumption of NaOH (alkaline hydrolysis of the ester)

c Determined as the ratio of the obtained to the theoretical DS

SEM Analysis

Spherical, three-dimensional hydrogels were observed by using scanning electron microscopy (SEM, Figure 3). Hydrogels samples with three different DS values (11 %, 14 % and 19 %) are presented in micrographs A, B and C, respectively. As can be seen, no significant differences are found, suggesting that the difference in the DS is not evident in their superficial morphology. Moreover, in the micrograph D, a detail of the sphere surface is shown. The surface is nearly smooth with only a few irregularities, and large pores are not visible. The internal structure of the gel was also observed, no major differences being noticeable.

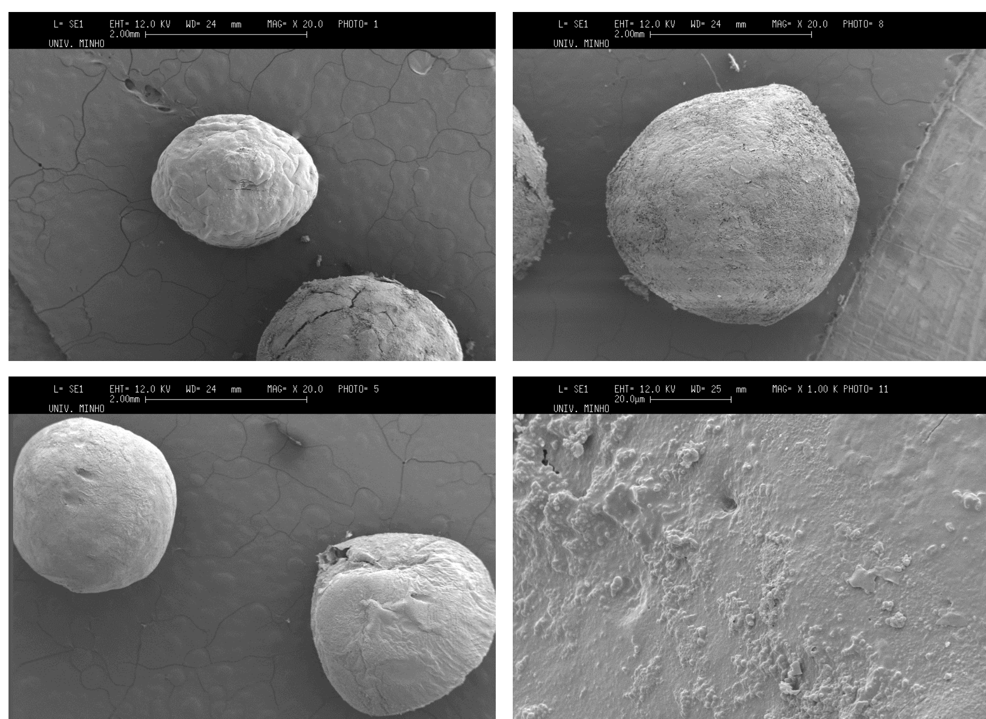

Figure 3. SEM micrographs of dextrin-VA spheres. A, B and C (magnification 20x) correspond to samples with different DS values, 11 %, 14 % and 19 %, respectively. D corresponds to a high detailed micrograph (magnification 1000x) of the sphere surface (DS=19 %).

GPC Analysis

GPC analysis of dextrin and dextrin-VA showed similar elution profiles. Chromatograms are presented in Figure 4. A single peak profile was found in dextrin chromatogram (A), corresponding to a molecular weight of approximately 45750 Da. The same profile was observed in all dextrin-VA samples (A, B and C) although in those samples the peak is slightly shifted to higher molecular weight (from 46000 to 48000 Da), probably due to the introduction of acrylate groups in dextrin structure. The increasing in the molecular weight seems to be related with increased DS values. The chromatogram D, which corresponds to the higher DS value (19 %), shows a more pronounced shift (48000 Da) than those in chromatograms C and B with DS values of 14 and 11 %, respectively.

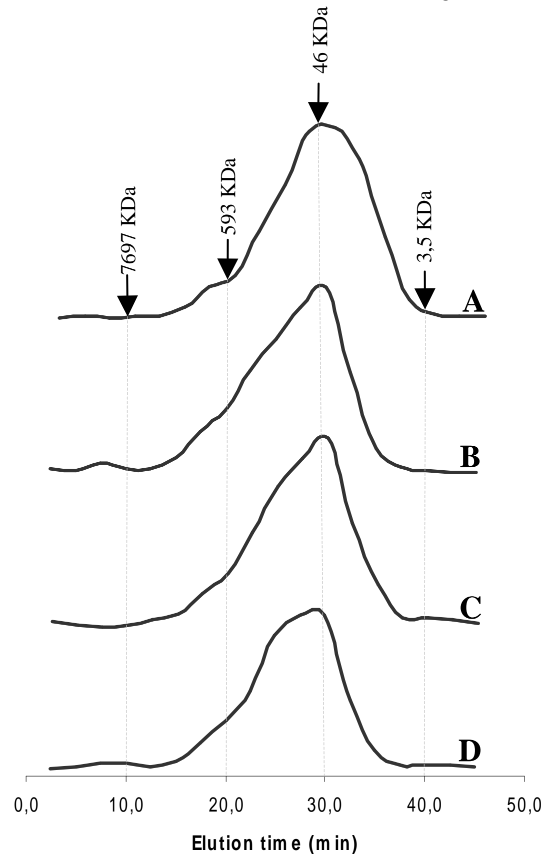

Figure 4. GPC chromatograms of dextrin (A) and dextrin-VA (B, C and D). Dextrin-VA samples have different DS values, 11 %, 14 % and 19 %, respectively.

Swelling behaviour of dextrin-VA hydrogels

Upon exposure to an aqueous solution, hydrogels reach an equilibrium swelling after a certain time. In this equilibrium state, the swelling and elastic retractive forces are in balance (Flory, 1953). The functionality and number of linkages in the hydrogel, as well as the monomer concentration, influence not only the swelling ratio, but also the erosion profile and the overall time for degradation. The influence of the DS on the swelling behaviour was studied in gels with two different initial monomer concentrations (Table 2). The swelling ratio profiles for the two monomer concentrations (33.3 and 44.5 %) were similar in an early incubation stage of about one week (until weight stabilization was observed). Both of them have increased for this initial period, after incubation in 0.01M citrate-phosphate buffer solution. This increase was attributed to the uptake of water molecules into the hydrogel matrix from the surrounding buffer solution. However, the difference in the initial

swelling values of the two different monomer concentration systems, is further evidence of differences in their initial network structure, because a more tightly crosslinked system will swell less than a more loosely one (Yeh *et al*., 1995). Accordingly, greater swelling values were found in less concentrate networks (3.37 to 3.58), compared with those obtained within the higher monomer concentration (1.91 to 1.94).

Table 2. Characteristics of the different dextrin-VA hydrogel samples

Gel	W_0[a] (% (w/v))	DS (%)[b]	Wcontent[c] (%(w/w))	SRE[d]
1		11.01	76.12 ± 0.51	3.58 ± 0.12
2	33.33	14.43	76.49 ± 0.71	3.43 ± 0.17
3		19.03	76.49 ± 1.36	3.37 ± 0.26
4		11.01	63.78 ± 1.33	1.94 ± 0.05
5	44.45	14.43	63.96 ± 1.07	1.91 ± 0.08
6		19.03	65.15 ± 2.04	1.91 ± 0.16

[a] Initial monomer concentration

[b] Degree of substitution defined as the amount of acrylate groups per 100 glucopyranose residues

[c] Water content after polymerization

[d] Swelling ratio at equilibrium

Several important characteristics of the swelling ratio are also shown in Figure 5. The equilibrium water content of these networks is very high. Since the degradation of these hydrogels is facilitated through hydrolytic cleavage of the ester bonds in the crosslinked structure, the network crosslinking density decreases with degradation, resulting in an increase in the swelling ratio (Martens *et al*., 2002).

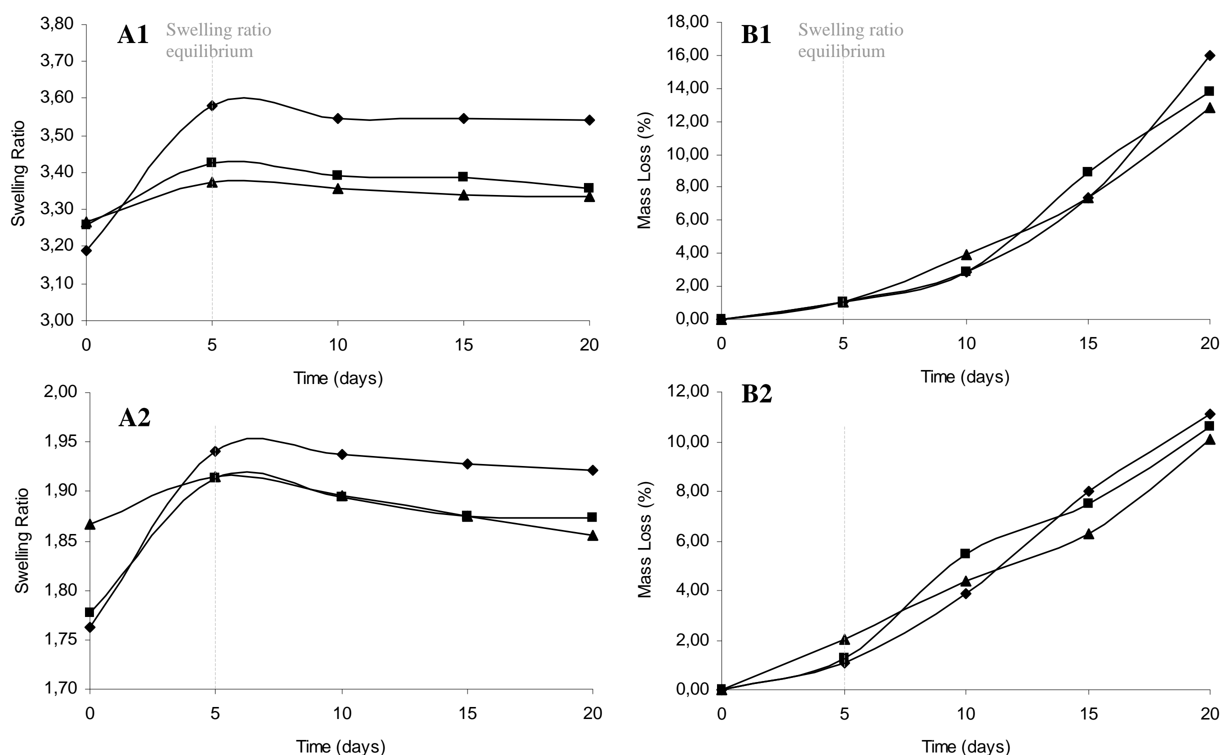

Figure 5. Swelling ratio (A) and mass loss (B) profiles of dextrin-VA samples with different DS values, 11 % (◆), 14 % (■) or 19 % (▲) and different initial monomer concentration, 33.3 % (A1and B1) or 44.5 % (A2 and B2) as function of degradation time.

However, the swelling ratio of all hydrogel samples started to decrease at day 8 (Figure 5 A). This observed trend of decreasing in the swelling values at a later stage suggests that the crosslinked hydrogels might undergo an erosion process with physical disintegration and mass loss (Lee *et al*., 2004). Martens and colleagues (2002) reported that if the degradation occurred via a homogeneous bulk erosion process, the swelling ratio would increase with incubation time because of loosened polymer network. Once, although hydrolysed, the polymer chains remain in the matrix, and the physical structure is not adversely affected. As result, the network becomes looser and continuously swells. Nevertheless, since, as already stated, the hydrolytic cleavage facilitates the degradation, the erosion events are controlled by the access to water. Consequently, some locals in the network might become preferentially degraded and eroded. Our results suggest that the erosion starts in the hydrogel borders. This process is described in Figure 6. Some polymer chains freely diffuse out of the network, after the hydrolytic cleavage of the ester bonds in the gel borders, leading to a decrease in the crosslinking density, thereby facilitating the release of some unbound molecules, which were entrapped in the matrix at the time of the polymerization. This erosion event is further cause of the mass loss and physical disintegration of the gel structure, being responsible for the decrease in the swelling values. Similar to the swelling behaviour, some specific features can be varied in the mass loss profile, resulting in variations in the overall time for degradation. This ability of manipulating the degradation times may be an attractive tool for tailoring release profiles of entrapped drugs, proteins, growing factors, among others (van Dijk-Wolthuis *et al*., 1997).

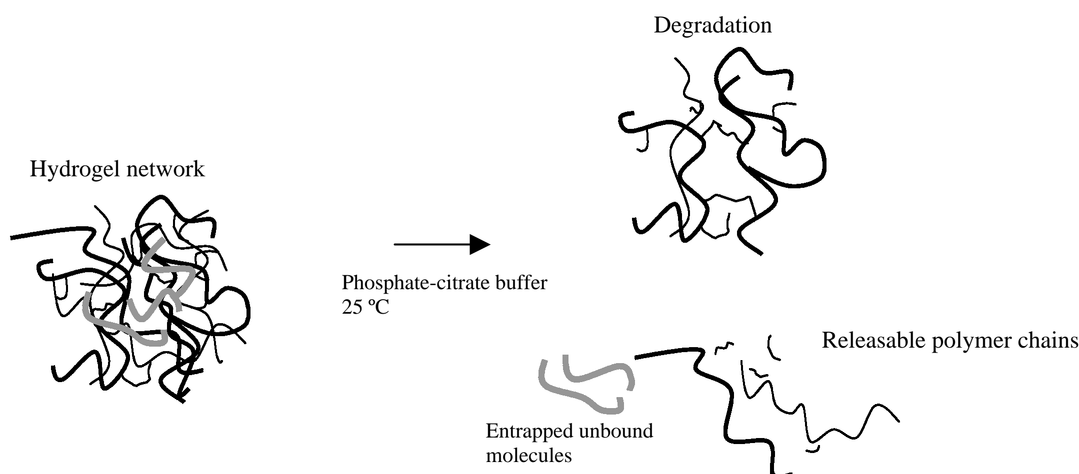

Figure 6. Schematic of the hydrogel degradation process. Each monomer can be connected to several chains in a range of conformations, in order to build the crosslinked structure. Additionally, while the polymerization step occurs, a small fraction of unbound molecules are physically entrapped in the matrix. Once a significant fraction of the ester bonds have been hydrolyzed, the polymer chains are eroded from the network. This erosion leads to a crosslinking density decrease, thereby facilitating the release of the entrapped molecules, resulting in a mass loss from the network.

Conclusions

Derivatized dextrin can be polymerized in aqueous solution by radical polymerization with APS and TEMED. The enzymatic procedure was shown to be flexible, enabling variations in the number of functional groups that are attached to the macromolecular structure (DS), by varying the VA concentration. Other features, such as the initial monomer concentration, can also be varied. These simple changes in the hydrogel formulation result in significant differences in the final network properties, such as the swelling ratio, erosion and mass loss profiles during degradation.

Combining the ability to tailor the hydrogel properties to fit a wide range of characteristics, with the biocompatibility of the starch-based materials, it is possible to obtain dextrin crosslinked networks which might be very attractive for biomedical applications.

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