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Reversible swelling behaviour of Diels–Alder clicked chitosan hydrogels in response to pH changes

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Abstract. Poly(propylene oxide)–poly(ethylene oxide)–poly(propylene oxide) (PPO–PEO–PPO) based bismaleimide (BMI) was employed as cross–linking agent for the synthesis of pH–sensitive clicked hydrogels by Diels–Alder (DA) reaction with furan–grafted chitosan in aqueous solution. The effect of the surrounding pH over the microstructure and the swelling ability of the hydrogels was evaluated depending on the initial composition. The results suggested that the hydrogels maintained the characteristic responsive properties of the original biopolymer even after the cross–linking reaction. The different macromolecular networks remarkably affected the final properties, especially when referring to pH–swelling sensitiveness and hydrogel porosity. In addition, the swelling parameters revealed that the hydrogels presented large liquid absorption capacity, showing excellent recovery properties and responsiveness at different pHs. The promising features of the ensuing hydrogels made them suitable as targeted pH–sensitive drug delivery systems.

Keywords: *tailor–made polymers, chitosan, cross–linking Diels–Alder reaction, pH–responsive hydrogels*

1. Introduction

'Smart' hydrogels attracted substantial interest in recent years, particularly pH–responsive ones, which show drastic changes in volume in response to external stimuli. Indeed, the design of scaffolds exhibiting environment–responsive faculties is crucial in many potential applications areas of 3D materials, such as biomedicine. The particular behaviour of pH–sensitive hydrogels makes them suitable as targeted drug delivery carriers, due to their ability to release drugs at a specific site of the body by conveniently changing their volume at different pH values [1]. Despite representing approximately 90% of all therapeutics used, oral drug delivery systems can be affected by adverse factors. According to this, the most attractive route of controlled release is by pH triggering, knowing that the pH gradient in the human gastrointestinal (GI) tract ranges from 1 to 7.5 [2] and, moreover, decreased pH values are found close to tumors or infarcted areas [3–6].

Several attempts, mainly focused on covalently cross–linked hydrogels, have been made in recent years to design materials with enhanced stimuli–sensitive performance to be used as drug carrier vehicles [7–10]. In this sense, our most recent investigations indicated that chitosan could be successfully cross–linked using Diels–Alder 'click' cycloaddition for hydrogel formation [11–13]. In view of the results achieved regarding their swelling capacity, porosity and antibiotic release pattern, it could be intuited that the referred hydrogels would exhibit satisfactory response when exposed to pH changes. However, up to now, little research has been reported on the influence of pH on this type of networked polymeric structures [14] and, to the best of our knowledge, this would be the first research reporting pH–responsivity values

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on chitosan–based hydrogels. Therefore, in the present study, the pH–response of biopolymeric hydrogels after the chemical cross–linking of furan–modified chitosan with a bifunctional maleimide cross–linker (BMI) is described.

In general, besides the consideration of cross–linking degree [12, 15, 16], the swelling behavior would be also determined by the intermolecular interactions in aqueous solution such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions, which depend on the macromolecular structure [17]. In the case of chemically cross–linked chitosan, two parameters would influence the swelling capacity of the hydrogels under study, namely i) the positive charges localized at the pendant primary amino groups of the main chitosan chain. Thus, under acidic conditions, the amino groups would protonate, provoking repulsive forces between them and promoting solubility, whereas chitosan is normally insoluble at alkaline and neutral pH due to the rigid crystalline structure and the strong intermolecular hydrogen bonding [18]. And, ii) the amount [12, 15], molecular weight [19] and hydrophilicity of the cross–linker [20–23].

At the same time, the resulting 3D microstructure of the hydrogels would also be a determining factor for the control of the swelling/shrinking ability and rate [24, 25]. Pores homogeneously distributed within the hydrogel are expected to result in fast–responsive materials, turning the response–rate into a direct function of the microstructure. Indeed, for a polymer network having an interconnected pore microstructure, absorption or desorption of fluids occur by convection, which is much faster than the diffusion process that dominates in nonporous hydrogels [26]. Considering the advantages that natural biopolymers provide in terms of biodegradability and low cost, among others, chitosan–based hydrogels were prepared by DA reaction with bismaleimides and their pH–responsiveness was assessed, focusing on the effect caused by the incorporation of different amounts of cross–linker on the microstructure and the pulsatile swelling of the materials. In this way, it was confirmed that the as–prepared hydrogels were of great potential for targeted drug delivery, as it was assessed that their behavior successfully adapted to the changes in the human body.

2. Experimental part

2.1. Materials

Medium molecular weight chitosan (Cs) was purchased from Sigma–Aldrich (Madrid, Spain). The average deacetylation degree of chitosan determined by ^{1}H NMR was 81% and the viscosity average molecular weight measured by an automated capillary viscometer (HAc 0.3 M/ NaAc 0.2 M, 25 °C) was 58 kDa. Furfural (Fu, 99%), sodium borohydride ($NaBH_4$, ≥99%), Jeffamine® ED 900 and sodium acetate trihydrate (CH_3COONa, ≥99%) were purchased from Sigma–Aldrich (Madrid, Spain). Acetic anhydride (Ac_2O), trimethylamine (Et_3N), PBS tablets (pH = 7.4), maleic anhydride (98 %), glacial acetic acid, sodium hydroxide solution (1 M) and hydrochloric acid solution (37%) were acquired from Panreac (Barcelona, Spain). Chloroform was purchase from Lab Scan Analytical Sicences (Gliwice, Poland). Acetone was acquired from Oppac S.A. (Noain, Navarra). Deuterium oxide for ^{1}H NMR was obtained from Merck (Darmstadt, Germany). Ethanol (96% v/v, extra pore) was supplied by Scharlau (Barcelona, Catalunya). Deionized water was employed as solvent and all the materials were employed as received.

2.2. Synthesis of hydrogels

The cross–linked hydrogels were obtained by reacting furan–modified chitosan (Cs–Fu) with a degree of substitution (DS) of 31% [11, 13] and previously synthesized bismaleimide (BMI). The hydrogels were prepared as detailed in our previous work [12].

In brief, a 1% chitosan solution in 2% aqueous acetic acid (HAc) was prepared and reacted with an excess of furfural for 5 h at room temperature under magnetic stirring. After that time, the formed imine groups were reduced by the addition of an excess of $NaBH_4$ under nitrogen atmosphere for 2 h. Cs–Fu was precipitated in NaOH (1 M), filtered, washed with water–ethanol–water, dialyzed against deionized water for 24 h and dried.

On the other hand, the bismaleimide (BMI) was synthesized by the modification of Jeffamine® ED 900 following two–step procedure. Bismaleamic acid (BMA) was obtained in the first step by the reaction between Jeffamine® ED and maleic anhydride. Then, the bismaleimide function was obtained by the

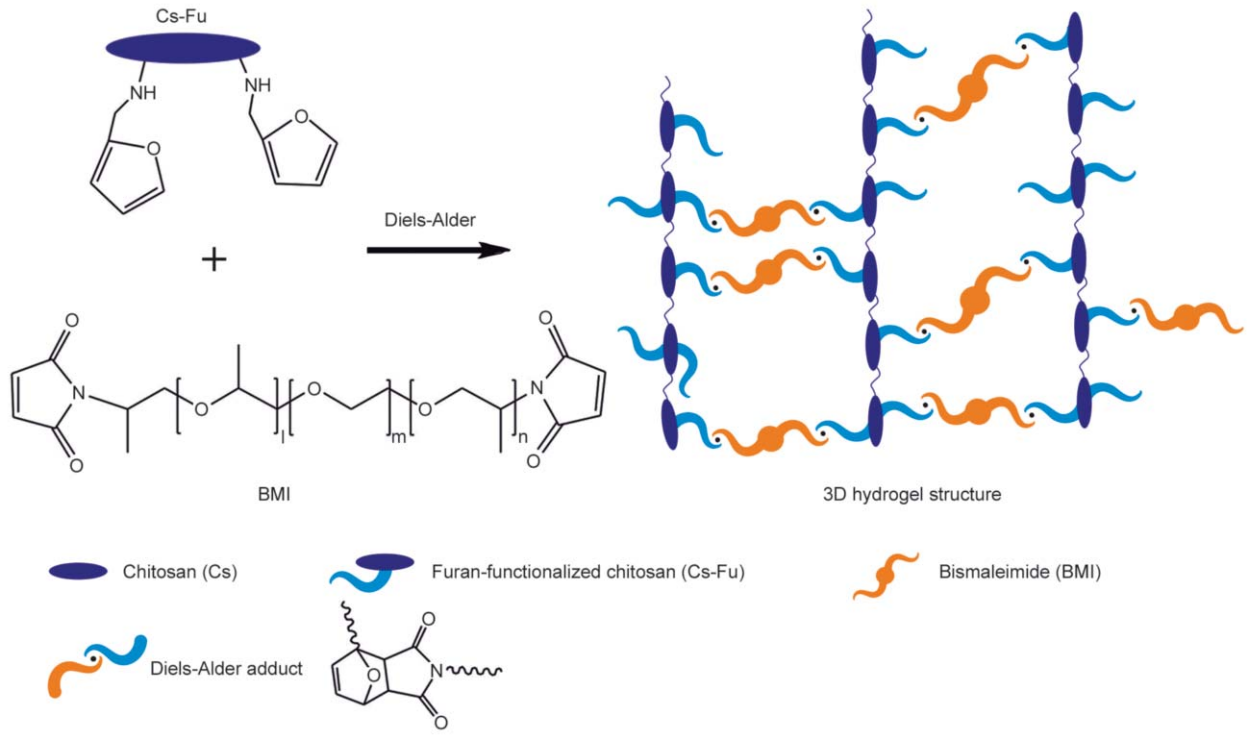

Figure 1. Schematic representation of hydrogel formation.

cyclization of BMA with triethylamine, sodium acetate trihydrate and acetic anhydride in 10 mL of acetone at 70 °C for 2.5 h at reflux. Finally, the cross–linking agent (BMI) was obtained after drying under vacuum at a temperature below 60 °C.

Clicked hydrogels were formed after the cross–linking of Cs–Fu with BMI by DA reaction in aqueous medium for 5 hours at 65 °C (Figure 1). Three different furan–to–maleimide equivalent ratios, 1:1, 1:2 and 1:3, were used hereby referred as CsFu:BMI 1:1, CsFu:BMI 1:2 and CsFu:BMI 1:3, respectively.

2.3. Methods

Scanning Electron Microscopy (SEM) experiments were performed by a JEOL JSM–6400 with a wolfram filament operating at an accelerated voltage of 20 kV and at a working distance of 15 mm. Freeze–dried samples were coated with approximately 20 nm of chromium using a Quorum Q150 TES metallizer. Measurements were performed with the initial samples and after being swelled in H_2O, HCl 0.1 M and PBS (pH = 7.4).

Atomic Force Microscopy (AFM) was performed in a Dimension ICON scanning probe microscope equipped with a Nanoscope V controller (Bruker), operating in Peak Force Tapping mode. An integrated silicon nitride tip/cantilever with a resonance frequency of around 70 kHz and a spring constant of 0.4 N/m was used, performing measurements at a scan rate of 0.5 Hz/s with 512 scan lines.

The insoluble fraction of the cross–linked materials was estimated as the gel fraction [%]. The hydrogels were immersed in PBS solution at 37 °C for 3 h (based on the equilibrium swelling times from previous swelling studies [12]). The gel fraction and the degree of swelling (*SW*) were determined using Equations (1) and (2), respectively; $W_{d,0}$ was the initial weight of sample in the dried state, W_s was the swollen weight of the sample and the weight of the final sample, dried until constant weight was W_d. The measurements were performed in triplicate:

$$\text{Gel}\,[\%] = \frac{W_d}{W_{d,0}} \cdot 100 \tag{1}$$

$$SW\,[\%] = \frac{W_s - W_d}{W_d} \cdot 100 \tag{2}$$

The response of the hydrogels to the environmental pH was studied by performing pulsatile swelling tests at 37 °C in NaOH 0.01 M (pH = 12) and HCl 0.01 M (pH = 2). Freeze–dried hydrogel samples (n = 3) were first swollen in the HCl solution for 20 minutes and their swollen weight was monitored every 7 minutes. Subsequently, the samples were

transferred to NaOH solution and the swelling was monitored following the same procedure. Finally, the swelling capacity of the samples in study at each pH was measured according to Equation (2) after freeze–drying. The pH–dependency of the swelling was studied in a total period of 2 h of incubation at both pHs.

3. Results and discussion

Recent studies had shown that the morphology of hydrogels based on chitosan was closely connected to local pH gradient and salt concentration [2, 24]. As it could be appreciated in Figure 2, the hydrogel suffered drastic diameter changes when exposed to different conditions, reducing almost to the half when it was allowed to air–dry and recovering the initial diameter after the immersion in water.

Salehi *et al*. reported that hydrogels that swell in response to external stimuli, such as pH or temperature, effectively open their pores for facilitate the diffusion of the encapsulated drug under predetermined conditions [27]. Hence, the microstructure of the hydrogels in study, swollen in different media for 24 h and freeze–dried, was analysed by scanning electron microscopy (Figure 3).

First of all, it should be highlighted that noticeable differences were appreciated between the different hydrogels in their initial state (as–prepared: Figure 3a, 3e and 3i). Despite the heterogeneity of the samples, it can be observed that CsFu:BMI 1:1 showed a more compact and closed microstructure, CsFu:BMI 1:3 presented a random distribution of pores (pore diameter ranging from 0.1 to 1.1 mm), whereas homogeneous porosity was found for the intermediate CsFu:BMI 1:2 sample with a mean pore size of about 0.5 mm. The higher porosity of hydrogels is usually had been related to a higher degree of cross–linking, which was in agreement with the results obtained in our previous work [12].

Besides, the images revealed that the swelling media and the final microstructure were markedly related. The samples were allowed to swell in water (H_2O), acidic (HCl 0.1 M) and phosphate buffer (PBS pH = 7.4) solutions until equilibrium was reached. In the case of CsFu:BMI 1:1, no large differences were observed in the morphology after swelling in the three different media, although the most compact structure appeared to obtain after immersion in PBS (Figure 3d). For CsFu:BMI 1:2 the initial highly interconnected porous microstructure was maintained after being swollen in water, but the microstructure was notably different in the two other samples. Indeed, after being swollen in PBS solution, a more compact and wrinkled surface with larger cavities was observed (Figure 3h). In case of the CsFu:BMI 1:3 sample, the porosity increased considerably after swelling in water (Figure 3j) compared to the original sample. More compact structures were observed for HCl and PBS swollen samples, although pits and grooves still appeared.

Whatever the type of cross–linking, networks formed by cross–linked chitosan usually has been reported to be porous [28–32], showing great resemblance to that of CsFu:BMI 1:1 hydrogel. In the case of study, by the implementation of the Diels–Alder reaction improved microporous structures were achieved for some of the compositions. Given the remarkable variation of the microstructure of CsFu:BMI 1:2 and CsFu:BMI 1:3 at different pHs, it could be possible to tune these microstructures for specific applications.

When comparing the three swelling media, it is worth noting that the hydrogels presented compacted

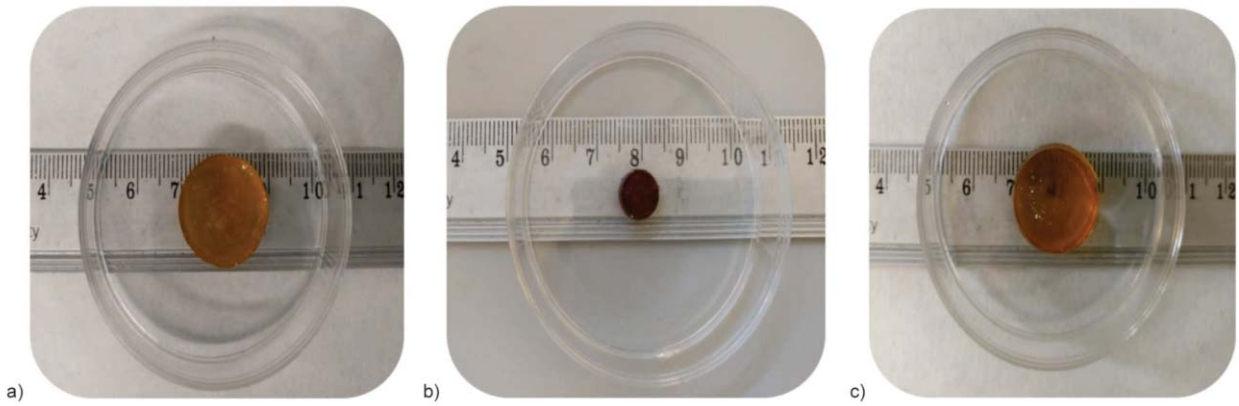

Figure 2. Transition of CsFu:BMI 1:2 hydrogel sample at different progressive stages: a) as-prepared, b) dried at room temperature and c) re-swelled in water.

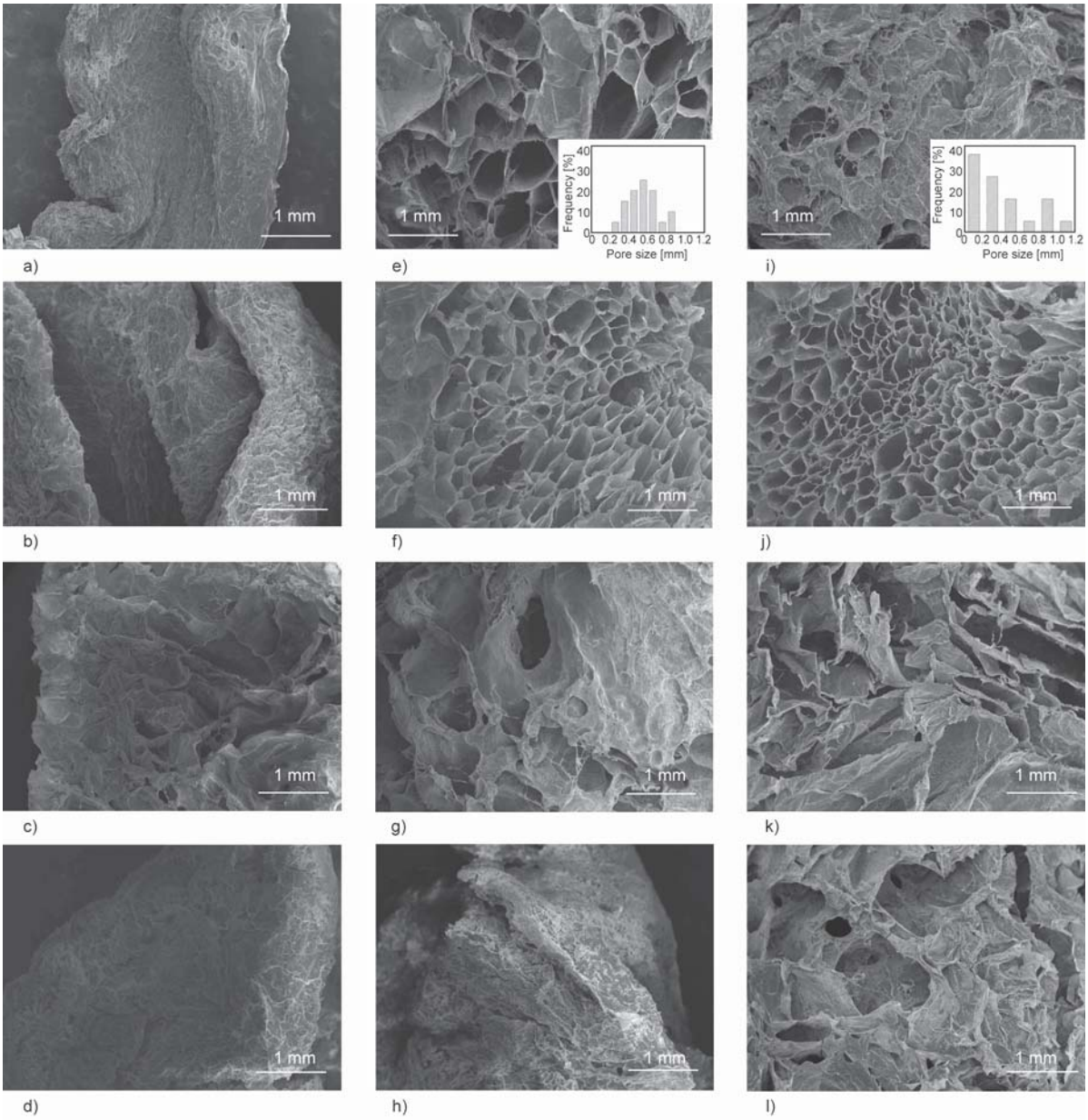

Figure 3. SEM images of the different hydrogel compositions: a) CsFu:BMI 1:1 as–prepared and after swollen in: b) H_2O, c) HCl and d) PBS; e) CsFu:BMI 1:2 as–prepared and after swollen in: f) H_2O, g) HCl and h) PBS; i) CsFu:BMI 1:3 hydrogel as–prepared and after swollen in: j) H_2O, k) HCl and l) PBS.

structures in PBS. As reported in literature, the electrostatic interactions between phosphate ions and the non–reacted amino groups remaining in the chitosan backbone could promote the shrinkage of the hydrogels in PBS solution [33]. This effect was less noticeable in the case of CsFu:BMI 1:3, probably due to of the presence of a greater amount of mixed or grafted BMI in the hydrogel. Regarding the almost neutral medium (H_2O pH = 6), it could be observed that the hydrogels maintained their initial morphology, although a slight change in the 1:3 ratio was observed probably as a consequence of the dissolution of the excess of BMI. In acid medium, where the $–NH_2$ groups of the chitosan would be protonated, more expanded networks were observed as could be expected. In the same trend of previously reported results for other polymeric hydrogels, changes in the pH could have an impact on the behavior of both the cross–linker and the polymer matrix, revealing the intramolecular structure of the hydrogels [25, 34, 35].

Surface roughness of hydrogels is an important issue as it could lead to better cell attachment on the hydrogel that is of crucial interest in some biomedical applications. The topographic images of the top surface of the hydrogels examined by AFM are shown in Figure 4. In terms of height, the samples

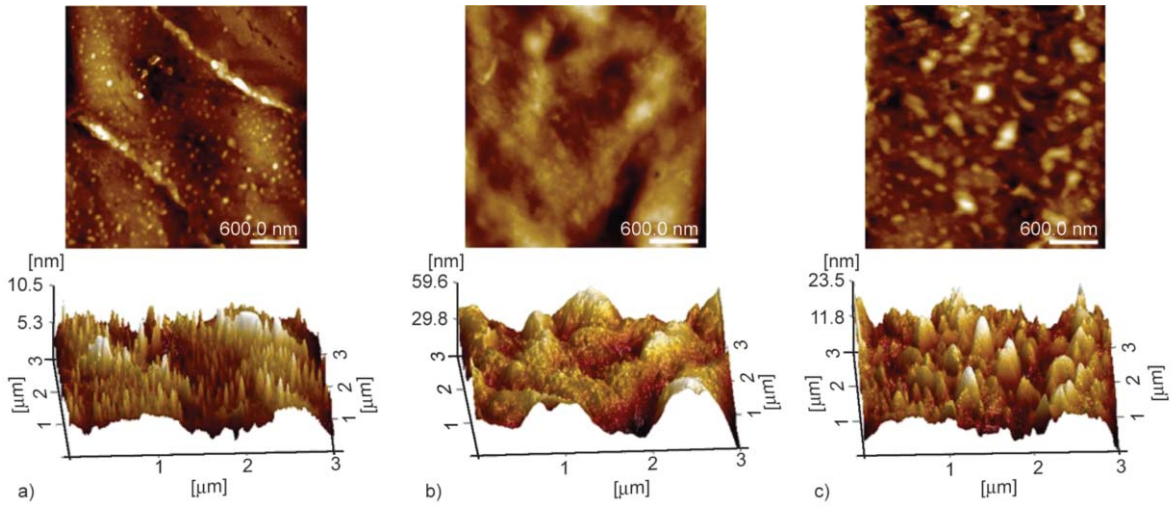

Figure 4. Height and 3D height topographic AFM images of a) CsFu:BMI 1:1, b) CsFu:BMI 1:2 and c) CsFu:BMI 1:3.

showed the characteristic appearance observed by other cross–linked chitosan hydrogels in previous studies [36, 37]. The images supported the rough surface of the hydrogels with different non–homogeneous areas. Furthermore, the roughness height parameters in terms of average roughness (R_a) and root mean square roughness (R_q) were obtained by AFM software at the nanometer scale (data shown in Table 1).

The increase in roughness could be originated by hydrogel cross–linking extent, as a correlation between cross–linking degree and roughness exists. Namely, the hydrogels with higher cross–linking degree tend to have rougher structures [37, 38]. As expected, higher roughness values were observed for CsFu:BMI 1:2 sample, supporting our previous results.

The pH sensitivity of chitosan–based hydrogels through the protonation of amino groups below the pK_a (6.2–6.5) [39] lead to volume changes of hydrogels (swell), whereas the opposite effect is normally observed when the pH increases (shrink) [40–42]. The degree of swelling at equilibrium in different media of these Diels–Alder cross–linked hydrogels was studied in our previous work [12], where this trend was clearly observed. However, in order to assess the suitability of the hydrogels as targeted drug delivery carriers a deeper study was performed exposing the materials to abrupt change of pH.

Figure 5 shows the swelling/shrinking behavior of the three chitosan–based hydrogels. The hydrogels were brought into HCl solution for 20 minutes (pH = 2) and subsequently transferred to a NaOH solution (pH = 10), for the same time. The alternative immersion was repeated three times so that an abrupt swelling/shrinking was ensured. The results evidenced that the hydrogels changed their ability to absorb solution when the environmental pH was altered. Namely, at basic pH the minimum swelling ratio was observed and the highest values were recorded at acidic environment in all cases, which is in agreement with the previous statements. Moreover, the effect of the amount of hydrophilic cross–linker in the hydrogels was also notable, not only in the values of swelling ratio, but also in the difference

Table 1. Topographical parameters of the chitosan–based hydrogels.

Sample	Roughness parameters	
	R_a [nm]	R_q [nm]
CsFu:BMI 1:1	0.8	1.0
CsFu:BMI 1:2	6.1	7.8
CsFu:BMI 1:3	2.7	3.5

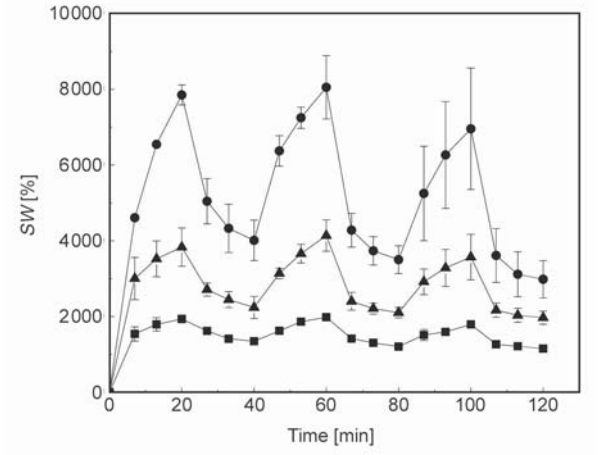

Figure 5. Swelling/shrinking pattern of hydrogels as a function of time at successive cycles at different pHs, ■ CsFu:BMI 1:1, ● CsFu:BMI 1:2, ▲ CsFu:BMI 1:3.

between the two pHs. Results further demonstrated that the shrinking rates were much higher than the swelling rates that seemed to be progressive.

CsFu:BMI 1:1 presented the lower degree of swelling and reduced ability to recover, which could be related to its compact structure. On the contrary, both CsFu:BMI 1:2 and CsFu:BMI 1:3 answered effectively to the pH changes, being more remarkable the response of CsFu:BMI 1:2. In order to quantify the pH–sensitivity of the hydrogels, the degree of the response was calculated as the difference between the maximum and the minimum swelling degrees achieved for each sample at the two pHs and related to the maximum value. The results were 34±0.11, 52±0.55 and 42±5.94% for CsFu:BMI 1:1, CsFu:BMI 1:2 and CsFu:BMI 1:3, respectively. According to the results, the highly porous microstructure of CsFu:BMI 1:2, previously assessed by SEM (Figure 3e), was likely to be the main factor affecting the pH responsivity. This behavior could be relevant for developing pH–sensitive controlled–release systems, due to the marked and quick response of the hydrogel to pH changes.

The hydrogels were allowed to swell for 3 hours in a neutral medium (PBS) at 37 °C, and the following results were calculated for each of the hydrogels: i) the gel content and ii) the equilibrium swelling value (Table 2). Based on this data, the sol fraction of each hydrogel was estimated Equation (3):

$$\text{Sol}\,[\%] = \frac{100 - \text{Gel}}{\text{BMI}_{\text{content}}} \cdot 100 \qquad (3)$$

where Gel refers to the gel fraction [%] calculated using Equation (1) and $\text{BMI}_{\text{content}}$ was the initial percentage of cross–linker in the hydrogels expressed in terms of mass.

Results revealed slight differences between the gel content values of the different formulations. Assuming that the sol fraction would be related to the non–reacted BMI (non–reacted furanic chitosan would not dissolve at pH 7.4), the sol fraction was calculated over the total amount of BMI in each hydrogel. As it could be concluded from the data, from CsFu:BMI 1:1 to CsFu:BMI 1:2, the further addition of BMI cross–linker turned out to be effective, promoting further cross–linking. Indeed, larger amount of cross–linker was incorporated in the final gel fraction of the CsFu:BMI 1:2 also resulting in the decrease of the final equilibrium swelling degree of the material. On the contrary, when comparing the CsFu:BMI 1:2 and CsFu:BMI 1:3 hydrogels, the gel fraction decreased and the final swelling degree increased, indicating that, in this case, further increase of the bismaleimide content did not result in higher cross–linked hydrogel even if its hydrophilic character enhanced the swelling capacity. Indeed, some authors claimed that using a great excess of cross–linker led to reducing cross–linking degree, with higher amount of the bismaleimide pendant from the main chain [43]. This conclusion was in agreement with our previous findings, where it was deduced that a great excess of BMI in CsFu:BMI 1:3 seemed to be grafted (or even mixed), but did not increase the cross–linking degree of the hydrogel. Nevertheless, in addition to the cross–linking degree, it had to be considered the effect of the phosphate ions in the PBS solution that probably interacted with the amino groups of chitosan, as previously stated in the morphological analysis.

4. Conclusions

pH–sensitive chitosan–based click hydrogels were prepared using different amounts of cross–linker by Diels–Alder reaction. The surrounding pH was found to have great impact in the microstructure of the hydrogels. In addition, the hydrogels not only presented responsive swelling capacity under acid or basic environments but also the response was found to be fast and it was repeated at least for three cycles. Thus, this strategy proved to be successful for the tuning of the final properties of the networks and their response to different environments in view of the targeted application.

Table 2. Swelling test results in PBS for 3 h at 37 °C for each hydrogel.

Sample	Cs–Fu content [wt%]	BMI content [wt%]	Gel/Sol fractions [%]	Sol. fraction ref. to BMI content [%]	$SW_{eq.}$ [%]
CsFu:BMI 1:1	55	45	63±2/37±2	83±4	260±34
CsFu:BMI 1:2	40	60	61±1/39±1	65±2	234±10
CsFu:BMI 1:3	30	70	57±6/43±6	62±8	267±4

Acknowledgements

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