Deamidation of Pseudopeptidic Molecular Hydrogelators and its application to controlled release

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ABSTRACT

Hypothesis

The incorporation of a succinic acid-derived moiety in amino acid derivatives would favor an intramolecular catalysis of a deamidation reaction. Such reaction would permit controlled disassembly of molecular hydrogelators and the use of the hydrogels for controlled release of actives.

Experimental

Low molecular weight hydrogelators containing a succinic acid-derived moiety were prepared by conventional organic synthesis procedures. Hydrogels were examined by electron microscopy and ^{1}H-NMR studies were carried out to evaluate the solubility in water of the hydrogelators and the deamidation reaction. Liberation of Rose Bengal entrapped in the hydrogels was monitored by UV-Vis spectroscopy.

Findings

Molecular hydrogels formed by pseudopeptidic derivatives of L-valine suffer a thermal deamidation reaction, leading to partial disassembly. The succinic acid-derived moiety present in the gelators is responsible of intramolecular catalysis of a deamidation reaction. Such neighboring group effect is reminiscent of biochemical processes such as protein deamidation and self-excision of inteins. It has been found that the thermodynamic equilibrium of the deamidation reaction is regulated by the efficiency of hydrogelation. As a proof of concept, the thermally promoted deamidation is applied to controlled release of Rose Bengal.

KEYWORDS

Molecular gels, self-assembly, controlled release, deamidation

INTRODUCTION

Molecular gels have received increasing attention in the last decades. In opposition to polymer-based gels, molecular gels are constituted by low molecular weight species. Self-assembly into fibrillar structures that percolate the solvent results in gel formation.[1] Extensive studies have been carried out to rationalize gel formation concluding that some design parameters such as the capability of forming 1-D aggregates and an appropriate solubility balance are key for the preparation of molecular gelators.[2-7] Different applications of molecular gels include controlled release, optoelectronic soft materials, catalysis and tissue engineering among others.[8-10] A wide structural variety of molecular gelators has been reported in the literature. Common structural units include hydrogen bonding groups such as amides, ureas, carbamates and, on the other hand, apolar moieties such as long alkyl tails or extended aromatic surfaces.[11] Particularly, amino acid, peptide and peptide-like motifs are very common units in molecular gelators.[12-14] The propensity of these building blocks to form intermolecular H-bonding and their chirality seem to represent key elements that provide with good self-assembly capabilities. The study of gel formation by peptides and related motifs has received especial attention due to the biological relevance of protein and peptide aggregation.[7, 15]

Self-assembled fibrillar networks from molecular gels can entrap bioactive substances that are liberated progressively in biomedical applications mainly related with to topic or subcutaneous drug release.[16] On this regard, release of actives can take place by passive diffusion out of the self-assembled network[17] or as a result of the progressive disassembly of the gel fibers.[16, 18, 19] Noteworthy, stimuli responsiveness of molecular hydrogels represents a major advantage in applications related to controlled release. For example, chemical stimuli such as pH changes of the presence of reactive species can trigger the release of entrapped species.[20]

It is reported that spontaneous degradation of proteins can take place in peptides and proteins containing asparaginyl and aspartyl residues. This process is an intramolecularly catalyzed nonenzymatic

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deamidation that takes place via succinimide intermediates, resulting in modified proteins.[21-23] As shown in Scheme 1, intramolecular attack of the peptidic nitrogen atom to the amide group in the side chain of asparagine affords a succinimide intermediate, which upon hydrolysis can produce aspartic or isoaspartic acid. A related chemical process is that found in inteins. Inteins are polypeptide sequences that are excised from the protein that contains them by a self-catalyzed protein-splicing reaction. A number of biotechnological applications based on intein excision have been developed such as splicing-dependent protein synthesis, tags for protein purification and labeling of proteins for NMR analysis among others. In the final step of the splicing reaction, an intramolecular attack leading to a succinimide unit and protein breakage takes place (see Scheme 1).[24, 25]
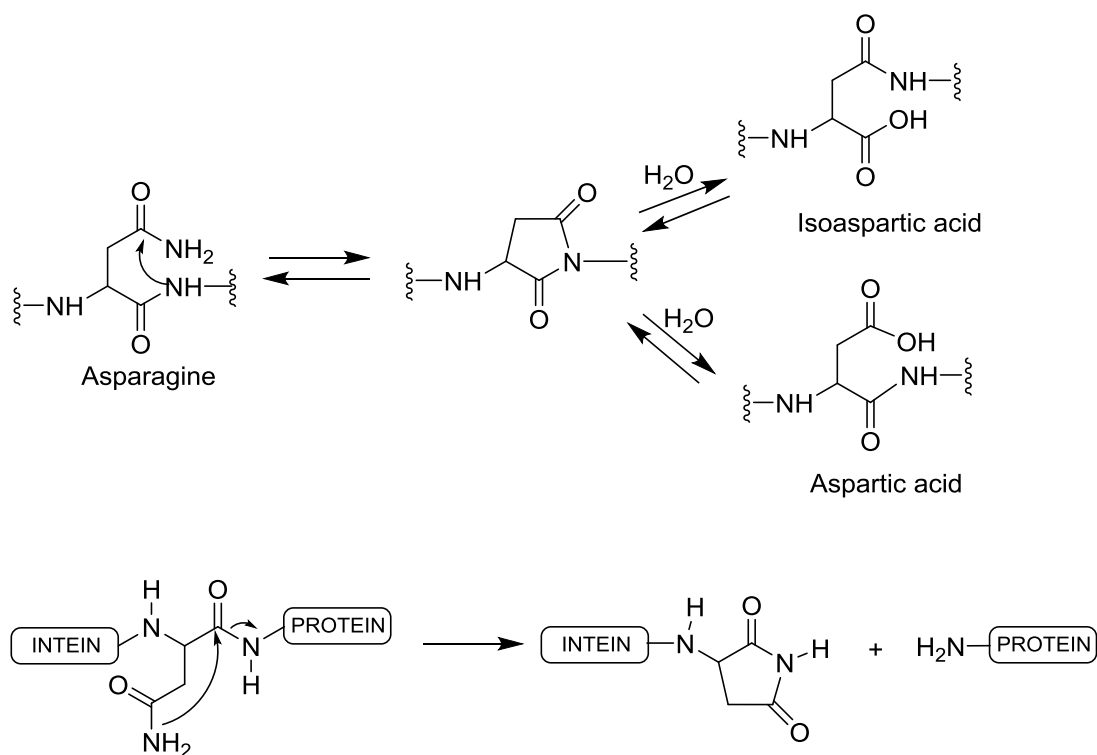
Scheme 1. Simplified mechanisms of protein deamidation (top) and self-excision in inteins (bottom).

Following our studies in the kinetics and thermodynamics of aggregation of peptide-related compounds,[26,27] here we report on how simple pseudopeptidic molecules experiment a deamidation reaction whose equilibrium state is modulated by the hydrogelation capabilities of the studied

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molecules.

MATERIALS AND METHODS

Synthesis

See full details and NMR spectra in the Supplementary Material.

Gelation studies

To determine the minimum concentration required to form a gel (mgc), 10 mg of the studied compound was weighted inside of a cylindrical glass vial (diameter = 1.5 cm), then 100 μL of DMSO was added and the system sonicated until a solution is formed. At this point 900 μL of distilled water were added. The closed vial was allowed to stand at room temperature until formation of gel (10 – 20 minutes). The formation of a gel was checked by turning the vial upside down. This procedure was repeated for different decreasing amounts of gelator (1.0 and 0.5 mg steps) until gel formation was not observed.

NMR spectroscopy

For the determination of solubility constants, the gels (20 mM for **SucValHex** and 10 mM for **GltValOct** and **SucValOct**) were prepared inside of a NMR tube using an internal standard for integration (HCOOH 0.5 %). PRESAT ^{1}H-NMR spectra were recorded at different temperatures (30 ºC to 85 °C, every 5 ºC), letting the system stabilize at the selected temperature for 10 minutes. Relative integration of gelator signals to the internal standard provided with the concentration of soluble gelator at the different temperature values.

For the study of the hydrolytic stability of the compounds, several batches containing 5 mg of each compound were suspended in 1 mL of deuterated D_2O and sonicated for 10 minutes. Analysis of the samples at different time intervals was performed by ^{1}H-NMR. To achieve full solubility of the samples after the stipulated time, solid powdered NaOH was added until pH = 12**.**

UV-Vis spectroscopy

For the study of the release of Rose Bengal, 2.5 mL of hydrogels formed by **GltValOct** (6 mM) and **SucValOct** (7.2 MmM) were prepared inside a vial of 8 mL using as solvent a 0.2 mM solution of Rose Bengal in water. Then, gels were washed by addition of 2.5 mL of water and centrifuged at 6000 rpm during 30 minutes, the supernatant water was discarded. This procedure was repeated several times until the supernatant water was colorless. Finally, 2.5 mL of distilled water were placed on the top of the gels, the vials were sealed and allocated inside of a thermostatic bath at 60 °C. Aliquots of 1.4 mL were analyzed at different time intervals by UV-Vis, monitoring the absorbance of Rose Bengal at 548 nm. The aliquots were placed back into the vials after each measurement.

Electron microscopy

Transmission electron microscopy micrographs were taken on a JEOL 2100 microscope equipped with a CCD camera (11 MP). The corresponding fresh gels were applied directly onto 200 mesh carbon coated copper grids. Excess of solvent was carefully removed by capillary action using filter paper. The grids were immediately stained with one drop of phosphotungstic acid 1 % for 1 min. Excess stain was removed by capillarity.

RESULTS AND DISCUSSION

Two molecules composed by an aliphatic tail (hexyl and octyl respectively), L-valine and succinic acid as structural units were initially prepared with the purpose of exploring their hydrogelation capabilities (see **SucValHex** and **SucValOct** at Scheme 2). Molecular gelators containing the succinic acid-derived moiety have been described previously in our group: a bolaamphiphilic compound,[17] a derivative of phenylalanine[28] and some organogelators.[29] The preparation of compounds **SucValHex** and **SucValOct** is simple and efficient. Acylation of the corresponding alkylamine with C-activated L-valine was

followed by N-acylation of the L-valine residue with succinic acid anhydride.
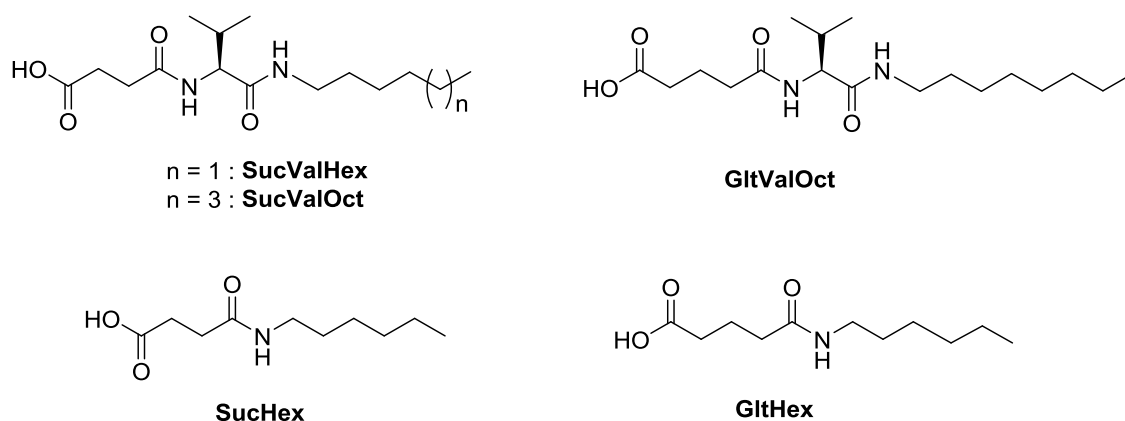
Scheme 2. Structure of the studied compounds.

Study of the gelation capabilities of **SucValHex** and **SucValOct** was performed at room temperature by addition of water to a DMSO solution of the gelator (10% DMSO in the final sample). Translucent to transparent hydrogels were formed with minimum gel concentration values (mgc) of 18 mM and 6 mM respectively for **SucValHex** and **SucValOct.** The higher efficiency of gelation of **SucValOct** in comparison to **SucValHex** should be ascribed to the more hydrophobic character of the former molecule (calculated clogP values are respectively 3.4 and 2.3). Transmission electron microscopy (TEM) images of the corresponding xerogels revealed an entanglement of self-assembled fibrillar objects observed commonly in molecular gels (Figure 1).
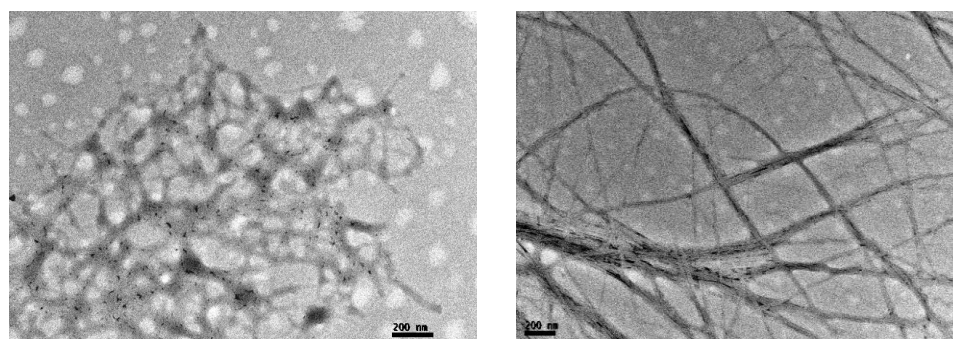

Figure 1. Transmision electron microscopy images of xerogels from **SucValOct** (left) and **SucValHex** (right). The bar length is 200 nm.

Therefore, VT-NMR studies of the solubility of the gel network were performed by integration of the ^{1}H-NMR signals of free gelator that coexists with the fibrillar network (NMR-silent) at different temperatures.[30] As it can be seen in Figure 2, the solubility of both **SucValHex** (ca. 1 mM) and **SucValOct** (ca. 0.03 mM) is poorly temperature dependent, being almost constant in the range 30-60 ºC. Above 60ºC a moderate solubility increase is detected, reaching values which are far below the mgc value at 25 ºC. Thermodynamically, this behavior indicates a practically null enthalpic component in the aggregation-dissolution equilibrium of the gel network, being the system entropically controlled. Such behavior has been reported previously in hydrogels and reflects the dramatic relevance of hydrophobic forces in comparison to intermolecular H-bonding for aggregation processes taking place in water.[31]
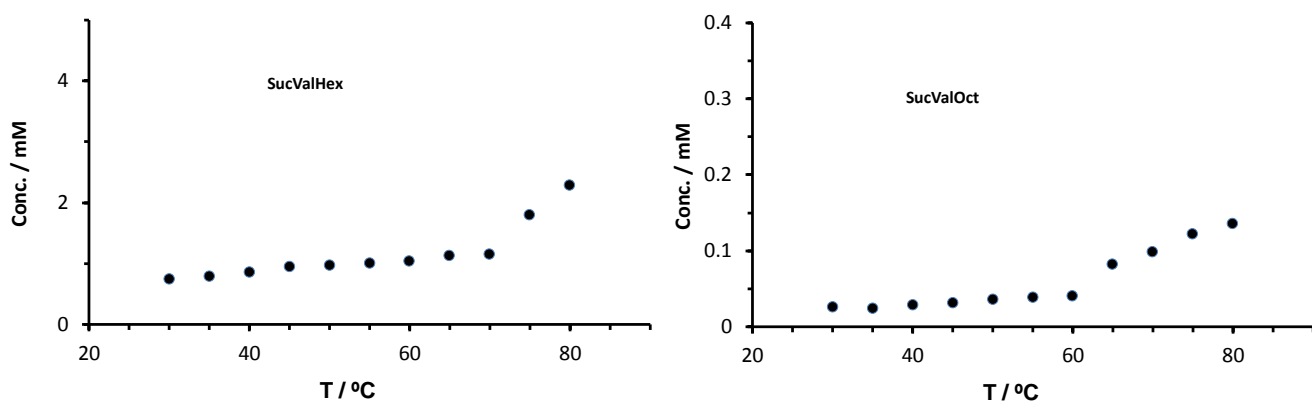

Figure 2. Variation of the solubility in water-DMSO (9:1) of the hydrogels formed by **SucValHex** and **SucValOct**.

Upon heating hydrogels of **SucValHex** and **SucValOct** for several hours at 80ºC some decomposition was detected by ^{1}H-NMR. It was hypothesized that a hydrolytic process, related to protein deamidation or self-excision, could be taking place. To evaluate this possibility, the simple model compound **SucHex,** which corresponds to succinic acid monohexylamide, was studied (see Scheme 2). After 18

hours at 80 ºC, **SucHex** is almost completely hydrolyzed affording hexylamine and succinic acid as revealed by ^{1}H-NMR analysis of the reaction.
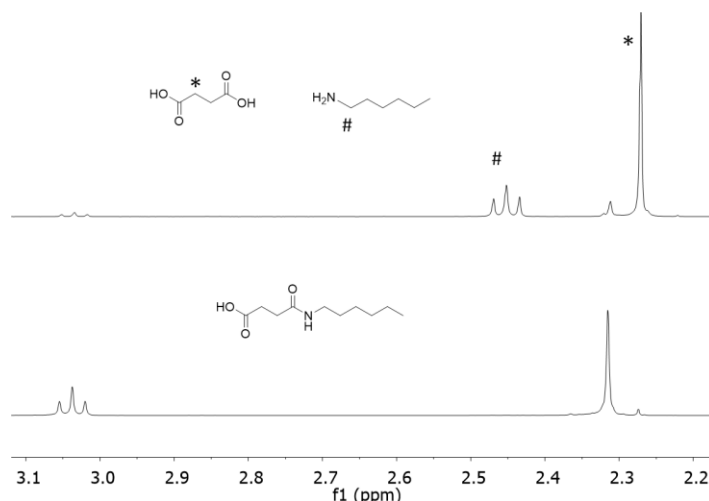

Figure 3. Partial ^{1}H NMR spectra of **SucHex** before (bottom) and after (top) heating at 80 ºC in D$_2$O for 18 hours.

<mark>Considering the robustness of amide bonds, its lability in the studied compounds is remarkable.</mark> This result can be rationalized considering a neighboring group effect of the carboxylic acid of the succinic acid-derived moiety. Intramolecular attack of the carboxylic acid to the amide carbonyl group would yield a tetrahedral intermediate that evolves to the excision of the molecule into the corresponding amine and succinic anhydride, which would be subsequently hydrolyzed into succinic acid (see proposed tentative mechanism at Scheme 3). To gain support for the proposed neighboring effect the analogue molecule **GltHex**, glutaric acid monohexylamide, was studied (see Scheme 2). This molecule was found to be completely stable to hydrolysis in the same conditions for several hours. Such dramatic difference when compared to **SucHex** supports the intramolecular catalysis of the deamidation reaction. It seems reasonable that the presence of an additional methylene unit in **GltHex** affords less thermodynamically stable cyclic intermediate than in the case of **SucHex** for entropic reasons. A precedent in the literature related to this reactivity described an intramolecular nucleophilic catalysis in the hydrolysis of monophenyl esters of succinic and glutaric acid. It was found that the formation of intermediate

anhydrides in the ester hydrolysis reaction was 230 times faster in the succinate ester when compared to glutarate ester.[32]
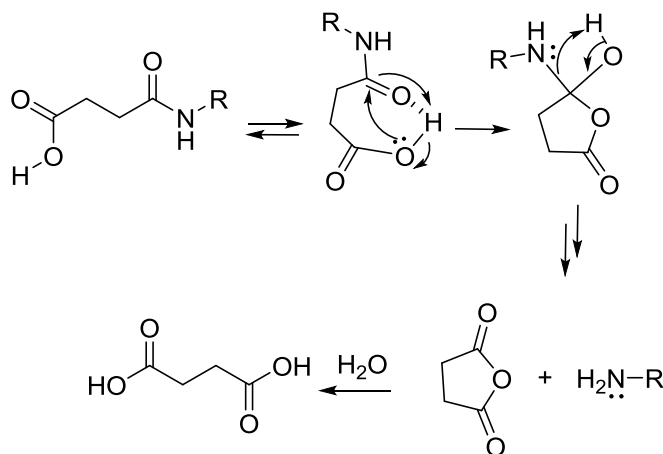
Scheme 3. Simplified mechanism proposed for the deamidation reaction.

^{1}H-NMR analysis of **SucValHex** and **SucValOct** after treatment at 80 ºC for 18 hours revealed that the same type of reaction observed for **SucHex** was taking place. It is assumed that the deamidation reaction occurs exclusively in the free molecules of gelator and not in the aggregates of the fibers. The stabilization towards hydrolysis in gel fibers has been reported in a few cases.[19, 33] As seen in Figure 4, after thermal treatment some signals of the new products such as the one corresponding to the chiral proton of the valine moiety or that from the free succinic acid are visible. Additionally, it was checked that after 18 hours the reaction progress was stabilized, reaching a thermodynamic equilibrium. As expected, the glutaric acid-derived analogue of **SucValOct**, namely, **GltValOct** (see Scheme 2) showed no degradation at all after thermal treatment for several hours.
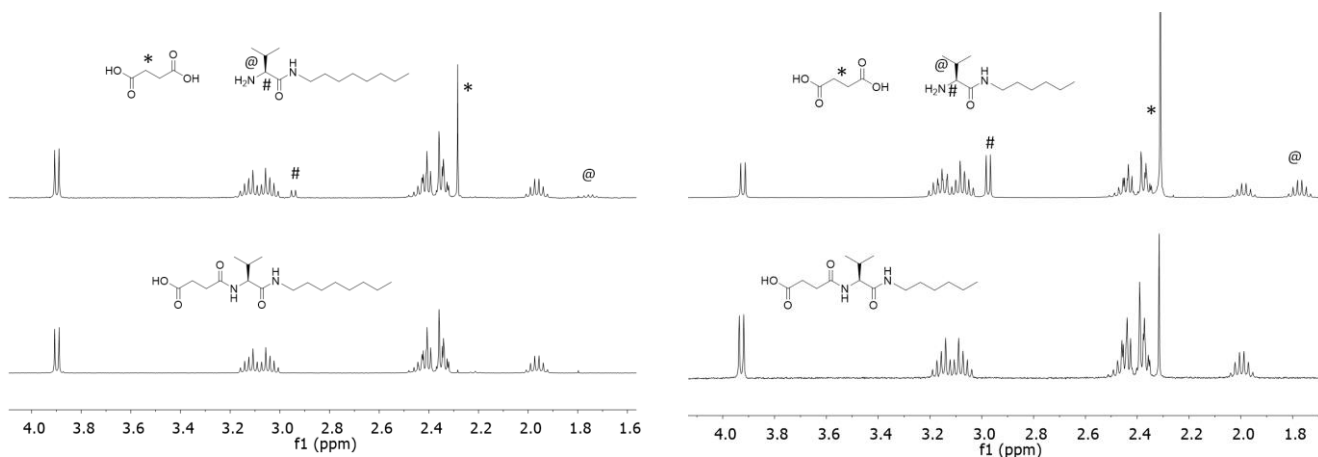
Figure 4. ^{1}H-NMR spectra of **SucValOct** (left) and **SucValHex** (right) before (bottom) and after (top) heating at 80 °C in D$_2$O for 18 hours.

In the case of **SucValHex** and **SucValOct** the reaction is far from completion in opposition to the case of **SucHex**. A thermodynamic equilibrium is reached which affords respectively 53% and 17% overall conversion for **SucValHex** and **SucValOct.** On the other hand, **SucHex** hydrolysis degree is 90%. As shown in Scheme 4, it is proposed that the feasibility of succinic acid conversion back to succinic anhydride allows an equilibrium to be reached. The existence of succinic anhydride in equilibrium with succinic acid in aqueous solution has been demonstrated in the literature.[34] The low hydrolysis degree at equilibrium of **SucValHex** and **SucValOct** when compared to **SucHex** can be explained considering their different hydrogelation capabilities. As outlined in Scheme 4, the aggregation of the hydrogelators would compete thermodynamically with amide hydrolysis. Therefore, the gelation efficiency can modulate the extent to which deamidation reaction occurs.
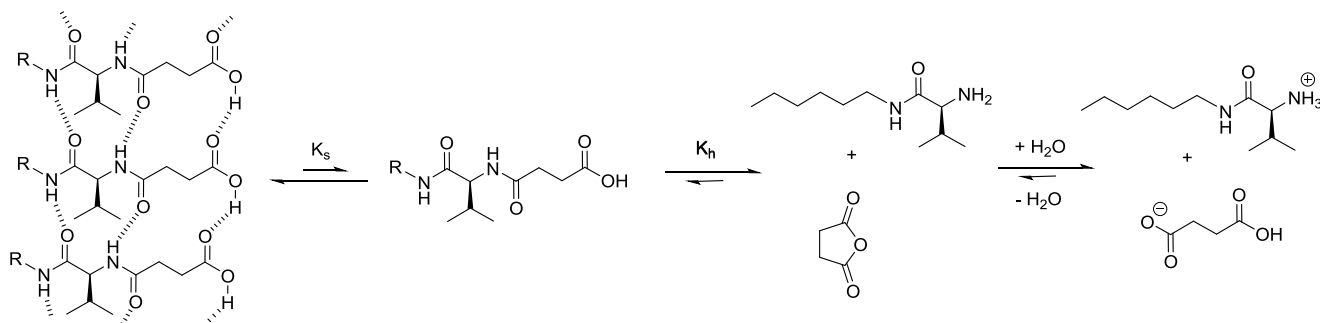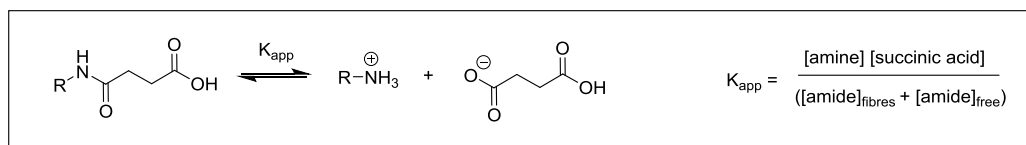
Scheme 4. Equilibria present in the studied system.

The correlation between gelation and deamidation efficiency can be simulated considering a system as that described in Scheme 4. A solubility constant regulates the thermodynamics of aggregation (K_s) and a hydrolysis constant (K_h) is associated to the deamidation reaction. For convenience, an apparent constant K_{app} is defined which considers the overall conversion of the gelator (free and aggregated) to the hydrolysis products (see Scheme 4). Figure 5 shows the simulated dependence of the overall hydrolysis constant (K_{app}) with the solubility of the gelator (log K_s) for a model system similar to those studied experimentally calculated using HYSS2009, a program for the simulation and speciation of chemical equilibria.[35] For the simulation the constant K_s was varied keeping a K_h value of 0.7, which is a reasonable guess for the studied system according the experimental results in Table 1 (discussed later) although the exact value does not modify the trend shown in Figure 5. An exponential dependence of the deamidation degree (K_{app}) on the solubility of the species is observed, therefore, lower solubility, namely, more efficient hydrogelation, results in stabilization of the hydrogelator and reduced hydrolysis.
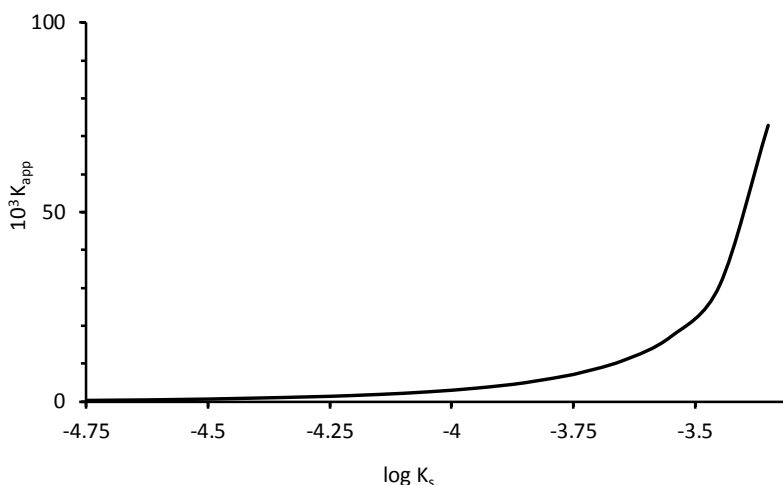
Figure 5. Simulation of the relationship between hydrogel solubility ($\log K_s$) and apparent deamidation constant (K_{app}) using a K_h value of 0.7 (see Scheme 4 for the definition of K_{app} and K_h).

The experimental results are summarized in Table 1. There, the apparent deamidation equilibrium constant, K_{app}, is compared to the solubility of the compounds. Compound **SucHex**, which is fully soluble and shows no aggregation in the concentration range assayed, presents a K_{app} value of 170, which is roughly one order of magnitude higher than that of **SucValHex**. Noteworthy, **SucValHex** presents a K_{app} value notably higher that than of **SuValOct** (9.6 and 0.6 respectively). Such difference can be correlated with their hydrogelation capabilities (mgc values are 18 mM and 6 Mm respectively) and the notable differences in their solubility at 80 ℃ (2.3 and 0.15 mM respectively).

Table 1. Comparison of solubility data and equilibrium constants for the deamidation process at 80ºC in D_2O. All the experimental data come from NMR analysis.

Compound	Solubility at 80 ℃ / mM[a]	[amide]$_0$ / mM	[amide]$_{eq}$ / mM	Yield of amine / %	10^3 x K_{app}
SucHex	> 25	25	2.5	90	170
SucValHex	2.3	16	7.5	53	9.6
SucValOct	0.15	15	12.4	17	0.6

From an applied point of view, the lability of the molecular hydrogelators could find application for the controlled release of actives. It was envisaged that thermal deamidation could potentially be used for controlled progressive release of species entrapped in the hydrogels. This approach would represent an interesting alternative for thermally triggered release for molecular hydrogels whose assembly is weakly temperature sensitive such as those reported here (no significant change in solubility with temperature, as shown in Figure 2). To test this idea Rose Bengal, a well studied photoactive molecule capable of producing singlet oxygen, was loaded in a hydrogel of **SucValOct**. The system was heated for several hours and the release of Rose Bengal monitored by UV-Vis (Figure 6). The experiment was carried out at 60ºC, a temperature that preliminary assays revealed to be appropriate for observing significant results during a period of a few hours. The detailed influence of the temperature on the release profile is left for future work.
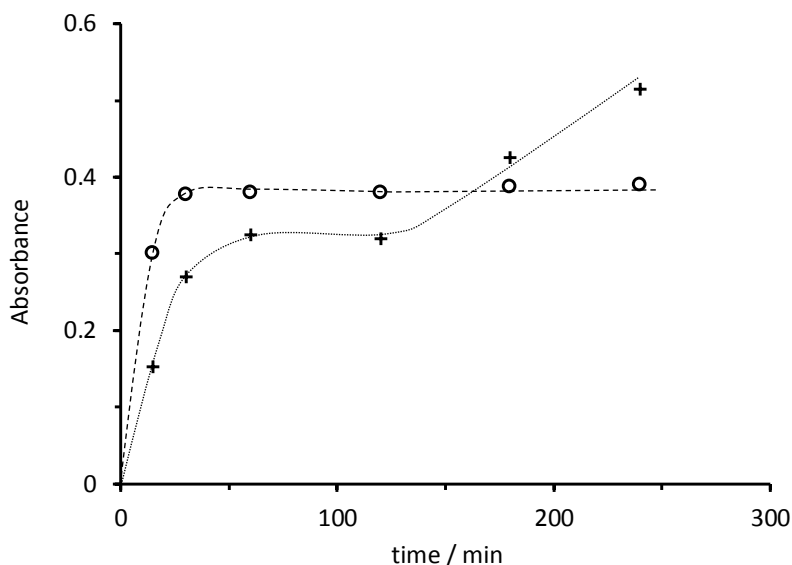

Figure 6. UV-Vis evaluation of the liberation of Rose Bengal (0.2 mM) entrapped in hydrogels formed by **SucValOct** (crosses) and **GltValOct** (circles). Dotted and dashed lines are used as a guide for the eye.

An initial release burst is observed, associated to the dye retained in the macroporous structure. Then, after some minutes, a steady release of tightly adsorbed Rose Bengal was observed because of 14

deamidation-promoted gel disassembly. Noteworthy, this effect is not observed for the analogue hydrogelator **GltValOct** (mgc = 5 mM), which, as mentioned above, is insensitive to deamidation and only liberates in an initial burst the dye weakly attached to the hydrogel.

Conclusions

The reported succinic acid-derived hydrogelators experiment a deamidation reaction as a result of intramolecular catalysis. Such reactivity is related to that observed in protein deamidation taking place at peptide bonds which involve asparaginyl or aspartyl residues and to self-excision observed in inteins. The deamidation reaction is found to be reversible and the equilibrium constant heavily dependent on the hydrogelation efficiency of the studied molecules. The results reveal how the intrinsic reactivity of a pseudopeptidic molecule can be finely tuned by controlling its hydrogel formation capabilities. Additionally, the studied system provides insight into the use of hydrogelation processes to develop smartly regulated dynamic systems with biochemical reminiscence. On the applied side, thermally promoted deamidation is shown to permit controlled release of actives entrapped in the hydrogel such as Rose Bengal. This strategy allows circumventing the temperature insensitiveness of hydrogels in the context of controlled release applications.

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