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Chemoselective cross-linking of alginate with thiol-terminated peptides for tissue engineering applications

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ABSTRACT

In this study we chemospecifically functionalized alginate with thiol-ended peptides for tissue engineering (TE); a carbodiimide linker and the disulfide exchange scheme were used. First carboxyls of alginate were activated by introducing N-hydroxysuccinimide (NHS) ester groups; these react with primary amines of the heterobifunctional reagent 2-(2-pyridyldithio)ethyleneamine (PDEA). Thiol-reactive alginate (alginate-S-S-py) forms as cross-linking intermediate. The degree of pyridyldithio-functionalization is modified through variable PDEA concentration and it was determined using UV–VIS spectroscopy and proton nuclear magnetic resonance. The applicability of the alginate-S-S-py as platform for the chemoselective coupling of thiol-ended peptides was tested with glutathione as model peptide. Other SH-terminal peptides were successfully cross-linked. Moreover, the peptide–alginate keeps its gel-forming ability when treated with Ca^{2+} cations, leading to functionalized hydrogels that can be further used to obtain different multicomponent systems. The developed chemoselective strategy of functionalizing alginate with thiol-ended peptides could enhance the potential of this polymer for TE.

1. Introduction

Alginates are widely used in tissue engineering (TE) applications ranging from bulking agents, drug delivery systems, cells carrier, and model extracellular matrix (ECM) (for review see: Alexander, Hyun, & David, 2006; Lee & Mooney, 2012).

This polysaccharide belongs to the family of unbranched binary copolymers containing 1-4 glycosidically-linked β-D-mannuronic acid (M) and its C-5 epimer, α-L-guluronic acid (G). Sodium alginate is extracted with a diluted alkali solution from brown algae and is not a random polysaccharide; depending on the algae source, it consists of blocks of similar and strictly alternating residues (i.e. M-blocks, G-blocks, and GM-blocks) (for details see review in Moe, Draget, Skjåk-Bræk, & Smidsrød, 1995). Sodium alginate is soluble in aqueous solutions and forms stable gels at room temperature in the presence of non-cytotoxic concentrations of certain bivalent cations (i.e. Ba^{2+}, Ca^{2+}) through ionic interaction with G groups. This enables hydrogels to be formed, often with viable cells embedded in the gel by cross-linking in non-cytotoxic conditions (Klöck et al., 1997). Advantageous properties like high biocompatibility and nonimmunogenicity made alginate suitable as biomaterial (Shapiro & Cohen, 1997). However, this polysaccharide is unable to specifically interact with mammalian cells (Smetana Jr, 1993). It is widely admitted that cell adhesion can be promoted by decoration of the polymer backbone with cellular adhesion species (Alexander et al., 2006; Rowley, Madlambayan, & Mooney, 1999). On the other hand it is generally accepted that short aminoacid sequences successfully mediate cellular events. A common example consists in the use of arginine glycine aspartic acid (RGD) sequence and its subtypes to promote cell adhesion (Kreeger, Woodruff, & Shea, 2003; Rowley et al., 1999; Simmons, Alsberg, Hsiong, Kim, & Mooney, 2004). It is unlikely that materials modified only with the ubiquitous linear RGD signal will lead to controlled responses of a specific cell type in complex environments (e.g. in vivo) (Healy, 1999). Consequently, other adhesive peptides that can be recognized by the cell membrane need to be used for the design of biomimetic materials for TE. In this context, another amino acid sequence used as heparin binding domain, Lys-Arg-Ser-Arg (KRSR), was demonstrated to enhance osteoblast specific attachment after 4 h while no significant attachment of endothelial cells and fibroblasts was

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observed (Dee, Andersen, & Bizios, 1998). An interesting result of the above mentioned study was that maximum cell proliferation was achieved when both RGD and heparin-binding domain were immobilized using 1:1 molar ratio. This observation was supported by a study using a different heparin-binding domain equivalent, Phe-His-Arg-Arg-Ile-Lys-Ala (FHRRIKA) (Rezania & Healy, 1999). It was demonstrated, that RGD modification alone could not promote focal contact formation while the heparin binding domain synergistically promoted osteoblastic differentiation and mineralization. Furthermore, early studies by Dalton, McFarland, Underwood, and Steele (1995) and Woods, Couchman, Johansson, and Hook (1986) demonstrated that a more "complete" cell response (e.g. cell attachment, spreading, formation of discrete focal contacts, and organized cytoskeletal assembly) was obtained by providing the cell with both the cell-binding (–RGD– containing sequence) and heparin binding domains of fibronectin.

In this context we expect that coupling bioactive peptides to alginate would specifically promote localized cellular events on the functionalized areas, while avoiding non-specific adhesion due to the inert unmodified alginate. On the other hand, with respect to the coupling chemistry, carbodiimide-based strategy was used to link RGD or other peptides to alginate, by the formation of a covalent amide bond between an activated carboxyl group of alginate and a terminal amine of the peptide chain (Bidarra et al., 2011; Rowley et al., 1999). Another option is the coupling of peptides to partially oxidized alginate using sodium cyanoborohydride. This mechanism is based on the reductive amination of aldehydes from alginate with the terminal group of the peptides (Bouhadir, Hausman, & Mooney, 1999).

In this context, we developed a new strategy to chemospecifically immobilize bioactive peptides on alginate in the aim of overcoming the lack of cell activity on this material. The method consists in a two step procedure (as described in Scheme 1). First, the carboxyl group on the alginate backbone was modified by the heterobifunctional reagent 2-(2-pyridyldithio)ethyleneamine (PDEA) and a coupling intermediate was formed. The degree of modification is controlled through the concentration of PDEA. Then, a disulfide exchange between the 2-pyridyldithio sequence incorporated on the alginate and the thiol-terminated peptide was performed. Peptide-grafted alginates preserved the gelling capacity generating hydrogels under mild conditions. This would further allow the development of more complex materials based on combinations of various peptide–alginates. The biocompatibility of the alginate based bioconjugation intermediates was assessed by in vitro assays. Moreover, a preliminary evaluation of calcium hydrogels based on alginate–c(RGD) demonstrated the potential of the bioconjugated alginate–peptides as biomimetic materials able to specifically interact with osteoblastic cells.

2. Experimental

2.1. Materials

Pharmaceutical grade, low viscosity sodium alginate rich in α-L-guluronic residues (approx. 70% of G-block content) was purchased from Medipol SA (Lausanne, Switzerland). 1-Ethyl-(dimethylaminoprolpyl) carbodiimide (EDC) and N-hydroxysulfo-succinimide (sulfo-NHS) were supplied from Sigma–Aldrich (Steinheim, Germany). 2-(2-Pyridyldithio) ethyleneamine (PDEA) was purchased from Biacore AB (Uppsala, Sweden). Glutathione reduced (GSH) was supplied from Acros Organics (NJ, USA) and used as received. The cystein terminated peptides CGGKRSR (KRSR), CGGFHRRIKA (FHRRIKA), and CGGc(RGDfK) (further abbreviated in this manuscript c(RGD)) were prepared and properly characterized by Institute des Biomolecules, Institute Max Mousseron, Universite de Montpellier, France and used as received. Analytical grade 2-mercaptoethanol (ME) was purchased from Merck (Darmstadt, Germany) and used as received. All the salts necessary to prepare phosphate buffer saline (PBS) at pH 7.2 and 8.5 were supplied by Sigma–Aldrich. MilliQ water was used for the buffers as well as for the dialysis. Dialysis membranes Spectra/Por® 7 (MWCO 12,000–14,000 Da) were supplied from Polylab (Antwerpen, Belgium).

Ca-alginate gels for the biological experiments were prepared using calcium carbonate $CaCO_3$ received from Acros Organics (NJ, USA) and D-glucono-δ-lactone (GDL) from Sigma–Aldrich (Steinheim, Germany).

For the cell viability tests, fibroblasts, obtained from 7 day old chicken embryo's, were first cultured in Minimum essential medium (MEM) with Hanks salts (from Gibco BRL (Life Technologies, Merelbeke, Belgium)) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.5% (v/v) penicillin–streptomycin and 1% (v/v) Fungizone® all from Gibco BRL (Life Technologies, Merelbeke, Belgium) in culture flasks purchased from Greiner bio-one (Wemmel, Belgium) and incubated at 37 °C in the presence of air with 5% CO_2. Dulbecco's modified Eagles medium (DMEM) without phenol red, supplemented with 10% (v/v) FBS was received from Gibco BRL (Life Technologies, Merelbeke, Belgium). 3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich (Steinheim, Germany). MTS kit consisting of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and an electron coupling reagent phenazine methosulfate (PMS) in a dilution 1:20 was obtained from Promega (Leiden, The Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich (Steinheim, Germany). Other culture materials, culture plates and test tubes were obtained from Greiner bio-one (Wemmel, Belgium).

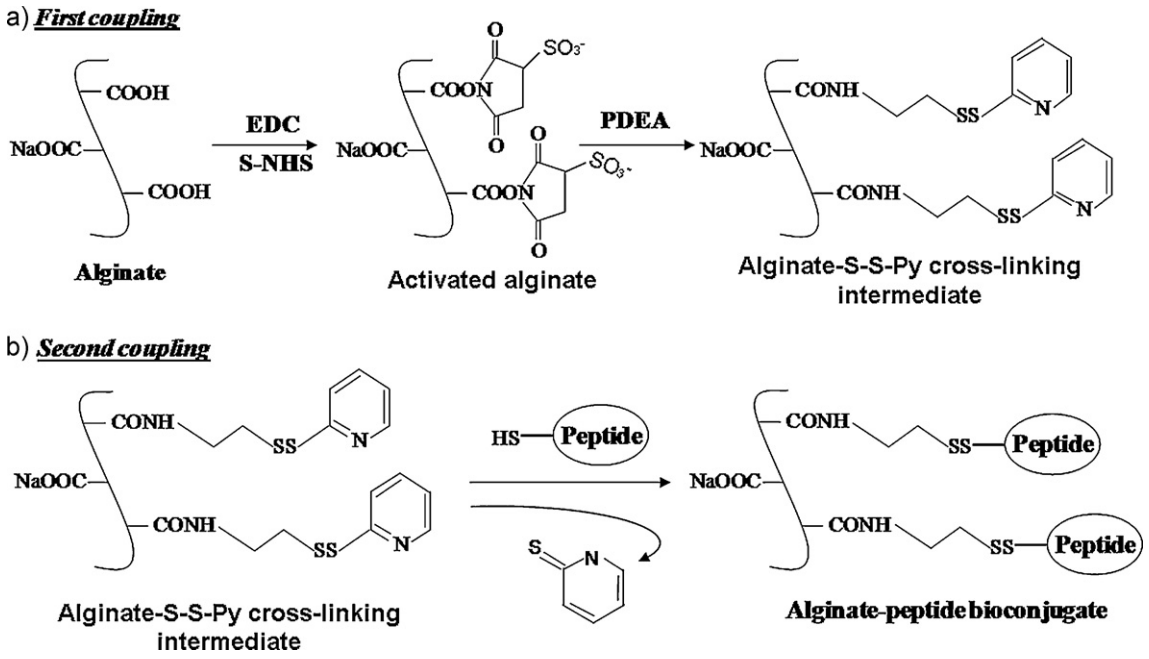
2.2. Synthesis

Alginate–peptide bioconjugates were prepared following a two steps procedure. First, alginate was chemically modified utilizing aqueous carbodiimide chemistry. EDC, a water-soluble carbodiimide, was used to form amide linkages between amine containing heterobifunctional reagent PDEA and carboxylate moieties on the alginate polymer backbone (Scheme 1). EDC activation was performed in the presence of sulfo-NHS. Further, thiol disulfide exchange between 2-pyridylthio sequence from alginate-S-S-py conjugation intermediate and sulfhydryl group from the thiol-terminated peptide was performed, as indicated in Scheme 1. 2-Pyridinethione was released during the reaction.

2.2.1. Alginate modification using PDEA

The chemistry was performed using 1% (w/v) alginate solution in 0.1 M PBS at pH 7.2. Sulfo-NHS was dissolved in alginate solution at a ratio of 1:2 to EDC; the amount of EDC added to the reaction mixture corresponded to 5% of all uronic acids available for the reaction (Rowley et al., 1999). In order to prepare alginate-S-S-py intermediates with various PDEA-content, several molar ratios between the activated carboxyl groups in alginate and the amine groups of PDEA were used, namely 1:0; 1:0.50; 1:0.67; 1:1; 1:2 and 1:5. As an example, the synthesis of alginate-S-S-py bioconjugation intermediate corresponding to a molar ratio of 1:1 between activated carboxyls in alginate and amines in PDEA is described below.

500 mg dry sodium alginate powder was dissolved in PBS (pH 7.2), to a final concentration of 1% (w/v). 13.6 mg sulfo-NHS was added to the alginate solution and it was dissolved during continuous stirring. 24.0 mg EDC dissolved in 1 mL MilliQ water was quickly added to the reaction mixture. 27.8 mg PDEA in 0.1 M PBS pH 8.5 was added after 5 min. The reaction time was set to be 2 h. The



Scheme 1. Reaction scheme of the (a) alginate modification with PDEA and (b) thiol disulfide exchange between alginate-S-S-py conjugation intermediate and thiol-terminated peptides.

obtained alginate-S-S-py product was purified by dialysis against MilliQ water for 24 h and freeze-dried until constant weight.

2.2.2. Preparation of alginate–peptide conjugates

In order to preliminary test the efficiency of the conjugation intermediates to form peptide-grafted alginates through thiol disulfide exchange, their reaction with a SH-ended model peptide, GSH, was followed. Stock solution of 0.1% (w/v) reduced GSH was prepared by dissolving the peptide in degassed PBS (pH 7.2). In a 50-mL two-necked flask equipped with condenser and magnetic stirrer, 0.3% (w/v) aqueous solution of alginate-S-S-py (obtained through dissolution in 0.1 M degassed PBS, pH 7.2) was poured. Next, GSH stock solution was dropwise added, at different concentrations corresponding to different ratios of PDEA in alginate and GSH in the range from 0 to 140%. The reaction is schematically presented in Scheme 1b. The reaction product was dialyzed against MilliQ water for 24 h and the final alginate–GSH bioconjugate was freeze-dried until constant weight. All solvents were carefully degassed before using.

The study continued with the preparation of other three alginate–peptide bioconjugates. The syntheses consisted in the disulfide exchange between alginate-S-S-py conjugate and thiol-terminated peptides, namely KRSR, FHRRIKA, and c(RGD), respectively, using the protocol described above. Coupling of the three peptides was performed for a selected molar ratio between the thiol-reactive end group in alginate-S-S-py and peptidic —SH group, namely 1:1.2 (i.e. 20% molar excess of the peptide was used).

2.2.3. Alginate gel preparation

Alginate gels for cell culture tests were prepared as previously described by Kuo and Ma (2001). Briefly, alginate or/and alginate–peptide bioconjugates were dissolved in MilliQ water to reach final concentration 2% (w/v). 1 M $CaCO_3$ slurry was prepared by suspending 5.0 g $CaCO_3$ in MilliQ water. 180 μL of this slurry was added to the viscous alginate or alginate–peptide solution (200 mg) and was Vortex-mixed for 1 min. Next, 0.6 M GDL was freshly prepared and 1515 μL of this solution was added to the alginate-$CaCO_3$ suspension during intensive stirring with magnetic stirrer. The alginate-based $CaCO_3$-GDL suspension was poured in 24 well-plates to obtain 1 mm high circular gels. The well-plates were stored in high humidity at 4 °C for 48 h, before cell culture tests. All compounds were sterilized in dry form, by ethylene oxide (UZGhent). The gels were prepared under sterile conditions.

2.3. Physico-chemical characterization

2.3.1. Characterization of alginate-S-S-py conjugation intermediate

2.3.1.1. 2-Pyridinethione assay. Dry alginate-S-S-py intermediate was dissolved in 0.1 M degassed PBS (pH 7.2) to obtain 0.3% (w/v) solution. 50 μL of 5.0 M aqueous ME solution was added to 2 mL of this solution, in a UV–VIS cell. The release of 2-pyridinethione following the reaction of with ME was measured at 343 nm, the characteristic wavelength of this compound (Ramanathan et al., 2001). A spectrophotometer Uvikon XL Bio-Tek Instruments UV–VIS was used. The conjugation efficiency of alginate-S-S-py was further calculated based on the calibration curve constructed using PDEA solutions of well known concentrations in the range of 0–0.06 mg/mL (prepared in PBS, at pH 7.2); the measurement was performed after ME treatment.

2.3.1.2. ^{1}H NMR analysis. Solutions for proton nuclear magnetic resonance (^{1}H NMR) analysis were prepared by dissolving 3.0 mg dry alginate or alginate-S-S-py conjugate in 600 μL D_2O. ^{1}H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, operated at 60 °C.

Five different samples of sodium alginate and PDEA mixtures were prepared by dissolving alginate powder in D_2O (5 mg/mL) together with an exact amount of PDEA powder in the range from 0 to 1.3×10^{-2} mg/mL. Three characteristic signals corresponding to the heterocyclic protons of PDEA were observed in the ^{1}H NMR spectra of alginate-S-S-py mixtures in the interval from δ 7.70 to 9.0 ppm. These signals, as expected, distinguished from the signals of the alginate. The mole fraction (F_G) of the 2-pyridinethione was calculated using Eq. (1):

$$F_G = \frac{I_1 + I_2 + I_3 + I_4}{4/I_A} \quad (1)$$

where I_1, I_2, I_3, I_4 are integrated peak areas corresponding to 2-pyridinethione residue at 8.88, 8.25, 8.16 and 7.75 ppm and I_A is an integrated peak area of guluronic H1 proton at 5.46 ppm. Using known concentration of PDEA in different alginate-S-S-py mixtures, the calibration curve was constructed. The degree of substitution (DS) was calculated based on the obtained calibration curve.

2.3.2. Characterization of alginate–peptide bioconjugates

2.3.2.1. UV–VIS analysis of alginate–peptide coupling. The success of the peptide coupling was first investigated through UV–VIS spectrometry, at 343 nm. The same equipment as presented above was used. 3 mg alginate-S-S-py was dissolved in 2000 μL PBS pH 7.2 and then reacted with the appropriate volume of peptide dissolved in PBS pH 7.2 (to a concentration of 1 mg/mL). Experiments were performed using PBS pH 7.2 as control. The kinetic of the reaction was recorded in time drive mode. The amount of consumed PDEA residues during the coupling reaction is converted into amounts of newly formed S-S-peptide and free 2-pyridinethione, respectively, which can be quantified through the increase of the specific UV absorbance at 343 nm. The reaction efficiency was expressed as percentage of alginate–peptide formed, with respect to total PDEA residues in the alginate-S-S-py conjugate. All solvents were carefully degassed before using.

2.3.2.2. ^{1}H NMR analysis of the alginate–peptide conjugates. All alginate–peptide bioconjugates together with the pure peptides were characterized by ^{1}H NMR spectroscopy. 3 mg of the dry alginate–peptide bioconjugate or 10 mg of the pure peptide were dissolved in 600 μL D_2O; ^{1}H NMR spectra were recorded using the same equipment as described above.

2.4. Biological experiments

2.4.1. Toxicity of the biopolymers

MTT test was used to measure the viability of living cells via their mitochondrial dehydrogenase activity (Mosmann, 1983). Alginate, and alginate-S-S-py powders were dissolved in MEM with Hanks salts supplemented with 10% (v/v) FBS, for the MTT assay. The stock solution of the biopolymers was prepared by dissolving 1 mg dry unmodified alginate or alginate-S-S-py conjugate in 1 L of culture medium. The stock solution was sterilized by filtration over a 0.22 μm Syrfil-MF filter, purchased from Whatman Inc. (Germany) and further diluted. The concentration range of the biopolymers was in range from 1 to 100 ppm. The cell viability tests were performed by plating 40,000 chicken embryo fibroblasts (7 day old embryos) in 96 well-plates and incubated for 24 h in a humidified incubator at 37 °C, in the presence of 5% CO_2, until they reached subconfluence. The culture medium was replaced with 200 μL culture medium containing different concentrations of the biopolymers. The fibroblast cells were cultivated in biopolymer solution for 24 h. After this incubation period, the medium was replaced by MTT solution prepared by dissolving 5 mg MTT in 1 mL distilled water filled up to 10 mL with culture medium. The culture plates with MTT solution were incubated for 4 h on a shaker system at 37 °C in the dark. Formazan crystals entrapped in living cells were dissolved in a 1% (v/v) Triton X-100 lysis buffer, prepared by adding appropriate amount of Triton X-100 to the 0.4 N HCl containing isopropanol. The absorbance of the formazan solution was measured after 30 min incubation at 580 nm on a Universal Microplate Reader EL 800 spectrophotometer, Bio-Tek Instruments UV-VIS spectrophotometer. MTT experiments were carried out in triplicate on all samples and % viability was calculated as the absorbance of the test cultures divided by the absorbance of the control cultures multiplied by 100.

2.4.2. Cell adhesion experiment

2.4.2.1. Cell adhesion on alginate hydrogels. MC3T3-E1 (subclone 14) cells (ATCC) were routinely grown in monolayer culture in α-MEM glutaMAX-1™ (Gibco BRL) containing 10 vol % fetal bovine serum (FBS heat inactivated, EC approved) and 1 mM sodium pyruvate (Invitrogen) at 5% CO_2/95% air, 37 °C.

The cultured cells were trypsinized with trypsin-EDTA and washed twice with PBS. For the cell cultivation, 100,000 cell/well were plated in 0.5 mL culture medium in 24 well-plates containing alginate gel (2%) or alginate–c(RGD) bioconjugate conjugate hydrogel (0–14.4 mg c(RGD)/g Alg) prepared 48 h before the cultivation test.

After 24 h, alginate and modified alginate hydrogels were imaged under an inverted phase-contrast microscope (Type U-RFL-T, Olympus, Aartselaar, Belgium).

After visualization, the culture medium was changed and non adhered cells were removed. The % viable and attached cells after 24 h of cultivation on the hydrogels was determined using the MTS assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Cat. No. G5421, Promega). 40 μL MTS solution was added, and the well-plates were incubated at 37 °C in the dark on a mechanical shaker for 4 h. After incubation, 200 μL of this medium was transferred into a 96-well plate and the absorbance of the formed formazan product was measured using a UV-VIS spectrophotometer at 490 nm. MTS experiments were carried out in triplicate on all samples and % cell adhesion was calculated as the absorbance of the test cultures divided by the absorbance of the control cultures multiplied by 100.

2.4.2.2. Cell adhesion on alginate dropplets. Alginate–c(RGD) bioconjugate based hydrogel at a peptide concentration of 14.4 mg/g alginate was tested in an additional cell adhesion experiment using MC3T3 osteoblast cells. 12 well plates were filled with a layer of unmodified alginate (800 μL). After hydrogel formation, 10 μL modified alginate were deposited as dropplets on top of the unmodified alginate hydrogel. 100,000 cell/well were plated in 2 mL culture medium in each well. The cell behavior of the MC3T3 cells on the hydrogel was followed for 1 day by morphological observation by phase-contrast microscopy. Viability of the cells adhered on the alginate dropplets was also evaluated using a live fluorescent staining. Calcein AM (TebuBio, Bouchout, Belgium) was dissolved in DMSO at a concentration of 1 mg/mL. Two microliter of this stock solution was added to 1 mL of PBS solution. Cells were washed twice with PBS, followed by 10 min incubation in the Calcein AM work solution. After rinsing twice with PBS, the hydrogel dropplets were evaluated under a fluorescent microscope (Type U-RFL-T, Olympus, Aartselaar, Belgium).

3. Results and discussion

3.1. Alginate-S-S-py conjugate

3.1.1. Preparation and characterization of the alginate-S-S-py conjugate

The alginate-S-S-py conjugates were prepared by coupling of PDEA a heterobifuntional reagent, via its amine group with carboxylates of the alginate backbone using EDC in the presence of sulfo-NHS as coupling agent (first coupling shown in Scheme 1). The PDEA content in the alginate-S-S-py conjugate, expressed as the degree of substitution (DS), was determined using the 2-pyridinethione assay. This method allows the accurate quantitative evaluation of 2-pyridinethione formed following the reaction of alginate-S-S-py with reducing species like ME. The amount of 2-pyridinethione is equivalent to the PDEA-content in the conjugate. The absorption spectrum of the non-modified alginate and

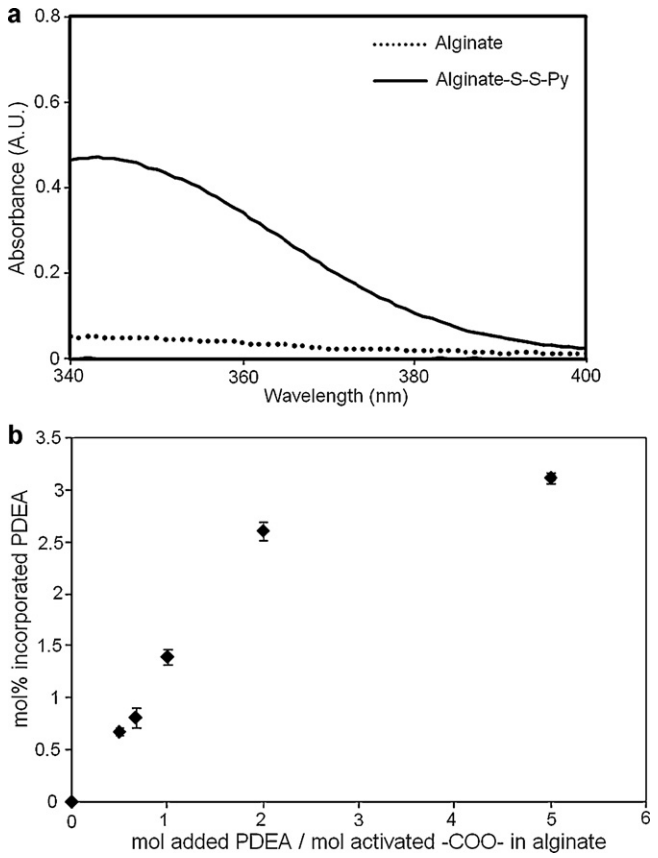


Fig. 1. (a) UV spectra ($\lambda = 343$ nm) of the alginate and alginate-S-S-py treated with ME; PBS was used as blank; (b) Master curve of the alginate-S-S-py synthesis, $n \geq 3$.

alginate-S-S-py before and after the reduction reaction with ME are shown in Fig. 1a. The observed increase in the absorbance, due to the 2-pyridinethione release, was only observed in case of alginate-S-S-py conjugate.

Several alginate-S-S-py conjugates with different degree of substitution in the range between 0 and 5% were prepared using different molar ratios of the PDEA amine groups to activated carboxyl groups in alginate. The concentration of the PDEA obtained in the sample was calculated based on a calibration curve. The DS of PDEA incorporated on the alginate was expressed as the % of modified carboxylates calculated as:

$$\mathrm{DS}\% = \frac{\text{Equiv. of incorporated PDEA}}{\text{Equiv. of modified carboxyls} + \text{non-modified carboxyls}} \times 100 \quad (2)$$

The PDEA incorporation increased with increasing amount of PDEA added and reached a maximum around DS = 3% (approx. 40% of all activated carboxyl groups were modified). Consequently, on one macromolecular chain approximately 30 carboxyl groups were modified and were able, in the following step, to react with the thiol-terminated peptides.

Based on these results a master curve of the PDEA modification was constructed (Fig. 1b).

The qualitative and the quantitative evaluation of the sodium alginate conjugation with PDEA were performed by ^{1}H NMR analysis. The ^{1}H NMR spectra of the non-modified alginate, pure PDEA and alginate-S-S-py conjugate in D_2O at 500 MHz and 60 °C were recorded.

The typical ^{1}H NMR spectrum of the non-modified alginate is shown in Fig. 2a. Three characteristic signals corresponding to the diagnostic peaks of alginate were observed (Chandía, Matsuhiro, & Vásquez, 2001; Laurienzo, Malinconico, Motta, & Vicinanza, 2005; Pluemsab, Sakairi, & Furuike, 2005; Tako, Kiyuna, Uechi, & Hongo, 2001; Zhao et al., 2007). The signal at 5.46 ppm was assigned to

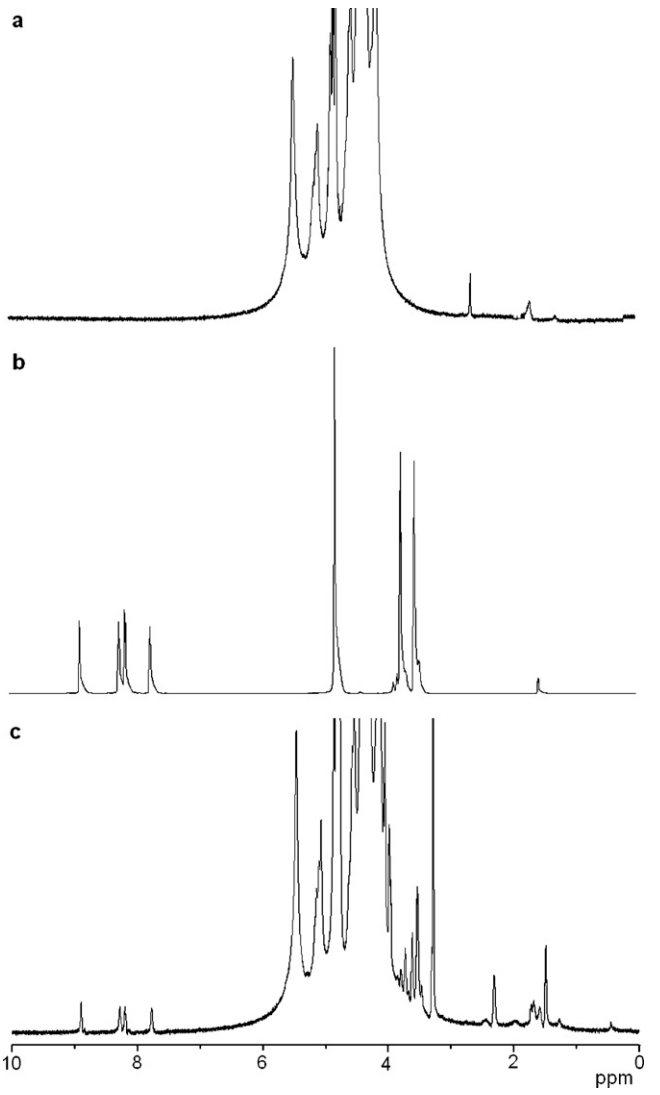


Fig. 2. ^{1}H NMR spectra recorded in D_2O at 500 MHz, 60 °C: (a) non-modifies alginate, (b) PDEA, and (c) alginate-S-S-py conjugate. Presence of 2-pyridinethione protons (7.0–9.0 ppm) is seen in (c).

G-1 (H1 proton of guluronic residue), 5.06-position was assigned to M-1 and GM-5 (H1 proton of mannuronic acid residues and H5 proton of mannuronic acid residue in having neighboring G residue, respectively) and that of 4.87-position was assigned to GG-5 (H5 proton of guluronic acid residue having a neighboring G residue). From these peaks, the monomer composition and fractions of nearest neighbors along the copolymer chain can be calculated. In the ^{1}H NMR spectrum of the pure PDEA (Fig. 2b) 4 characteristic signals, assigned to the heterocyclic protons of the pyridyl ring in PDEA molecule, were observed at 8.88 respectively 8.25, 8.16 and 7.75 ppm. These signals differ from those characteristic for the alginate protons. The signals at 3.76 ppm and 3.54 were assigned to the ethyl group of the PDEA molecule. A typical ^{1}H NMR spectrum of the alginate-S-S-py conjugate was obtained (Fig. 2c) with the attributed structure. The presence of peaks attributed to protons of pyridine at 8.88–7.76 ppm in the PDEA-modified alginate ^{1}H NMR spectrum confirms the expected derivatization.

^{1}H NMR analysis of the alginate-S-S-py conjugates allows a quantitative analysis using the calibration curve. The samples were prepared in D_2O and the ^{1}H NMR spectrum of all samples was recorded at 500 MHz and 60 °C. The concentration of the alginate

Table 1
Amount of PDEA in alginate-S-S-py conjugates as determined by NMR and UV measurements.

Sample	—COOH:—PDEA (molar ratio)	DS [%]	
		^{1}H NMR method	UV–VIS method
1	1:0.5	Not detectable	0.68 ± 0.04
2	1:0.66	Not detectable	0.81 ± 0.10
3	1:1	0.98 ± 0.14	1.40 ± 0.08
4	1:2	1.14 ± 0.32	2.61 ± 0.09
5	1:5	3.81 ± 0.21	3.12 ± 0.05

was the same in all samples (5 mg/mL). The amount of the PDEA varied in range from 0 to 3 mg/mL. The ratio between integrated peak areas of the peak at 5.46 ppm (H1 proton of guluronic residue) and the peak at 5.06 ppm (H1 proton of mannuronic acid residues) were calculated for each sample. The DS's calculated from NMR compared to those calculated from UV measurements are summarized in Table 1.

The DS's determined by ^{1}H NMR analysis presented in Table 1 were in reasonable agreement between the values of the DSs obtained from the UV–VIS spectrophotometric determination for samples 3–5. In case of samples 1 and 2 with low degree of substitution, the ^{1}H NMR analysis was not sensitive enough to detect the specific diagnostic peaks of the pyridyl ring.

3.1.2. Long-term stability of alginate-S-S-py conjugate in PBS

The long-term stability of PDEA-modified alginate was followed by spontaneous 2-pyridinethione release in 0.1 M PBS (pH 7.2) within 24 h. 3.0 mg modified polymer was dissolved in 2 mL PBS and the absorbance of the solution was recorded in time drive mode (Fig. 3a). As a control, another sample prepared from the same stock solution was analyzed before and after ME treatment. In both cases PBS was used as a blank. The absorbance of the alginate-S-S-py solution showed a slow continuous increase. When comparing the absorbance of the alginate-S-S-py solution after 24 h with the sample treated with ME only 2% of the 2-pyridinethione present in the alginate-S-S-py was released.

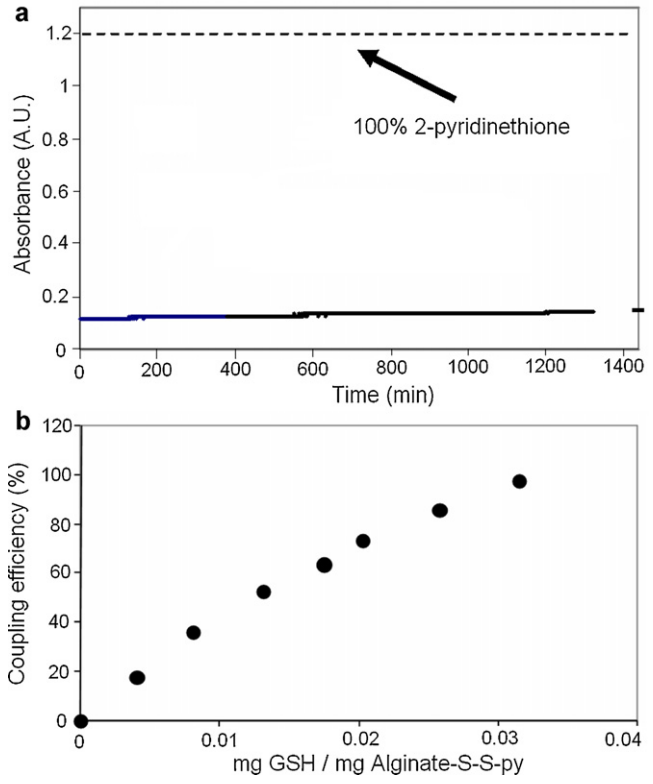


Fig. 3. (a) Long-term stability of alginate-S-S-py in phosphate buffer at pH 7.2. Kinetic curve of the spontaneous 2-pyridinethione (py-2-thione) release determined by UV–VIS spectrophotometry at 343 nm. (b) Coupling efficiency of the alginate-S-S-py intermediate with reduced GSH at different concentrations, $n \geq 3$.

3.2. Peptide conjugation

3.2.1. Model peptide conjugation with alginate-S-S-py

As a model peptide glutathione (GSH), a tripeptide containing a cystein group, was used in its reduced form (Reduce-Imm reduction kit from Pierce). In all experiments PDEA-modified alginate with DS = 1.4% was used as the substrate for the peptide conjugation. In case all PDEA side group react with the thiol-terminated peptide, on each macromolecule approximately 20 peptide molecules will be immobilized. The solution of the alginate-S-S-py for the conjugation was prepared by dissolving the solid white product in degassed 0.1 M phosphate buffer at pH 7.0. The stock solution of GSH (1 mg/mL) was prepared in the same buffer. Different amounts of GSH in the range from 0 to 140%, with respect the equivalents of S-S-py groups in the alginate-S-S-py conjugate, were added dropwise to the polymer solution kept under argon atmosphere. The progress of the reaction and was investigated by recording the changes in UV at $\lambda = 343$ nm absorbance of the solution caused by the 2-pyridinethione release.

The reaction of GSH and the PDEA-residue was fast, the absorbance reached a constant value after 60 min. Based on the kinetic curves of all samples, and the absorbance of the bioconjugate solution after 60 min was taken as the final value. The efficiency of the reaction was calculated as the amount of the released amount of 2-pyridinethione to the theoretically available PDEA residue in the sample to during the disulfide exchange. A maximum efficiency of the reaction of 92.3% ± 0.4, was achieved by adding a 40% molar excess of GSH with respect to the PDEA moieties. The amount of released 2-pyridinethione increased with increasing the amount of GSH; then it was converted into coupling efficiency whose dependence on the GSH/intermediate ratio is shown in Fig. 3b. The obtained values proved the efficient immobilization of the peptide stating that the synthesized conjugation intermediate alginate-S-S-py is suitable for specific cross-linking with thiol-ending peptides. On the other hand the results show that it is possible to control the amount of bound peptide through the modification of peptide concentration.

The ^{1}H NMR spectra of the alginate-S-S-py conjugate, pure GSH and alginate–GSH bioconjugate were analyzed for characteristic peaks. As shown in Fig. 4, peaks corresponding with the pyridyl group (interval 7–9 ppm) were not longer visible in the alginate–GSH bioconjugate (black circled in Fig. 4). Alginate has a very complex ^{1}H NMR spectrum overlapping with the characteristic peaks corresponding to GSH. However, a new peak at 2.9 ppm that could be attributed to GSH was observed (marked as A in the spectrum-Fig. 4), confirming the presence of the GSH in the alginate–GSH product.

3.2.2. Conjugation of the selected thiol-terminated peptides

Three alginate–peptide bioconjugates were synthesized and characterized using UV–VIS spectroscopy and ^{1}H NMR analysis as described before for alginate–GSH conjugate. The kinetic of the conjugation reaction was followed in time drive mode by increased absorbance of the reaction solution. The amount of 2-pyridinethione formed during the reaction showed a continuous increase for approximately 20 min, with a fast release rate in the first 10 min. After 20 min the concentration of the 2-pyridinethione became constant. For all alginate-S-S-py peptide bioconjugates reactions were fast and had similar kinetics. The DS expressed as

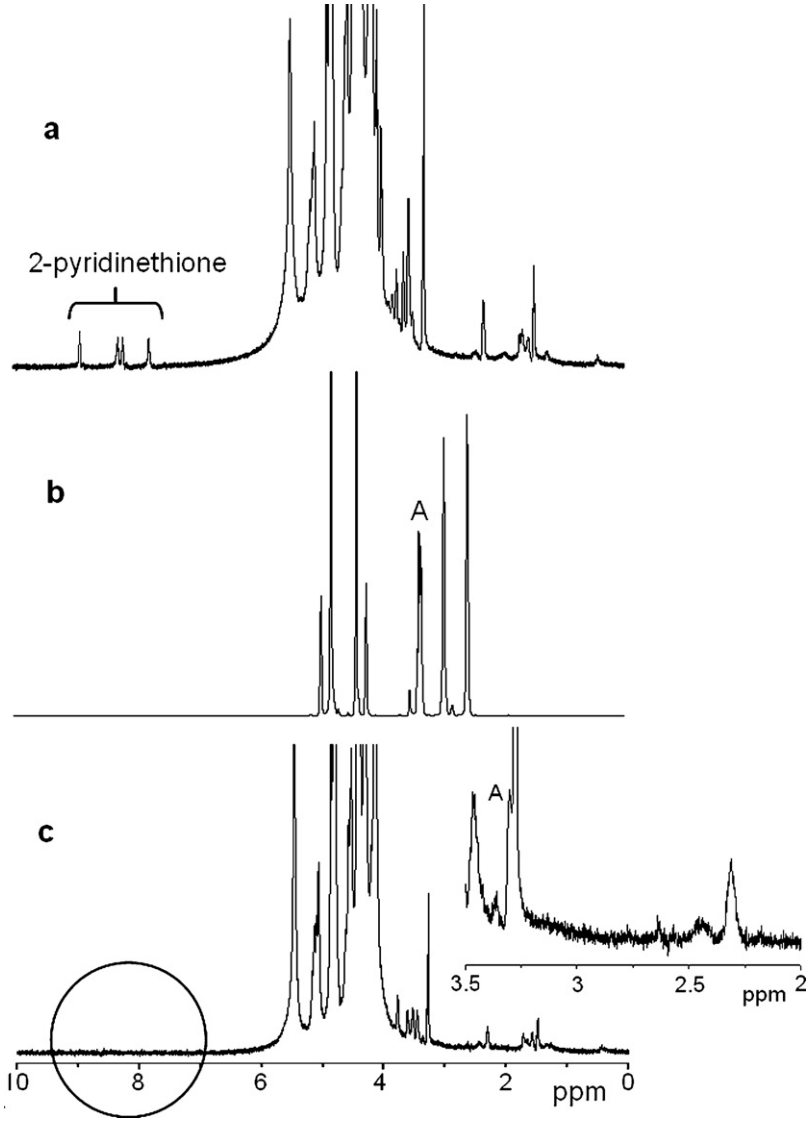


Fig. 4. Characteristic ^{1}H NMR spectra of (a) alginate-S-S-py conjugate, (b) reduced GSH and (c) alginate–GSH bioconjugate in D_2O at 500 MHz, 60 °C.

the percentage of coupled PDEA moieties versus total PDEA content in alginate-S-S-py intermediate was calculated. The coupling yield for the specific alginate–peptide conjugates were respectively 91.1% ± 1.2 for alginate–c(RGD), 91.2% ± 3.6 for alginate–FHRRIKA and 91.5% ± 1.1 for alginate–KRSR bioconjugate.

The ^{1}H NMR spectra of the alginate–peptide bioconjugate were compared with the spectra of alginate-S-S-py and of the corresponding peptides. Signals characteristic for each peptide were identified. Disappearance of the characteristic signals for the pyridyl protons in all the alginate-S-S-py conjugates was observed and this was due to the disulfide exchange between the thiol-terminated peptide and the alginate intermediate (Fig. 5b, d and f). Moreover, a triplet signal at 7.78–7.67 ppm appeared, allowing qualifying the peptide content in the alginate–c(RGD) bioconjugate. For the bioconjugate alginate–FHRRIKA, two intensive new peaks at 7.75 ppm and at 7.60 ppm corresponding to phenylalanine (Phe), and one triplet around 1.30 ppm corresponding to isoleucine confirmed the presence of the peptide in the bioconjugate. Alginate–KRSR conjugate does not have any characteristic signals in the region above 5.46 ppm (Fig. 5f). The entire signals were overlapping with those from the alginate-S-S-py conjugate. However, the disappearance of the pyridyl protons was again observed; several signals forming a broad peak in the range from 1.24 ppm to 2.23 ppm correspond to arginine and lysine in the alginate–KRSR bioconjugate.

3.3. Toxicity of the biopolymers

The novel alginate materials were evaluated to ensure their biocompatibility, comparative to native alginate. This investigation was needed as a first assessment of the toxicity of eventual residual conjugation intermediate remaining in the final bioconjugation products. The effects of unmodified alginate and alginate-S-S-py conjugate on the survival of chicken embryo fibroblast cells was measured using the MTT method performed according to the guidelines of ISO 10 993 for biological evaluation of medical materials.

As shown in Fig. 6a, exposure of the cells to unmodified alginate or alginate-S-S-py conjugate did not affect the cell viability, even at concentrations up to 100 ppm. At the same concentration the cell viability of the 2-pyridinethione was still 70%.

It should be stressed that after the disulfide exchange between the alginate-S-S-py intermediate and the peptides, the amount

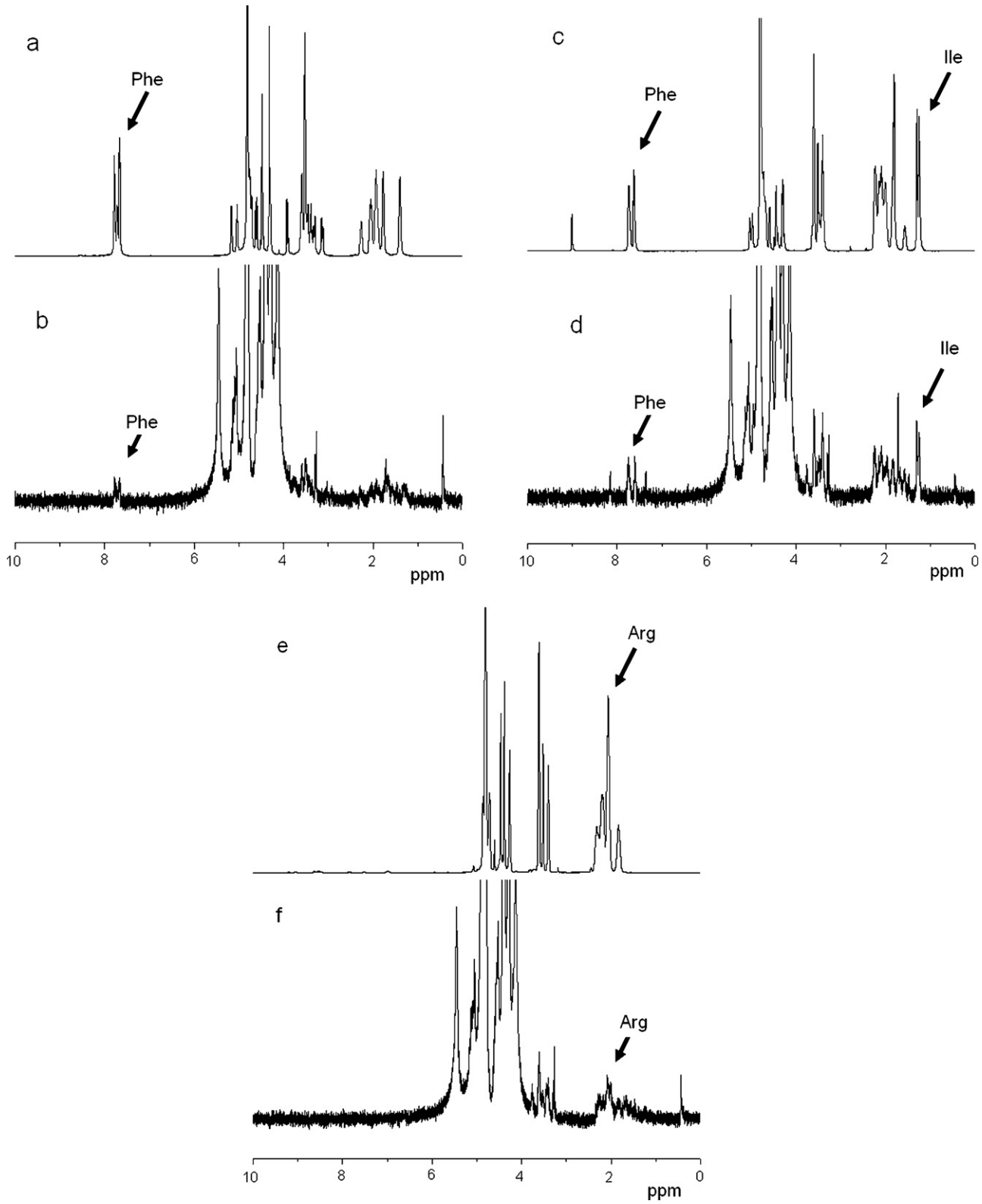


Fig. 5. Characteristic ^{1}H NMR spectra of (a) c(RGD) peptide and (b) alginate–c(RGD) bioconjugate; (c) FHRRIKA peptide and (d) alginate–FHRRIKA bioconjugate; (e) KRSR peptide and (f) alginate–KRSR bioconjugate, recorded in D_2O at 500 MHz, 60 °C.

of non reacted S-S-py group in the conjugate stayed below pmolar concentration. Accordingly, these results are extremely important since they state for no further risk of alginate-S-S-py-related toxicity in the covalently modified peptide–alginate bioconjugates.

3.4. MC3T3 cell adhesion tests

Cell adhesion experiments with MC3T3 osteoblastic cells were performed to illustrate the increased biological interaction of cells with the covalently modified alginates. Osteoblastic cells were

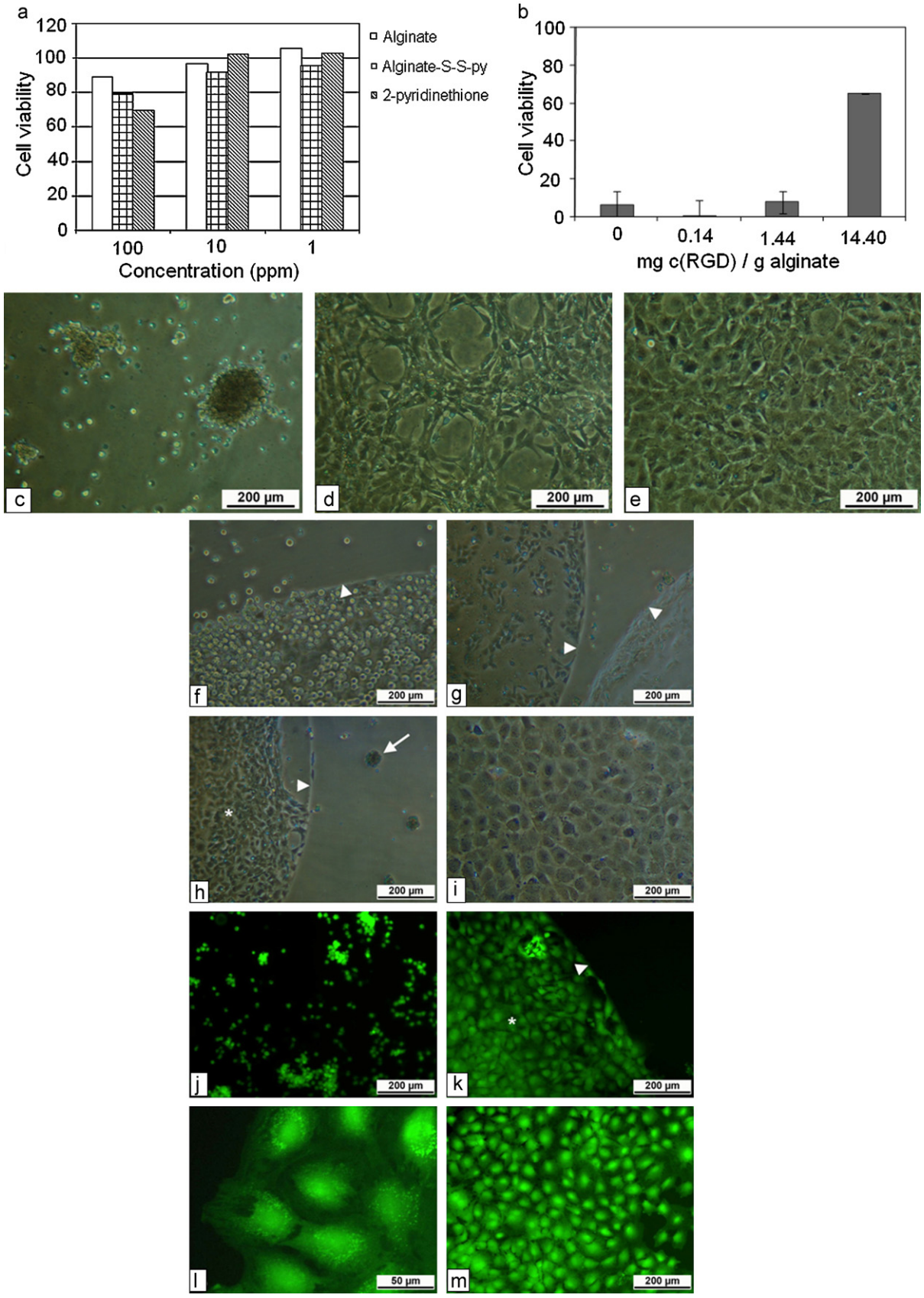


Fig. 6. (a) Cell viability tests performed by MTT assay on unmodified alginate, alginate-S-S-py conjugate, and 2-pyridinethione, $n \geq 3$. (b) Percentage of viable cells relative to the culture plate control after 24 h on alginate hydrogels with increasing RGD concentration. Phase-contrast micrographs of MC3T3 cells 24 h post-seeding cultured on: (c) unmodified alginate hydrogel, (d) RGD-modified alginate (14.4 mg c(RGD) concentration/g alginate) and (e) tissue culture plate (control), after 24 h. Cells on modified alginate hydrogel droplet after (f) 1 h, (g) 4 h and (h) 24 h compared with (i) control. Viability of cells seeded on alginate droplet (j) and modified alginate droplet (k, l (detail)) compared with (m) control. Cells were stained with Calcein AM. *Annotations*: arrowhead, border of the modified alginate droplet; asterisk, confluent monolayer; arrow, cluster of cells.

cultivated for 24 h on the surface of alginate–c(RGD) bioconjugate based hydrogels prepared at three different peptide concentrations 0.14, 1.44 and 14.4 mg/g dry alginate. Fig. 6b illustrates the amount of viable cells adhered on the surface of alginate gels as determined by MTS assay after 24 h incubation. The presence of viable MC3T3 cells is proved by a significant increase of formazan absorbance on the alginate–c(RGD) bioconjugate based gel surface with the highest peptide content. MC3T3 cells seeded onto c(RGD)-coupled alginate hydrogels (14.4 mg c(RGD)/g alginate) were able to attach and were well spread after 24 h (Fig. 6d) comparable with the control (Fig. 6e), while on unmodified alginate cells were unable to adhere and formed cell clusters (Fig. 6c).

In a next step, cell adhesion on alginate gels compared to modified alginate gels was followed in a competitive assay. In this experiment, a layer of non-modified alginate hydrogel was prepared first and poured into the wells. After hydrogel formation, droplets of modified alginate solution were placed on the top of the formed hydrogel. The cell behavior on the alginate gels was followed 1 h, 4 h and 24 h post-seeding. After 1 h, round cells were observed on the modified alginate droplet and the non-modified alginate gel (Fig. 6f). The arrowhead is showing the edge of the modified alginate hydrogel. After 4 h of cell cultivation, the osteoblast cells preferably adhered to the hydrogel droplet (modified alginate gel) rather than to the non-modified alginate gel. Non-attached round cells were observed on the alginate gel between the alginate–peptide bioconjugate droplets (Fig. 6g). After 24 h cultivation, cells on the modified alginate droplet formed a confluent monolayer (Fig. 6h) compared to control cultures (Fig. 6i). The border of the modified alginate droplet covered with cells can be clearly seen, while non-adhered cells were visible on the alginate surface (Fig. 6h).

Adhered cells on the alginate hydrogels were also examined by fluorescence microscopic analysis. After 24 h of incubation, viable cells were observed on alginate (Fig. 6j) and modified alginate hydrogels (Fig. 6k and l) comparable with the control (Fig. 6m). These fluorescent images also confirm the difference in cell morphology of cells cultured on alginate versus modified alginate hydrogels. Cells on alginate hydrogels do not attach and form clusters (Fig. 6j). Cells on modified alginate hydrogels attach and show a well spread morphology (Fig. 6k and l) compared with control cultures (Fig. 6m). Based on these results we can conclude that MC3T3 cells preferably adhere to the surfaces with c(RGD)-covalently immobilized onto alginate.

3.5. Discussion

Alginate polymers meet many requirements for ideal ECM materials, in that they are well characterized, are amenable to sterilization and storage, and may be chemically modified with simple chemistries (Ratner, 1996). Alginate lacks interaction with mammalian cells. An ECM-like derivative prepared from this polymer could be an ideal biomimetic material to promote cell–peptide interactions. Biological responses of the cells cultivated on different substrates are dependent on several factors, but mainly on the spatial distribution or concentration of peptides incorporated into the biomimetic materials and the spacer for peptide modification. Basically, two different methods of the covalent binding of the peptides could be performed: (1) binding of the peptides to the surface of the materials, (2) binding of the peptides on the macromolecular backbone before the more complex material preparation. When chemical modification of the alginate in bulk was performed, a prediction of the available number of peptides on or through the material was more complex. In the present study, we have chemically modified alginate by incorporating osteoblast specific peptides onto the macromolecular backbone using a new alginate-S-S-py intermediate. This new bioconjugation intermediate was synthesized by the direct reaction between the activated $-COO^-$ groups of the alginate using EDC/NHS chemistry and the $-NH_2$ groups of the heterobifunctional reagent PDEA. The amount of the PDEA residues introduced in the alginate could be controlled by the conjugation protocol described before. Using alginate-S-S-py intermediate a very specific and controllable interaction of the alginate macromolecule with selected thiol-terminated peptides could be performed. Reproducibility of the experiment as well as the master curve formed after reaction of various molar ratios between PDEA and activated carboxyls leaded to valuable results confirming coupling of reasonable amount of PDEA with alginate. The alginate-S-S-py intermediate showed a promising potential for peptide coupling chemistry, because they could specifically interact with various types of thiol-terminated peptides. Moreover, these peptides reacted via the free $-SH$ groups, which forced the orientation of the peptide with the cell-active group away from the backbone. The disulfide exchange between alginate-S-S-py and the peptide leaded to the release of 2-pyridinethione which could be quantitatively determined via UV–VIS analysis, as well as ^1H NMR analysis.

The final product was a biologically active compound containing a controlled amount of peptide. By preparing different alginate–peptide bioconjugates a more complex material could be easily prepared by mixing single bioconjugates at well selected concentration ratio. This method is far more practical than preparing polymer conjugates in one step containing several peptides on one substrate. Consequently, the biomimetic materials–cell interaction in the presence of several peptides could be more accurately studied. The alginate–peptide bioconjugates containing active substances bound to the alginate backbone via the disulfide bridge can be stable or can be cut for controlled release based on the application field.

To maintain the biological activity of synthetic peptide sequences upon immobilization, the modified peptide should be flexible and experience minimal steric hindrance. Hern and Hubbell (1998) showed that cells adhered to the RGD modified surface containing PEG spacer (MW 3400) at low surface density (0.01 pmol/cm^2), although the surface modified with RGD without the spacer exhibited limited cell adhesion even at higher surface density (1 pmol/cm^2). Although numerous studies attempt to draw general principles on the level of modification that induces optimal cellular responses, the desirable peptide density and length of spacer may vary depending on the specific cell types or properties of biomaterials. In our work, we have studied the coupling efficiency of the alginate-S-S-py on the model peptide, GSH. The success of the coupling of alginate-S-S-py with GSH was confirmed both directly, through analyzing the structural changes of the obtained product, alginate–GSH versus the reacting species, as well as through the demonstration of the 2-pyridinethione release during peptide coupling. The amount of the coupled peptide was proportional to the amount of the peptide added to the alginate-S-S-py solution. In the next step, three osteoblast specific peptides were used for bioconjugation with the alginate intermediate. The structure of all peptides used in this study contained a cystein at the N-terminus. Two sequences of glycine were introduced as a spacer, to assure the flexibility of the peptides after their covalent coupling onto the macromolecular backbone. Finally, each peptide obtained also specific sequences, which predicted their different function in the biomimetic material–cell interaction (Dee et al., 1998; Healy, 1999; Rezania & Healy, 1999). The selected peptides KRSR, c(RGD) and FHRRIKA were successfully coupled on the alginate. The major advantage of small cyclo-peptides like c(RGD) is their resistance to proteolysis and their ability to bind with higher affinities to integrin receptors than linear RGD (Bogdanowich-Knipp, Jois, & Siahaan, 1999). The coupling reactions are very fast, clean, reproducible and

simple to perform. ^{1}H NMR and UV–VIS spectroscopic procedures were set up to control the syntheses and to characterize the obtained products. The degree of alginate modification can vary according degree of substitution and can lead to different densities of chemically bounded peptides, depending on the application.

The chemical study ended with the preparation and characterization of the peptide–alginate bioconjugates. The experiment was further continued with prelimimary biological investigation of the studied materials. In a first step, cytotoxicity of alginate-S-S-py intermediate was studied with respect to of alginate, and 2-pyridinethione. MTT is a water-soluble tetrazolium salt that can be converted to a water-insoluble purple formazan via the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria (Slater, Swyer, & Sträuli, 1963). The amount of formazan formed can be determined spectrophotometrically and serves as an estimate of the number of mitochondria and hence the number of living cells in the sample (Denizot & Lang, 1986). The tests performed via MTT showed no toxicity on chicken embryo fibroblasts for the tested materials within the concentrations they will be present in a biological system. These experiments highlight the possibility of the use of bioconjugates in in vivo conditions.

Osteoblasts are anchorage-dependent cells that must adhere to the substrate surface prior to undergoing subsequent cell functions such as proliferation, synthesis of collagen and other extracellular matrix proteins or others. Adhesion of osteoblastic cells to substrate surfaces is mediated through the specific interactions of cell-membrane receptors with selected peptide sequences within extracellular matrix proteins (for example fibronectin, collagen, etc.). Rowley and Mooney (2002) compared modified and unmodified alginates showing, that the unmodified alginate could not support myoblast proliferation. Increasing RGD ligand density on high guluronic acid (G) containing gels increased markers of differentiation, but on high mannuronic units (M) containing gels no effect was observed. Wang et al. in their work have shown, that the ability of the bone marrow cells from mature Albino Wistar rats (BMC) to proliferate on high molecular weight G-type alginates may be related to the mechanical properties of those materials and the BMCs ability to adhere and subsequently migrate over the surface. Minimal ligand spacing for cell adhesion in two-dimensional cultures was determined to be 440 nm (Massia & Hubbell, 1991). The preliminary biological study performed in the present work provided evidence that the amount of attached MC3T3 cells on modified alginate hydrogels increased with increasing peptide concentrations. The highest amount of attached cells was reached when cultivating cells on the alginate–c(RGD) bioconjugate based hydrogels with a peptide concentration of 14.4 mg/g dry alginate. The specific adhesion on modified versus non-modified hydrogels was demonstrated by the preferred attachment of cells on modified alginate droplets in contrast to the unmodified alginate layer on the bottom of the multiwell. In these bioconjugates the RGD content was 10 times higher than the GRGDY content in the modified alginates described by Rowley et al. (1999). The alginate–c(RGD) bioconjugate has shown very promising results for further studies of the specific peptide–cell interactions in vitro as in vivo.

4. Conclusions

To obtain alginate-based biomimetic materials, a new specific alginate–peptide coupling strategy was developed. The study started with the preparation of a thiol-reactive alginate intermediate which will selectively react with thiol-terminal peptides. The success of the reactions was investigated through two methods, UV–VIS spectrophotometry as well as by ^{1}H NMR analysis. The extent of modification of the polysaccharide depends on the activation of carboxyls and on the concentration of PDEA used in the synthesis. In vitro cytotoxicity tests demonstrated that the new alginate based intermediate was non-toxic in a wide range of concentrations. GSH was used to prove the coupling efficiency of the conjugation intermediate. Three different alginate–peptide bioconjugates were further synthesized with efficiency above 91%. This proves that the method developed here provides bioactive alginate that can specifically interact with osteoblastic cells. Hydrogels were also prepared from alginate–c(RGD) bioconjugate; the surface coating of alginate with alginate–c(RGD) showed a promising cell-activity. We consider that alginate-based biomimetic matrices with multiple peptide signals promoting specific cell interaction may be obtained using this conjugation, and can be further combined to generate coatings or complex hydrogel compositions useful in tissue regeneration. However, this will be further developed, since it was beyond the aim of this work. We estimate that the alginate–peptide bioconjugation method presented in this study is a promising approach for the development of new biofunctionalized alginate-based materials.

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