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An acetal-based polymeric crosslinker with controlled pH-sensitivity

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Abstract

Even though collagen hydrogels have been used in many biomedical applications due to their unique characteristics, they lacked in mechanical stability and resistance to enzymatic degradation. Aiming at the elimination of these drawbacks, collagen hydrogels have been crosslinked with a variety of synthetic crosslinkers all of which presented increased toxicity, which resulted in their limited use in biomedical devices. In order to overcome this limitation, a branched copolymer based on N-hydroxysuccinimide (NHS) activated poly (ethylene glycol) (PEG) groups was synthesized by *in situ* deactivation enhanced atom transfer radical polymerization (DE-ATRP) and used as crosslinker for collagen hydrogels. The NHS-PEG polymer was modified with labile acetal linkages (acetal-NHS) which made it responsive to pH-alterations and its responsiveness was studied at various pH. The activated acetal-NHS polymeric crosslinker demonstrated long-term stability at neutral pH but underwent hydrolysis at acidic pH, showing strongly pH-dependent degradation properties. By using the acetal-NHS polymeric crosslinker, collagen hydrogels with controlled degradation profiles were fabricated. Both the acetal-NHS crosslinker and the crosslinked collagen hydrogels exhibited excellent cytocompatibility.

1. Introduction

In the last few years, pH-responsive systems demonstrated great potential in the biomedical field as nano/micro drug carriers for targeted and controlled delivery of pharmaceutical substances as well as in tissue regeneration. Such potential is due to the numerous pH gradients existing in both normal and pathophysiological conditions.[1] For example, tumor and inflammatory tissues have an environment that is slightly more acidic than that of blood and normal/healthy tissue. In addition, the intracellular vesicles of cells involved in the endocytosis mechanism, i.e. endosomes and lysosomes, are also acidic.[2-5] Therefore, it is advantageous to design materials that are stable at neutral pH (ca. 7.4) but sensitive to environments with lower pH values. pH-sensitive systems normally contain acid-cleavable functional groups/linkages such as hydrazones, orthoesters, and acetals/ketals. Among these, acetals/ketals compounds and their polymers are promising candidates for the development of acid-sensitive systems and many research groups [1, 6-10] have in fact developed acetal polymer gels/liposomes for acid-labile drug delivery or gene vectors. However, for the majority of pH-sensitive systems the materials are basically composed of synthetic polymers, and this raises concerns about degradation products. Nevertheless, biodegradable materials[11-14] are highly desirable as they produce biocompatible degradation products that are not accumulated in the body. As an important natural biodegradable polymer, collagen is a major constituent of the connective tissue [15-17] including dermis, blood vessels, tendons, and ligaments. Because of its weak antigenicity, collagen has, for many years, been used in hard/soft tissue regeneration, wound dressings and in the treatment of skin defects.[18-23] The collagen fibers in connective tissues provide the desired mechanical stability and proteolytic resistance when *in vivo* lysyl oxidase mediated crosslinking takes place.[24] However, reconstituted collagen lacks sufficient strength and disintegrates upon handling or collapses under the pressure exerted from surrounding tissue *in vivo*.[25] To improve its stability, synthetic crosslinking agents such as glutaraldehyde, N– (3 – dimethylaminopropyl) – N'– ethyl carbodiimide hydrochloride (EDC), and EDC with N-hydroxy-sulfosuccinimide (EDC-NHS) have been used.[26-

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[28] However, these agents have been shown to have associated toxicity which limits their applications in the fabrication of bio-medical materials.[29, 30] Therefore, different approaches using non-toxic chemical crosslinkers have been developed, usually based on poly(ethylene glycol) polymers,[25, 31, 32] one of which is poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG). Researchers have shown the ability of such molecules to crosslink collagen in the absence of cell toxicity and have explored their properties for use in drug release.[31, 33] Generally, the use of crosslinked collagen hydrogels or nano/micro-gels in controlled release and tissue regeneration applications is based on enzyme degradation. However, other environmental stimuli such as pH or redox potential have also been used to trigger the dissociation of gels,[34] facilitating similar applications. To date, there are no reports about how to obtain controlled stability in collagen gels consisting of acetal compounds such as acid-labile crosslinkers. Thus, in this study a new branched polymer with acid-cleavable acetal groups was developed as a pH-sensitive protein crosslinker. This technique involves the formation of covalent bonds between proteins by using multifunctional reagents containing reactive groups, (i.e. N-hydroxysuccinimide) that react with functional groups (i.e. primary amines) of various amino acid residues from the collagen molecules. The controlled degradability of the crosslinker due to its pH-responsive behaviour, renders this material useful for applications of controlled and sustained delivery. Its water solubility it's a further advantage since it can be used in a variety of eco-friendly reactions in contrast to numerous crosslinkers that are soluble in organic solvents. Finally, by combining the above characteristics with its biocompatibility and its reduce toxicity, the crosslinker presented in this study constitutes a perspective candidate for *in vivo* applications.

2. Materials, instrumentation and synthetic methods

2.1. Materials

Poly (ethylene glycol) methyl ether acrylate (PEG-A) (Mn=454), p-methoxybenzaldehyde (p-MBA) and 2-hydroxyethyl acrylate (HEA) were dried for 24 h before use using molecular sieves (4 Å). Methacryloyl chloride (97%), N-hydroxysuccinimide (NHS, 98%), ethyl 2-bromoisobutyrate (EBriB, 98%), pentamethyldiethylene-triamine (PMDETA, 99%), triethylamine (TEA, 99%), p-toluene sulfonic acid monohydrate (TsOH), copper(II) chloride ($CuCl_2$, 99%), Sodium hydroxide (NaOH), Ethylenediaminetetraacetic acid (EDTA), 2,4,6-Trinitrobenzene sulfonic acid (TNBS), Tris-(hydroxy methyl) amino methane hydrochloride (Tris-HCl – pH 7.4), chloroform-d (99.8%), 2-butanone (HPLC grade), n-hexane (ACS reagent grade), acetic acid glacial (99%) and dichloromethane (DCM, ACS reagent grade) were used as received. For cell cultures, phosphate buffer saline (1x) (PBS) and Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% pen strep were used. Collagen type I was used for the fabrication of the hydrogels and was extracted from bovine tendon and collagenase was extracted from clostridium histolyticum. All the reagents were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Instrumentation

The polymers were characterized by gel permeation chromatography (GPC) using a Varian 920-LC GPC system equipped with a RI detector and the columns (30 cm PolarGel-M) were eluted using dimethylformamide (DMF) and calibrated using PMMA standards. All calibrations and analysis were performed at a flow rate of 1 ml/min at 50 °C. The final product was characterized using a Jeol NMR spectrometer at 400 MHz in which deuterated chloroform ($CDCl_3$) was used as solvent. The degradation studies were carried out using a Nanodrop-2100 UV-Vis spectrophotometer. The samples were freeze-dried using a Labconco freezone benchtop freeze-dry system. For the biological evaluation, a Thermo Scientific Varioskan Flash Plate Reader was used to measure the absorbance at 550 nm and 595 nm

2.3. Synthesis of the acid cleavable divinyl acetal monomer

Acetal monomer was prepared in accordance with a reported method [35] with some modifications (**Scheme 1**). Briefly, the monomers p-MBA (0.1 mol) and HEA (0.8 mol) were added into a single-neck round bottom flask, with TsOH (0.015 mol) as catalyst and molecular sieves as drying agents. The mixture was stirred in an ice bath overnight and the reaction was stopped by adding TEA (0.021 mol). The molecular sieves were removed by filtering the solution under reduced pressure. The reaction mixture was extracted using an aqueous solution of potassium carbonate (K2CO3, 0.1 M), and finally purified using a basic alumina column with n-hexane/ethyl acetate mixtures of 5/1 as eluents. Yield: 43%. The obtained product of di(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane was characterized by 1H NMR in CDCl3 (ppm): 3.7 (t, 4H), 3.8 (s, 3H), 4.3 (t, 4H), 5.6 (s, 1H), 5.8 (d, 2H), 6.1 (q, 2H), 6.4 (d, 2H), 7.4 (d, 2H).

2.4. Synthesis of the NHS-monomer

The NHS-monomer (**Scheme 2**) was also prepared in accordance with a reported method, with some modifications. [35, 36] Briefly, NHS (15 mmol), TEA (18 mmol) and DCM (300 mL) were added to 500mL round-bottom flask and placed in an ice-bath. While stirring methacryloyl chloride (14mmol) was added dropwise to the solution using a dropping funnel. The flask was removed from the ice-bath and stirring continued for 24 h at room temperature. The

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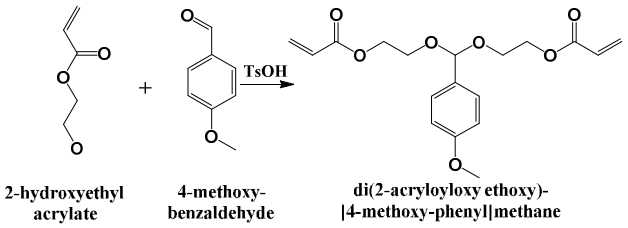
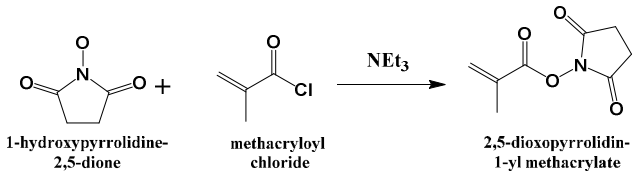
mixture was filtered to remove the solid salt produced and the organic phase was washed three times with saturated sodium bicarbonate water solution followed by distilled water.



Scheme 1. Synthetic procedure of the acetal monomer

The organic layer was dried using anhydrous magnesium sulfate, filtered, and the solvent was evaporated to render a white solid. Yield: 77%. The obtained product 2,5-dioxopyrrolidin-1-yl methacrylate was characterized using 1H NMR in CDCl3 (ppm): 6.4 (H, -H2C=C), 5.9 (H, -H2C=C), 2.9 (4H, OSuH), 2.06 (3H, -CH3).



Scheme 2. Synthesis of NHS monomer

2.5. Synthesis of the acetal-NHS polymer via DE-ATRP

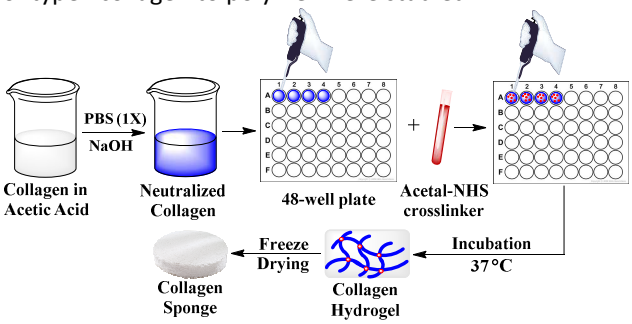
In a typical process, the polymers were prepared in a two-neck round bottom flask. PEG-A (8.17 g, 18 mmol, 90 equiv), EBriB (78 mg, 0.4 mmol, 2 equiv), bisacrylate acetal monomer (0.35 g, 1 mmol, 5 equiv), NHS-monomer (0.183g, 5mmol, 5 equiv), 2-Butanone (10 ml), CuCl2 (0.1 mmol, 0.5 equiv) and PMDETA (17.3 mg, 0.1 mmol, 0.5 equiv) were added into the flask and oxygen was removed using argon flow through the solution for 30 minutes. L-ascorbic acid (0.02 mmol, 0.1 equiv) was then added into the flask under positive pressure of argon and subsequently the flask was immersed in a preheated oil bath at 50 °C. The solution was stirred at 800 rpm and aliquots were taken at different time points. The polymer was purified using dialysis for 48 h in an excess amount of acetone with TEA.

2.6. pH-dependent degradation of the acetal-NHS polymeric crosslinker

The pH-dependent degradation of the acetal-NHS crosslinker was investigated at pH 3.0, 5.0 and 7.4 using UV-Vis spectrophotometry. Briefly, 20 mg of the acetal-NHS branched polymer were dissolved in 0.2 ml acetone/water (1/1, volume) solution and the pH was adjusted using acetic acid (0.5 M). Aliquots of 5 µl of solution were collected at predetermined time intervals and the concentration of the hydrolyzed product was calculated by measuring the absorbance of the sample solutions at 280 nm.

2.7. Synthesis of crosslinked collagen hydrogels and sponges

Collagen type I solution (7 mg/ml in acetic acid) was diluted to a final concentration of 5 mg/ml with PBS (1X), neutralized to pH 7.0 by using NaOH (0.5 M) and then placed in a 48-well plate. The acetal-NHS polymeric crosslinker that was synthesized in a previous step was added to the collagen solution and the formed gel was incubated at 37°C overnight to allow the gel to stabilize (**Scheme 3**). The gels were freeze-dried for 2 days to generate the collagen sponges. Different weight ratios of type I collagen to polymer were studied.



Scheme 3. Preparation of collagen hydrogels and collagen sponges

2, 4, 6-Trinitrobenzene sulfonic acid (TNBS) assay was used to determine the crosslinking degree of the collagen in accordance with a reported method.[36] A standard curve of known concentrations of glycine was used to quantify the free amine groups and the degree of crosslinking was calculated as described in equation 1.

$$Crosslinking\ degree = 1 - \frac{\#\ free\ amines\ of\ crosslinked\ sample/Mass}{\#\ free\ amines\ of\ non-crosslinked\ sample/Mass} \quad (1)$$

The collagen sponges were incubated at 37 °C with bacterial collagenase (extracted from Clostridium histolyticum) in 0.1 M Tris-HCl (pH 7.4) at a concentration of 10 U for 6h and 24h. The enzymatic reaction was terminated at the predetermined time points using EDTA (0.25 M) and the solution was then centrifuged at 4.000 rpm for 5min at 4°C. The supernatant was removed and the precipitate was washed three times with distilled water and lyophilized.

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Mass loss was calculated by comparing the initial mass (W0) with the mass measured at a given time point Wt, as shown in the following equation:

$$Mass\ loss = \frac{W_0 - W_t}{W_0} \qquad (2)$$

2.8. pH-dependent degradation of the crosslinked collagen

The collagen hydrogels were freeze-dried to make sponges which were immersed in solutions with different pH prepared by the addition of 1M acetic acid or 1M NaOH. The pH was checked every twelve hours and was adjusted accordingly to maintain the correct values. The degradation study was carried out using UV-Vis spectrophotometry by measuring the absorbance of the released collagen into the different solutions at 275 nm

2.9. Biological evaluation

2.9.1. Cytotoxicity assessment of the acetal-NHS polymeric crosslinker

3T3 mouse fibroblast cells were incubated at 37°C and 5% CO2 for 24h with various concentrations of the acetal-NHS polymeric crosslinker and AlamarBlue® assay was used to assess the cell's metabolic activity. The reduction percentage was calculated according to equation 3 where A_LW is the value of absorbance at lower wavelengths, A_HW is the value of absorbance at higher wavelengths and R0 is the correlation factor which can be calculated by dividing the absorbance of the oxidized form at the lower wavelength (550 nm) with the oxidized form at the higher wavelength (595 nm)

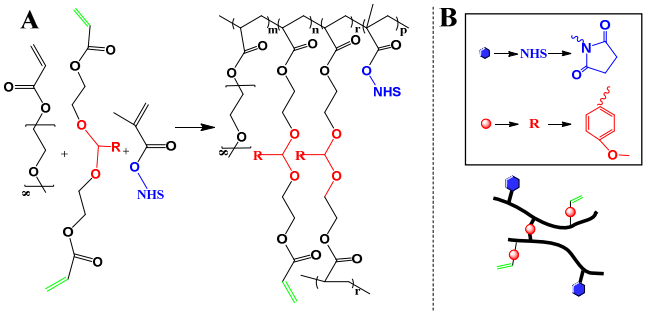
$$AR_{LW} = A_{LW} - (A_{HW} \times R_0) \times 100 \qquad (3)$$

2.9.2. Cytotoxicity assessment of collagen hydrogels crosslinked with the acetal-NHS polymer

Crosslinked collagen hydrogels with a weight ratio of polymer to crosslinker 1:2 were placed in a 96 well plate for cell culture. Subsequently, 3T3 fibroblast cells (in DMEM medium) were seeded on each gel surface (5000 cells/cm2) and left for 1 hour to allow cell attachment to the surface of the gels. After 1 h of incubation at 37°C and 5% CO2, 200 µl of DMEM, culture medium was added into each well and the culture was incubated under the same conditions for a further 24h. AlamarBlue® was employed to assess cell metabolic activity. Cells alone were treated as control (n=3).

3. Results and discussion

A number of factors need to be considered when designing a crosslinker with pH-sensitivity including biodegradability, high sensitivity in pH alterations and biocompatibility. Biodegradability can be controlled by adjusting the hydrophilicity of the crosslinker which is translated to an easy dissolution of the crosslinker molecules in water. This leads to a fast reaction of the functional groups of the crosslinker with the functional groups of the reacting reagent, i.e. collagen. High sensitivity in pH alterations allows the easy hydrolysis of the crosslinker and thus, in our case, of the fabricated crosslinked collagen gels. Finally, the role of biocompatibility is an especially important one because of the need to use these crosslinkers and/or hydrogels *in vivo*. In recognizing the above requirement, an ideal crosslinker would have, an acetal-NHS polymeric crosslinker with branched structure synthesized (**Scheme 4**) by the *in situ* deactivation enhanced atom transfer radical polymerization (DE-ATRP) method. This is a simple 'one-pot' reaction offering the advantages of a branched structure while avoiding the difficulties of dendrimer synthesis and purification. [37, 38]



Scheme 4. Controlled radical crosslinking polymerization through *in situ* DE-ATRP for the preparation of the pH-sensitive acetal-NHS polymeric crosslinker (left) and graphical representation of the branched structure with NHS groups (blue hexagons), acetal groups (red spheres) and pendant vinyl groups (green) (right).

The GPC trace results (Fig. 1A) demonstrate the successful polymerization process over time. In the first 2 hours of the reaction Mw is 9,660 g/mol, PDI is 1.30 and the total monomer conversion is 32.8% (Fig. 1A black curve) but as the reaction time increases to 3.5 hours (indicating a higher molecular weight in comparison to the polymer after 2 hours), Mw increases to 14,100 g/mol, PDI increases to 1.47, and the total monomer conversion is approximately 50% (Fig. 1A red curve). The above calculations were made after comparing the results of the measured polymers with PMMA standards of narrow distribution and well defined Mw and Mn. The growth and evolution of the polymer is time-dependent as can be seen from the previous results, thus demonstrating a well-controlled chain growth, via the DE-ATRP method. The 1H NMR spectrum (Fig. 1B) illustrates the double bonds and characteristic acetal proton peaks within the acetal-NHS polymeric crosslinker. The characteristic peaks at

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chemical shifts 6.4, 6.15 and 5.85 ppm are attributed to the vinyl functional groups and the chemical shift of 5.6 ppm is attributed to the acetal proton in the copolymer. Through the integration of the value of these peaks, it is possible to calculate the final polymer composition [7] which consists of 81.8% molar percentage of PEG-A, 11.1% acetal units, 8.0% NHS units, 4.0% vinyl groups and a branching degree of 7.1%.

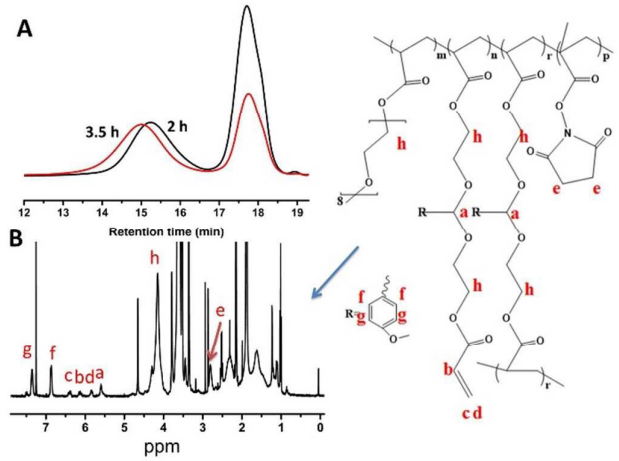

Fig. 1. **(A)** GPC traces of the polymerization of acetal-NHS hyperbranched polymer via DE-ATRP at different reaction times. Black curve: 2h; red curve: 3.5h. **(B)** 1H NMR spectrum of acetal-NHS branched polymer. **(C)** Typical molecular structure of the acetal-NHS polymeric crosslinker.

The calculated composition shows a deviation from the addition of monomer ratios, especially PEG-A, indicating a significantly lower value when compared to the feed ratio (90%). Such results can be explained by the weak reaction activity due to the long PEG side-chain. The results from GPC and NMR determine that the final polymer consists of 25 PEG-A repeating units, approximately 2 pendent vinyl groups, 2-3 branched units and 2-3 NHS groups. The number of branched units is similar to (that of) NHS units indicating that each branch of the polymer has only one NHS group. Generally, polymer degradation studies can be performed by investigating the reduction of molecular weight. If a typical branched structure was formed, exposure of the polymer to certain conditions, low pH in the present case, will result in cleavage of the branching units linked by acetal groups to form small fragments of the polymer, degradation scheme shown in Fig. 2A. To explore its hydrolysis, the polymer was dissolved into DMF and the solution pH was adjusted to 3.0 using 1 M acetic acid. After being hydrolysed for 2 days, GPC was used to measure the molecular weight. A decrease in the molecular weight occurred for the polymer (Fig. 2B, bottom), confirming that this structure consists predominantly of shorter branching chains. Mw value was decreased from 15 KDa (after purification) to 8.5 KDa (a near 2-fold decrease) after degradation, indicating that the polymer consisted of approximately 2 branched units, which is consistent with the 1H NMR results discussed above. The pH-dependent hydrolysis of the acetal-NHS crosslinker was then investigated at pH 3.0, 5.0, and 7.4. The extent of acetal hydrolysis was determined by analysing the by-product of P-MBA (Fig. 2A, top) using UV-Vis spectroscopy and measuring the adsorption peak at 280 nm at predetermined time intervals (Fig. 3).

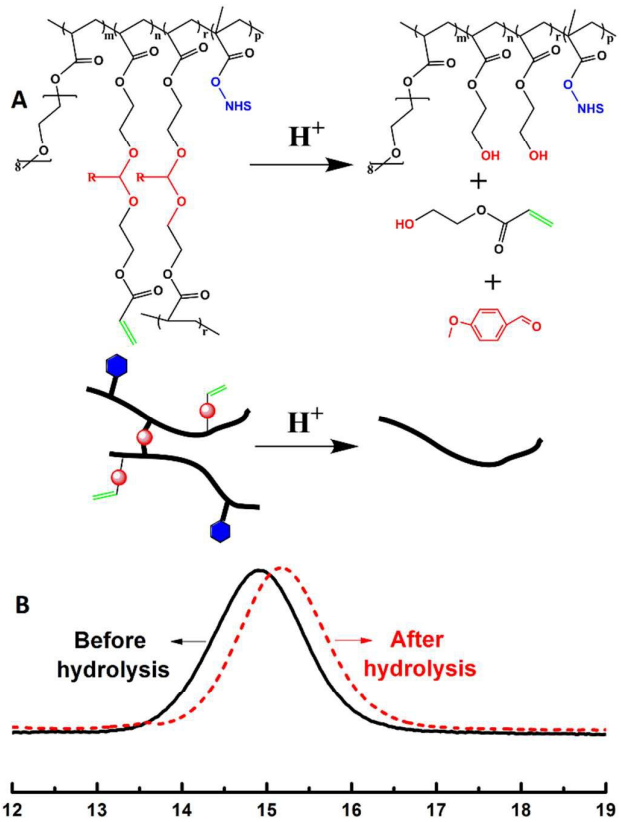

Fig. 2. **(A)** Degradation mechanism of the final polymer into 2-HEA and p-MBA, in acidic solution. **(B)** GPC trace of the branched polymer before and after hydrolysis, at pH 3 for 24h.

At pH 3.0, the polymer was completely hydrolysed in 24 h while at pH 5.0 a decrease in the hydrolysis rate could be observed. Approximately 50% of the polymer degraded after 50h at pH 5 but at neutral pH the branched polymer remained relatively stable for the same period of time. Studies for longer periods (120 hours) demonstrated that at neutral pH the polymer was hydrolysed at approximately 25% while at pH 5 it reached approximately 80%. The above results prove the high stability of the crosslinker at neutral pH and the controlled degradation depending on pH. The degradation studies of collagen sponges crosslinked with the acetal-NHS crosslinker were carried out by measuring the absorbance of the released collagen into

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solutions of various pH values, at 275nm [39, 40] (Fig. 4). In this study, p-MBA after hydrolysis could not be measured because its aldehyde group reacts with the free amines of the collagen thereby rendering it incapable of detection. The complete degradation of the polymer/collagen sponge was determined at pH 3.0 for 114 h. The results showed that the half-life of collagen/polymer was approximately 100h at pH 5.0 and 184h at pH 6.0 and it was relatively stable at pH 7.4.

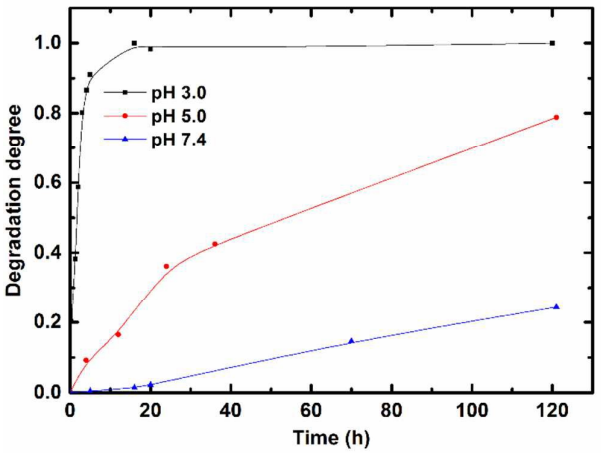

Fig. 3. Hydrolysis of acetal-NHS polymeric crosslinker at different pH value for various degradation times.

Collagen hydrogels were prepared by mixing various amounts of the polymeric crosslinker with collagen type I at 37°C. To determine the crosslinking degree of collagen, TNBS assay was used. [41] TNBS readily reacts with the primary amino groups of amino acids in aqueous solution at pH 8 to form yellow adducts while no coloured derivatives are formed when reacting with secondary amines. The results that are presented in Fig. 5A demonstrate a crosslinking degree of 37±3% when the weight ratio of the collagen to crosslinker is 1:1 and 47±2% when the ratio is 1:2. The crosslinking degree (46±2%) did not increase further when the weight ratio of collagen to crosslinker increased 1:4, showing that a maximum amount of crosslinking had been reached when using the 1:2 ratio.

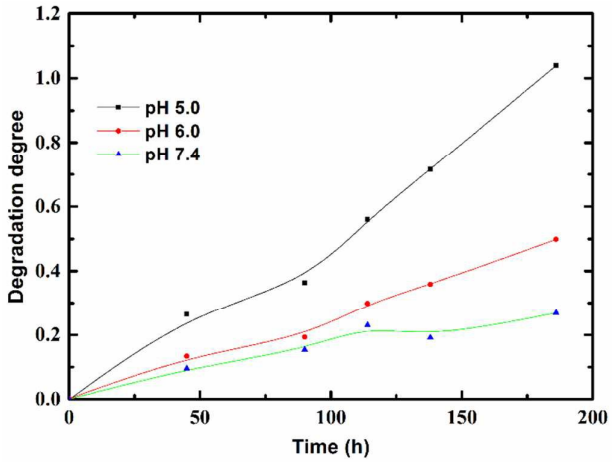

Fig. 4. Degradation of collagen sponge with acetal-NHS hyperbranched polymer crosslinker at different pH value at various time points.

The values presented in this figure are close to each other because in this experiment the amount of the crosslinker held constant while the amount of collagen was altered. By using this specific amount of the crosslinker, which was optimized after a series of experiments (data not given in this work) the degree of the crosslinking wouldn't change drastically. This is also one of the reasons why the collagen is not crosslinked 100% but approximately 46%. The reason why a specific amount of the crosslinker was used was because the use of a higher amount of the crosslinker would result in an undesired increased stiffness of the collagen due to an extending branching which would make it difficult to use. Non-crosslinked collagen was used as a control. Comparative studies were carried out using commercial non-degradable 4-arm polyethylene glycol succinimidyl glutarate (4S-StarPEG, Mw 10,000) as a crosslinking agent. The crosslinking degree of the collagen sponges was found to be lower at 1:1 weight ratio (48%, data not given here) compared to the acetal-NHS crosslinker, which could be because the NHS groups of 4S-StarPEG are located at the end of the PEG chain making it react more easily with free amines. Degradation by collagenase showed an efficient crosslinking reaction, leading to the formation of a stable hydrogel (Fig. 5B).

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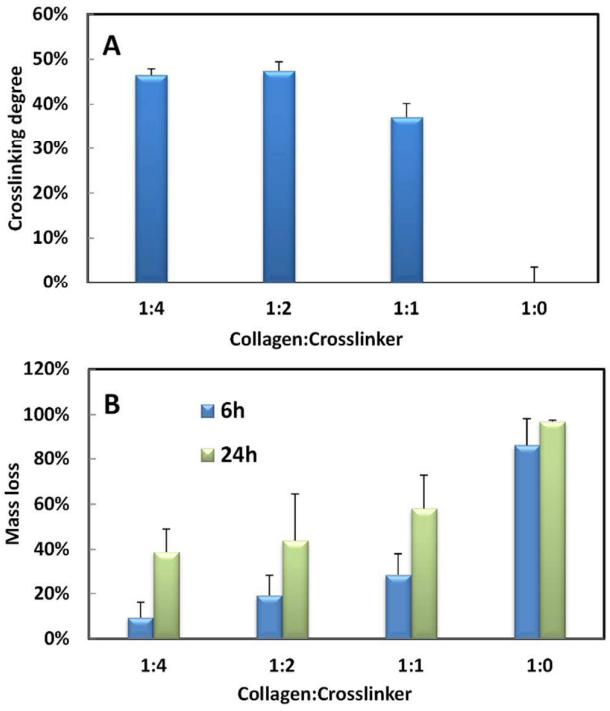

Fig. 5. **(A)** Quantification of the crosslinking degree of collagen sponges by the TNBS assay; **(B)** degradation of the crosslinked collagen sponges using collagenase, for different weight ratios of collagen to polymeric crosslinker. (n=3, mean±SD).

Almost full degradation (98%) was achieved when the collagen was exposed to collagenase for 24h without treatment with the polymeric crosslinker while 58%, 44% and 40% weight loss was observed for the weight ratio (collagen to polymeric crosslinker) of 1:1, 1:2 and 1:4 after 24 h, respectively. Cytotoxicity of the synthesized pH-sensitive crosslinker and of the subsequent pH-sensitive collagen hydrogel has been assessed using the alamarBlue® assay. The crosslinker has been tested in various concentrations up to 1.0 mg/ml. The metabolic activity of the cells remains at approximately 100% for concentrations up to 0.1 mg/ml and is slightly reduced at concentrations higher than 0.5mg/ml. At the maximum concentration of 1.0 mg/ml where the crosslinker was tested the metabolic activity of the cells reduced at approximately 80% concluding that the crosslinker presents reduced toxicity at high concentrations.

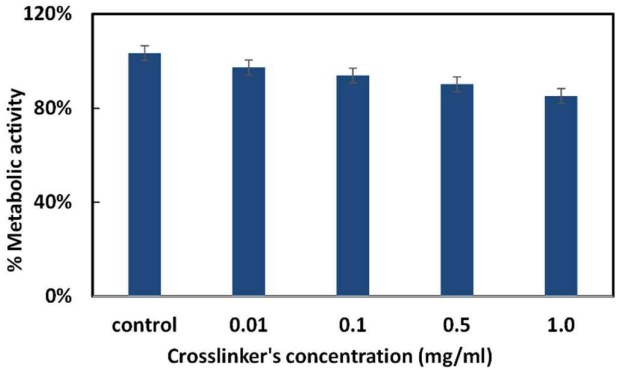

Fig. 6. Cells' metabolic activity of the polymeric crosslinker using alamarBlue® on 3T3 fibroblast cells (5,000 cells/well) for 24 h. No significant differences were noted between the different groups (n = 3, one-way ANOVA, $\rho < 0.2$). Data presented as the mean ± standard error of the mean

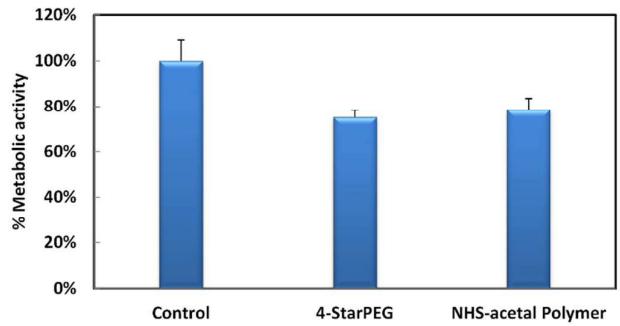

Fig. 7. Cell's metabolic activity of the crosslinked collagen hydrogels using the acetal-NHS polymeric and the 4-starPEG, as crosslinkers. The assessment was carried out using alamarBlue® on 3T3 fibroblast cells (5,000 cells/well) for 24 h (n=3, mean±SD).

The results are anticipated considering that, the crosslinker is based on a PEG segment which is used robustly in biomedical field to reduce the toxicity of various biomaterials (Fig. 6). A comparative study between the synthesized acetal-NHS crosslinker and the commercial 4-StarPEG demonstrated that the synthesized crosslinker is slightly more biocompatible than the commercial one rendering it a good alternative for the fabrication of crosslinked collagen hydrogels. We also believe that the results will be better if a lower amount of the polymeric crosslinker is used.

4. Conclusions

Summarizing, a pH-sensitive, PEG-based, and water-soluble polymeric crosslinker was successfully synthesized using the DE-ATRP method. The crosslinker was composed of NHS monomers that can react with primary amino groups, thus making it an attractive crosslinking material for a variety of synthetic and natural monomers, and was properly modified

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with acetal di-acrylates, the branching units of which can be cleaved at low pH. Hydrolysis studies demonstrated that the half-life of the polymeric crosslinker is closely dependent on pH and this dependency also affects the crosslinked polymeric hydrogels. *In vitro* studies showed that both the crosslinker and the collagen hydrogels were non-toxic and that the as prepared crosslinker demonstrated reduced toxicity when compared to the robust used 4-StarPEG crosslinker. The controlled degradation profiles that depend on the pH-sensitive units of the crosslinker render it a promising applicant for the fabrication of pH-sensitive drug delivery systems and tissue engineered structures for protein-protein interactions.[42-44]

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