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Hyaluronic acid conjugates of glycine peptides and L-tryptophan

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ABSTRACT

This work reports about the conjugation of glycine C-terminal ethyl and methyl ester peptides and L-tryptophan methyl ester with sodium hyaluronate in aqueous solutions using the peptide coupling agent DMTMM (or short DMT, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride). Detailed infrared (IR) absorbance and ^{1}H and ^{13}C (2D) NMR studies (heteronuclear multi-bond correlation spectroscopy, HMBC) confirmed covalent and regioselective amide bonds with the D-glucuronate, but also proves the presence of DMT traces in all conjugates. The ethyl ester`s methyl protons on the peptides` C-terminal could be used to quantify the degree of substitution of the peptide on the hyaluronate scaffold by NMR. The ester group also proved stable during conjugation and work-up, and could in some cases be selectively cleaved in water whilst leaving the amide bond intact as shown by potentiometric charge titration, NMR and IR. The conjugates did not influence the capability of human umbilical vein endothelial cells (HUVECs) to reduce MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) to a formazan dye, which points towards a low cytotoxicity for the obtained products. The conjugation method and products could be tested for tissue engineering gels or drug delivery purposes with alternative, biologically active peptides.

1. Introduction

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan with essential biological functions. The polysaccharide and its derivatives have been used extensively in biomaterial science to prepare gels and scaffolds for cell seeding, tissue growth and regeneration [1]. For these purposes, HA is very often derivatized chemically to alter its biological or physical properties including cross-linking and gelation. Very common chemical modifications of HA are the attachment of cross-linkable groups [2], fluorescent labels, or cell adhesive peptides (RGD) [3]. Peptide conjugation is also inspired by the fact that HA forms large proteoglycan complexes with heavily glycosylated proteins in the extracellular matrix of mammalian tissue, were the protein fractions are non-covalently bound [4]. Several methods exist to prepare polysaccharide peptide conjugates, but almost all use some kind of coupling agents or reactive spacer to increase the reactivity of the functional groups present [5–7]. Examples are brush-like structures [6,7], were the brushes are peptides protruding from the core polysaccharide chain [5], or block-like copolymers, in which the oligopeptides are linked to the polysaccharides´ reducing end via click-chemistry or similar approaches [8,9]. For brush-like structures of HA, very often conventional coupling agents are used to activate relatively unreactive carboxylates. This can either happen through the activation of the polymer backbone, or the activation of the smaller (peptide) molecule to be coupled. Especially in the latter case protecting groups for amino acid side chains would be necessary, and differences in the solubility of polysaccharide and peptide can complicate the conjugation [5]. In any case, a site specific conjugation with an unambiguously defined

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structure of the polymer chain is desired, but very difficult, if not impossible, to achieve with current chemical methods. The most commonly used coupling agent for carboxylates on polysaccharides might be EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide-hydrochloride, often in combination with *N*-hydroxy succinimide (NHS). It could however be shown that EDC cannot easily be removed from activated polysaccharides, [10] as it appears to form persistent covalent bonds with the polymers [11,12]. Authors therefore investigated DMT/DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride) for conjugation of amino group containing molecules, including amino acids, claiming less side reactions and cleaner products compared to carbodiimides [6,13]. Other works report the condensation of HA with furfuryl amine for subsequent cross-linking by a Diels-Alder cycloaddition reaction [2]. However compared to EDC, DMT is not the most often used coupling agent for the modification of hyaluronic acid [14] or polysaccharides in general [6]. An Elsevier Scopus© engine search with the keywords "DMTMM" and "hyaluronic" on February 12th, 2024 gave 128 hits. To contribute to the advances and a better understanding of a covalent hyaluronic acid modification with peptides using DMT, this work investigates the conjugation of the polysaccharide with mono- di-, triglycine esters, and tryptophan methyl ester through amide bonds (Fig. 1). L-tryptophan was chosen to additionally introduce a biocompatible and biobased fluorescent molecule to the polysaccharide [10]. The aim of this work is to better comprehend the conjugation efficiency, and the stability of the amide and ester bonds under the conditions employed. Conjugation of the peptides *N*-terminal to the carboxylate of HA in the presence of either methyl or ethyl ester protecting groups on the C-terminal, probably has several advantages compared to previously investigated structures [5]. The products were characterized by elemental analysis, attenuated total reflectance infrared (ATR-IR), 1D, 2D ^{1}H and ^{13}C NMR, UV–vis and fluorescence spectroscopy. The influence of the conjugates on the metabolic activity of human umbilical vein endothelial cells (HUVECs) was assessed using an MTS assay.

2. Experimental section

2.1. Materials

Sodium hyaluronate (HA, Mw: ~ 90 kDa), diglycine methyl ester hydrochloride (DiGly-OMe·HCl) and triglycine ethyl ester hydrochloride (TriGly-OMe·HCl) were purchased form Carbosynth, United Kingdom. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT), glycine methyl ester hydrochloride (Gly-OMe·HCl), L-tryptophan methyl ester hydrochloride (Trp-OMe·HCl), sodium chloride, methanol hydrochloride (1.25 M HCl), dimethyl sulfoxide (DMSO), deuterium oxide (D_2O, 99.9 %) were purchased from Sigma-Aldrich/Merck, Germany. Primary human umbilical vein endothelial cells (HUVECs) (1 × 106 cells), Medium 200, Low serum growth supplement (LSGS) were purchased from Thermo Fischer Scientific, Austria and CellTiter 96® Aqueous one solution cell proliferation assay was ordered from Promega, Germany. Cellulose dialysis membrane (Mw cut-off: 6 kDa) was purchased from Roth chemicals, Germany. Milli-Q water from a Millipore (MA, USA) water purification system (resistivity ≥18.2 MΩ cm, pH 6.8) was used for all aqueous solutions during these experiments.

2.2. Synthesis of hyaluronic acid conjugates

HA (3.2 mmol COONa, 1.287 g) was dissolved in 100 mL of Milli-Q water and stirred overnight. DMT (3.2 mmol, 0.886 g) and either nothing, or Gly-OMe·HCl (16 mmol, 2.01 g), DiGly-OMe·HCl (16 mmol, 2.92 g), TriGly-OEt·HCl (16 mmol, 4.06 g) or Trp-OMe·HCl (6.4 mmol, 1.63 g) were added to the HA solution. The reaction times were 24 h for HA DMT, 48 h for HA-Gly-OMe and 72 h for all other products. The native pH value of the reaction solutions is pH 4–6 due to the hydrochloride salts used. The reaction mixtures were then dialyzed for 1 day against 2 M NaCl solution and 3 days against Milli-Q water with the dialysate exchanged every 4 h, frozen for 2 days at −37 °C and lyophilized for 2 days at −37 °C and 103 mbar yielding well water soluble products in all cases.

The samples are labelled as follows: HA (sodium hyaluronate), HA-DMT (control reaction, no peptide), HA-Gly-OMe, HA-DiGly-OMe, HA-TriGly-OEt, HA-Trp-OMe for the respective peptides or amino acid ester derivatives of hyaluronate (Table 1).

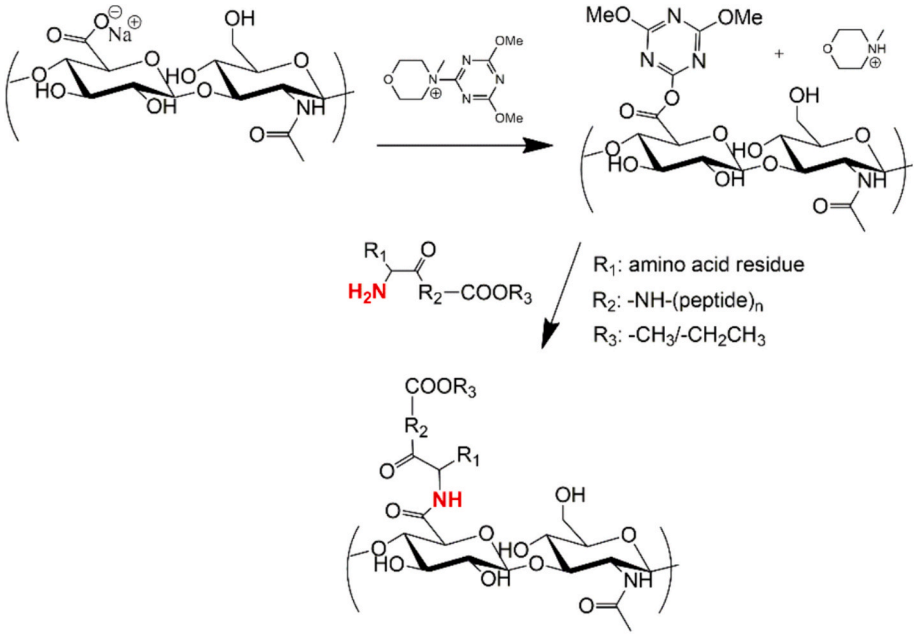

Fig. 1. Reaction pathway for the aminolysis of DMT activated hyaluronate using methyl or ethyl ester C-terminal protected amino acids/peptides, forming amide bonds with the polysaccharide.

Table 1
Sample list and their relative H/C/N mass content.

Sample	H%	C%	N%
$HA_{theor.}$	5.26	41.79	3.48
HA-DMT	6.13 ± 0.7	35.84 ± 0.18	2.89 ± 0.03
HA-Gly-OMe	n.d.	n.d.	n.d.
HA-DiGly-OMe	6.18 ± 0.00	37.35 ± 0.06	4.70 ± 0.09
HA-TriGly-OEt	5.02 ± 0.11	37.78 ± 0.01	6.24 ± 0.04
HA-Trp-OMe	6.23 ± 0.13	46.32 ± 0.04	6.60 ± 0.04

2.3. H/C/N mass content, infrared-, UV–vis- and fluorescence spectroscopy

An elemental analyzer vario MICRO cube, from Elementar Elementar Analysensysteme GmbH, Langenselbold, Germany was used to determine the C/N/H mass content of 2 mg samples in duplicates. Sulfanilamid was used as a calibration standard along with He as a carrier and oxygen as the combustion gas. ATR-FTIR spectra for all products in powder form were recorded using a Perkin Elmer Spectrum GX Series-73,565 FTIR-spectrometer with a scan range of 4000 to 650 cm^{-1}. All samples were subjected to 32 scans at a resolution of 4 cm^{-1}. UV − vis absorbance and fluorescence spectra were measured with an Infinite M200 (Tecan Trading AG, Switzerland) multimode microplate reader, at room temperature (excitation wavelength: 280 nm). The purified, isolated products were dissolved in Milli-Q water at a concentration of 0.5 mg mL^{-1}.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were acquired on an Agilent Technologies DD2 600 MHz NMR spectrometer equipped with 5 mm HCN cryoprobe, and on an Avance Neo 600 MHz NMR spectrometer equipped with 5 mm QCI cryoprobe. Samples were dissolved in deuterated water (D_2O). The temperature for the samples was set at 298 K. The Larmor frequency of proton and carbon nuclei was 599.66 MHz and 150.80 MHz, respectively. ^{1}H and ^{13}C NMR chemical shifts are reported in parts per million and referenced with respect to the deuterated trimethylsilylpropanoic acid sodium salt (TMSP-d_4 δ 0.0 ppm). All NMR data were processed using Mnova software (Mestrelab Research S.L.).

2.5. Potentiometric charge titration

pH-potentiometric charge titration of the samples was carried out with a two-burette automatic titrator T70 (Mettler Toledo, Switzerland), under inert atmosphere (nitrogen gas bubbling). 0.1 M HCl and 0.1 M KOH were used as titrants in the burettes. The analyte was prepared by dissolving the samples in MilliQ water (1.5 mg mL^{-1}) and adjusting the ionic strength to 0.1 M with a 3 M KCl solution. The analyte was titrated forward (acidic to alkaline) and backward (alkaline to acidic) between $2 < pH < 11$. The pH values were recorded with a combined glass electrode (InLab Reach, Mettler Toledo, Switzerland). All samples were measured thrice. Under the same conditions as the analyte, a blank HCl–KOH titration was performed. Plotting Q/m (pH) titration isotherms yielded the total quantity of charged functional groups (e.g., carboxylic groups). Where Q/m represents the number of charged functional groups per mass of sample in mmol g^{-1}. Further details on the data treatment technique and computations are described elsewhere [15].

2.6. MTS cell viability assay

Cryopreserved primary culture HUVECs were thawed in a water bath at 37 °C and seeded into cell culture flasks at a density of 2500 cells cm^{-2}. Cells were expanded until 80 % confluency at 37 °C in a humidified atmosphere of 5 % CO_2 in Medium 200 containing LSGS, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin (all Gibco™).

For the experiment, HUVECs were seeded in 3 individual 96-well plates at a density of 10^4 cells mL^{-1} at 37 °C. Sample solutions were prepared by dissolving 10 mg polymer in 10 mL cell culture medium. These samples were diluted to 3 different concentrations (150–15-1.5 μg mL^{-1}). After 24 h of incubation when cells reached 70–80 % confluence, the medium was removed and replaced by 100 μL of sample solution. All samples were prepared in triplicates and the cell culture medium used as negative and 5 *v*/v % formaldehyde as positive control. Cells were incubated with sample solutions for 24 h at 37 °C. For the MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) test Cell Titer 96®AQ_{ueous} one solution reagent was thawed completely in a water bath at 37 °C. 20 μl of solution was added into each well and incubated in 37 °C for 3 h. Absorbance was recorded at 490 nm using a Plate CHAMELEON™ Multilabel plate reader (Hidex Oy, Finland).

3. Results and discussion

3.1. H/C/N mass content

Because of the presence of nitrogen in HA, determining the degree of substitution from the nitrogen content in the samples is arithmetically not possible. However, an increased nitrogen content can be observed for all derivatives compared to the control reaction with DMT and the theoretical composition of HA (Table 1). The N content is the highest for HA-TriGly-OEt and HA-Trp-OMe, owing to the presence of higher relative masses of nitrogen in the substituent. The presence of the carbon rich aromatic indole ring of Trp-OMe is also indicated by a significant increase in the carbon content of HA-Trp-OMe.

3.2. Infrared spectroscopy

Fig. 2 depicts the ATR-IR spectra of all samples (left) and the unbound amino acid/peptide esters (right). The characteristic peaks for HA are visible at 3287 cm^{-1} for the hydroxyl valence vibration. The C—H stretching at 2888 cm^{-1} the C═O stretching of carboxyl group at 1605 cm^{-1}, and the C—O ring vibration of the saccharides at 1040 cm^{-1} are visible in all products. The spectra of HA-DMT has very similar peaks as HA and no significant peaks could be attributed to the presence of DMT. The carbonyl stretching vibration of the ester bonds of the glycine and L-tryptophan methyl esters, and the glycine peptides between 1727 and 1750 cm^{-1} also appear in the products and can be distinguished from carboxylates (HA 1605 cm^{-1}) and amide bond carbonyls (amide I, 1692 cm^{-1} DiGly-OMe, 1642 cm^{-1} TriGly-OEt). The amide bonds formed through the conjugation to HA can be addressed to the carbonyl signals between 1660 cm^{-1}–1650 cm^{-1} (amide I) in all products, especially visible for HA-Gly-OMe and HA-Trp-OMe. These signals overlap with the amide bonds within the two peptides in HA-DiGly-OMe and HA-TriGly-OEt. The typical amide NH vibration for monosubstituted amides in the two peptides around 3400 cm^{-1} are covered by the OH vibrations of the polymer backbone in the products. In HA-Trp-OMe the peak at 749 cm^{-1} can be assigned to the aromatic C—H bending of the indole ring [10]. These findings indicate the presence of the intact ester protecting group, and a covalent conjugation of the substituents to HA via amide bonds.

3.3. NMR

3.3.1. HA glycine peptide derivatives

3.3.1.1. ^{1}H NMR of HA glycine peptide derivatives. Fig. 3 shows the proton spectra of HA and all glycine related products. Most of the chemical shifts are observed in the region between δ_H 3.2 and 4.6 ppm, which are characteristic for the hyaluronan scaffold. Signals at δ_H

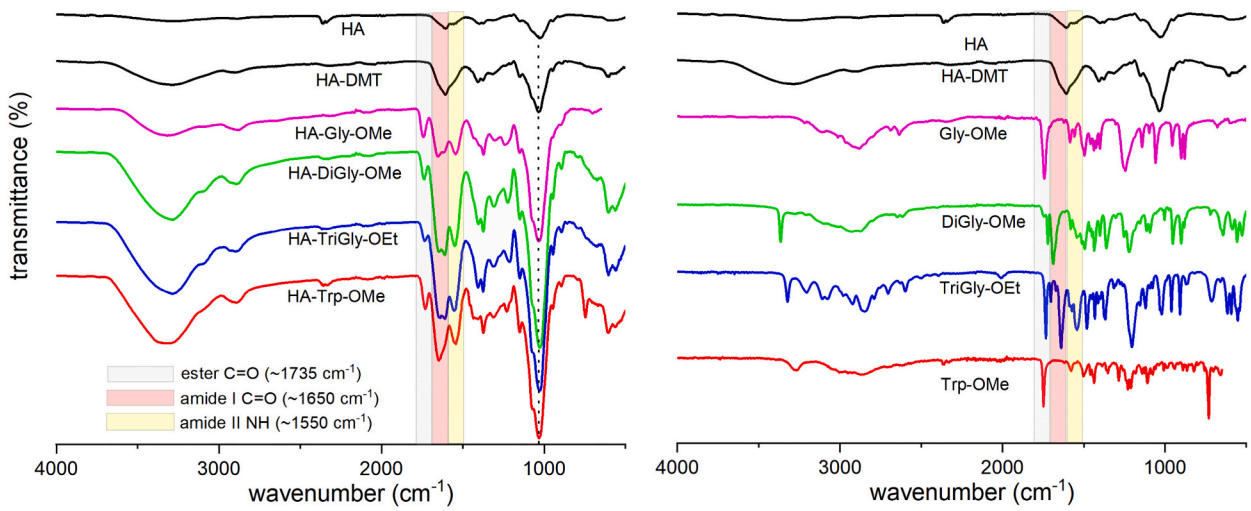

Fig. 2. ATR-IR spectra of sodium hyaluronate (HA), reacted with the coupling agent (HA-DMT) and the respective products (left, HA-Gly-OMe, HA-DiGly-OMe, HA-TriGly-OEt, HA-Trp-OMe) obtained from the reaction of glycine methyl ester hydrochloride (Gly-OMe), diglycine methyl ester hydrochloride (DiGly-OMe), triglycine ethyl ester hydrochloride (TriGly-OEt) and L-tryptophan methyl ester hydrochloride (Trp-OMe) (right). The regions of ester (C=O), amide I (C=O), and amide II (N—H) absorbance are labelled. Amide *I* (C=O) and amide II also occur in unmodified HA, and partially overlap with the carboxylate (C=O) of the polysaccharide.

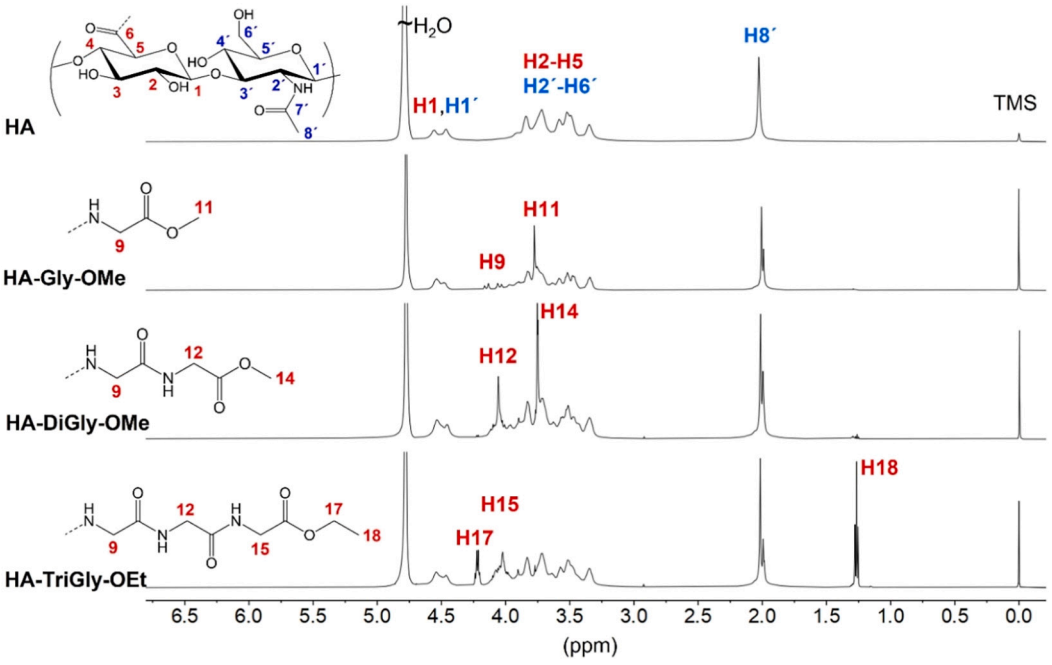

Fig. 3. ^{1}H NMR spectra (D_2O) of sodium hyaluronate (HA) and conjugated glycine products.

4.4–4.6 ppm are attributed to the H1/H1' anomeric protons of HA. The methyl protons on the *N*-acetyl HA moiety (H8) are clearly visible in all samples at δ_H 2.0 ppm. The sharp signal of the (Di)Gly-OMe methyl protons at δ_H 3.8 ppm (H11/H14) confirms the presence of the methyl esters. Moreover, the methylene H17 (δ_H 4.22 ppm) and the methyl H18 protons (δ_H 1.27 ppm) of the TriGly-OEt are present in the HA-TriGly-OEt sample. Assuming the absence of unbound peptide, the degree of substitution of the tripeptide in HA-TriGly-OEt can be conveniently calculated via integration of the *N*-acetyl -CH_3 and ethyl ester -CH_3 proton peaks (DS 0.38). The remaining anionic charges are obviously sufficient for the polymer to remain water soluble. This might change at higher degree of substitution, or the introduction of more hydrophobic amino acid residues into the peptides. It is however not possible to confirm a covalent bond to the HA backbone from the ^{1}H NMR spectra.

3.3.1.2. ^{13}C NMR of HA glycine peptide derivatives. Fig. 4 shows the ^{13}C NMR spectra of HA and all glycine related products with the following signals: *N*Ac carboxy carbonyl group (C7) at δ_C 178 ppm, D-glucuronate carbonyl group (C6) at δ_C 173–174 ppm, the anomeric carbons (C1´C1) 103 and 106 ppm, hyaluronic scaffold in the region between δ_C 70 and 86 ppm (C2-C5 and C3-C5). The *N*-acetylanhydroglucosamine methylene group (C6) at δ_C 63 ppm, the C2´ group at δ_C 57 ppm, the methyl ester group at δ_C 56 ppm, the glycine methylene group at δ_C 44 ppm and the *N*-acetyl methyl group at δ_C 25 ppm. The signals of the carbonyl region of the products become more complex with an increase in the chain length of the peptide (Fig. 5). The formation of an amide bond between the HA carboxylate (peak 6) and the substituents can be seen by an upfield shift of the HA carbonyl signal (peak 6a). Minor traces of the triazin ring of DMT are visible in the 170 ppm region of **HA-TriGly-OEt**.

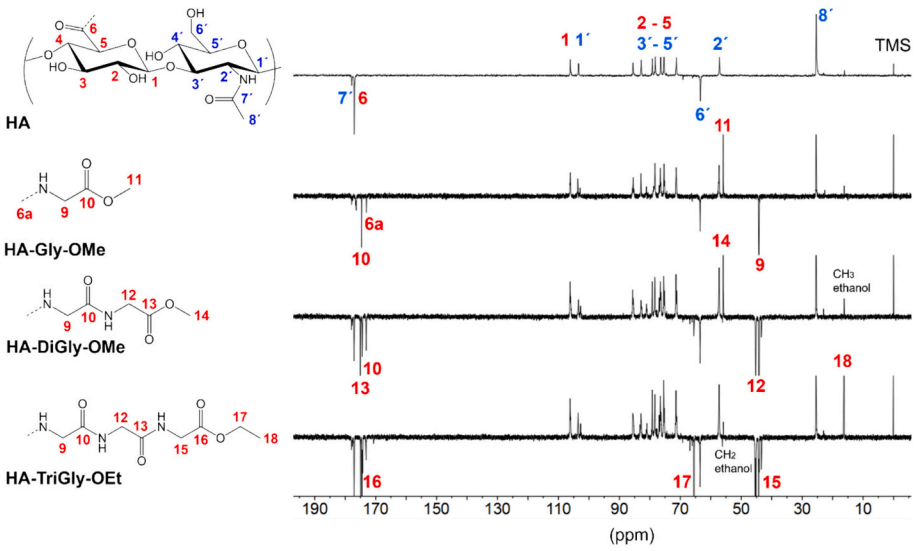

Fig. 4. DEPTQ-135 ^{13}C NMR spectra of sodium hyaluronate (HA) and conjugated glycine products in D_2O.

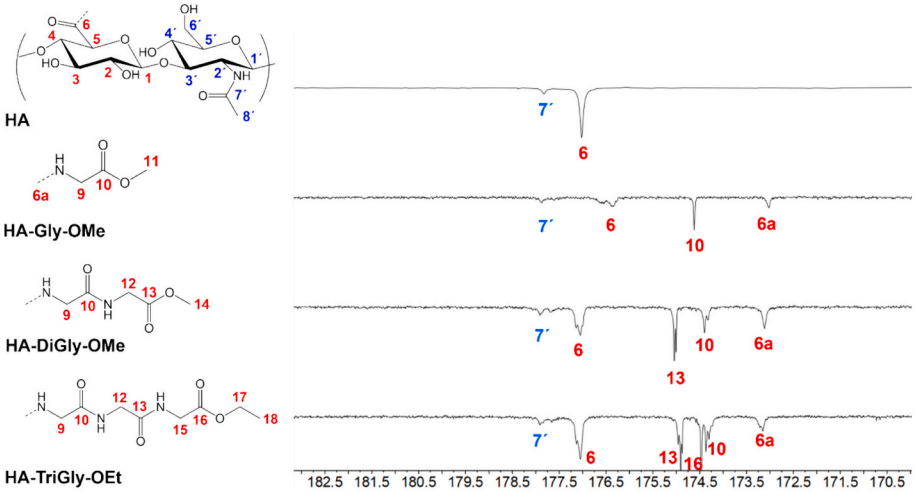

Fig. 5. Carbonyl region of the DEPTQ-135 ^{13}C NMR spectra of sodium hyaluronate (HA) and conjugated glycine products HA-Gly-OMe, HA-DiGly-OMe, HA-TriGly-OEt in D_2O.

Long-range correlation signals observed in the ^{1}H—^{13}C HMBC spectra support a covalent bond of the glycine peptides to the hyaluronan scaffold. The key correlation signals are marked with a dashed line, which shows correlation between the H9 methylene group of HA-DiGly-OMe and C6a as well as C10 carbon atoms (Fig. 6). In addition, HMBC correlation signals are visible for the C13 carbonyl and H14 methyl protons of HA-DiGly-OMe.

Similarly, 2D NMR data confirms covalent bonding of the triglycine ethyl ester, and accordingly the formation of HA-TriGly-OEt conjugates (Fig. 7). Dashed lines in this figure show the key correlation signals between H9 methylene group of the peptide with C6a and C10 carbon atoms (Fig. 7). For both products, ^{1}H signals of the triazine methoxy protons correlate with the carbon atoms of the triazin ring (170–171 ppm range) and likely with the C6a of HA. This indicates that free or HA bound DMT residues are present, which is very relevant with respect to the purity of the conjugates and the suitability of the coupling agent compared to EDC/NHS [13] . Under the conditions given, not all DMT could be removed.

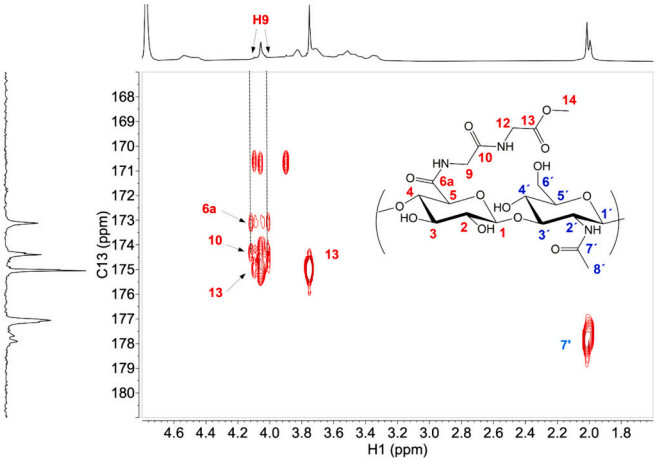

Fig. 6. ^{1}H—^{13}C HMBC spectrum of HA-DiGly-OMe in D_2O.

3.3.2. *HA tryptophan derivative*

The ^{1}H and ^{13}C NMR spectra of hyaluronic acid with covalently

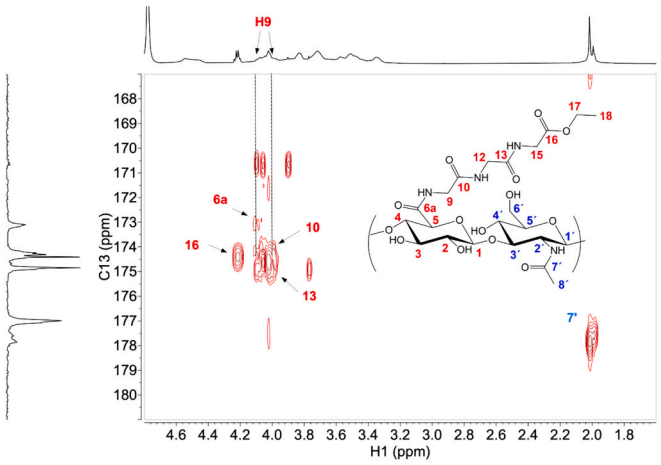

Fig. 7. $^{1}H—^{13}C$ HMBC spectrum of HA-TriGly-OEt in D_2O.

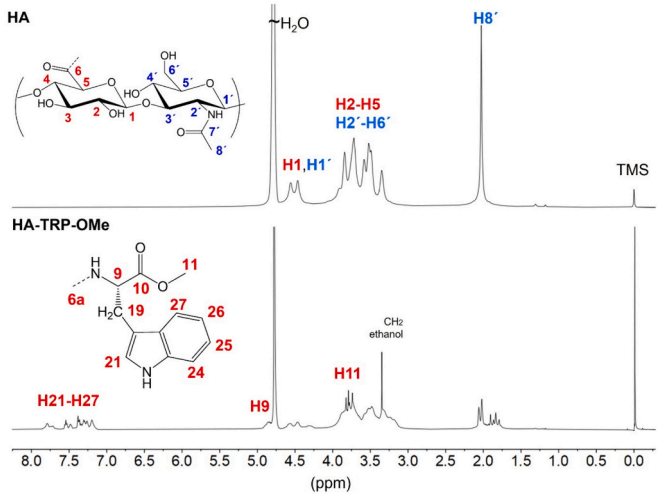

Fig. 8. ^{1}H NMR spectra of sodium hyaluronate (HA) and the L-tryprophan methyl ester conjugated HA-Trp-OMe in D_2O.

attached Trp-OMe, are shown in **Figs. 8 and 9**. Beside characteristic hyaluronan ^{1}H NMR chemical shifts between 3.35 and 4.56 ppm additional signals were observed in the aromatic region between 7.0 and 7.8 ppm, which correspond to proton nuclei of tryptophan residue (H21-H27), the methyl ester signal (H11) at 3.35 ppm and the methylene group (H19) of Trp-OMe at 3.79 ppm. The presence of a distinct splitting in the NAc signal, can be interpreted as different chemical environments in the polymer chain. Because of the presence of distinctive peaks from the tryptophan indole ring in the aromatic region, it was possible to calculate the degree of substitution of Trp-OMe in the sample. Integration of indole ring peaks between 7.0 and 8.0 ppm and relating to the *N*-Acetyl (NAc) methyl protons gives a DS Trp of 0.5. In addition, ^{13}C DEPT-135 NMR spectrum showed signals in the aromatic region (C20-C28; 110–140 ppm), which can be attributed to the Trp moiety, as well as characteristic ^{13}C DEPT-135 NMR signals as follows: NAc and Trp methoxy ester group (C7' and C10) at 178 ppm, HA amide (C6, peak 6a) at 173 ppm, methyl ester CH_3 group (C11) at 56 ppm, Trp methylene group (C19) at 29 ppm.

3.4. UV–vis and fluorescence spectroscopy

The UV–vis absorbance (230–400 nm), and the fluorescence emission spectra (λ_{ex}: 280 nm, λ_{em}: 310–400 nm) of the products are shown in Fig. 10. Significant UV–vis absorbance could be observed for HA-TriGly-OEt and HA-Trp-OMe. The reason for the unexpected UV-absorbance of the tripeptide conjugate is not clear, but could be attributed to the presence of DMT or some cyclization reaction of the peptide during coupling or workup. Peptides and proteins are frequently studied using fluorescence spectroscopy. Tryptophan, tyrosine and phenylalanine are well known fluorescent aromatic amino acids that can be used as probes to study conformation and dynamics [16]. The indole group is the primary reason for absorbance at 280 nm and emission at 350 nm. Any change in the tryptophan microenvironment effects the tryptophan fluorescence [17]. The conjugation technique used here leads to a water-soluble fluorescent HA derivative that can potentially be applied in imaging or for Förster resonance energy transfer (FRET) measurements [18]. Contrary to a lower solubility of L-tryptophan modified carboxymethyl cellulose reported previously [10], HA-Trp-OMe are well soluble products at this degree of substitution (DS 0.5, see chapter 3.4.2).

3.5. pH-dependent charge isotherms

Fig. 11 shows the forward and backward titration isotherms of the unmodified polysaccharide (A-B), the glycine methyl ester, the

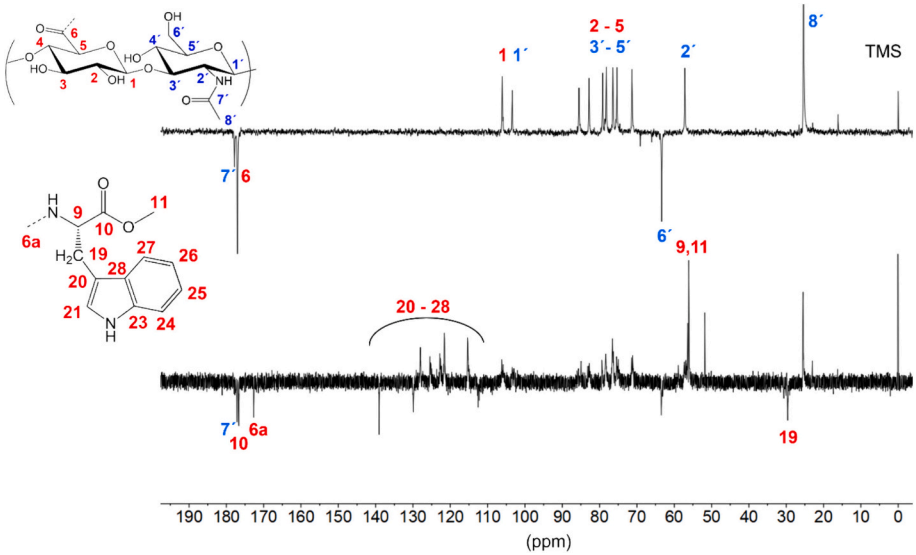

Fig. 9. DEPTQ-135 ^{13}C NMR of hyaluronic acid (HA) and HA-Trp-OMe.

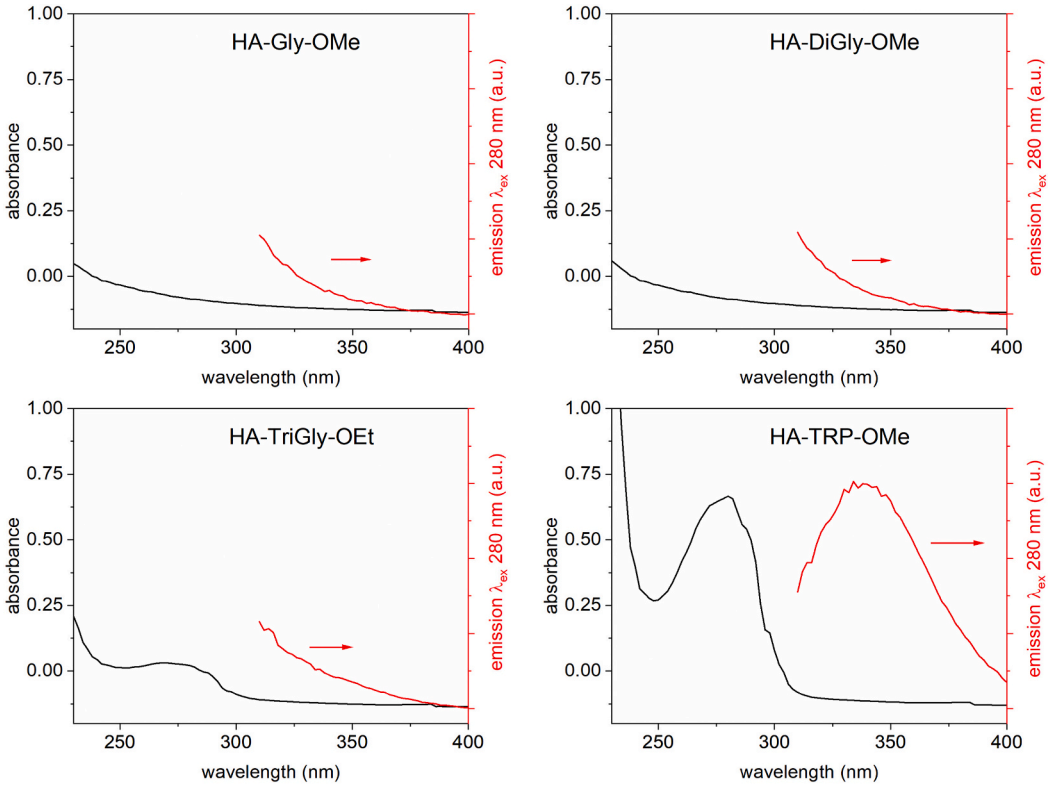

Fig. 10. UV–vis absorbance (left) and fluorescence emission spectra (right, excitation wavelength: 280 nm) of the four products HA-Gly-OMe, HA-DiGly-OMe, HA-TriGly-OEt and HA-Trp-OMe (0.5 mg ml^{-1} in water).

respective peptides (D—F), and the conjugation products (C and G-I). HA and HA DMT, ($pK_a \sim 3.3$) have a total amount of carboxylate charge of 1.6 and 2.3 mmol g^{-1} (sodium $HA_{theor.}$: ~ 2.5 mmol g^{-1}). The pK_a of the carboxylate is relatively low for this technique which requires reaching a plateau value in the acidic range to unambigously calculate the concentration of protonable carboxylates. Nevertheless a relative comparison between the samples gives interesting insights.

It is worth noting that the glycine methyl ester and the respective peptide esters hydrolyze at pH > 8 in water during the forward titration (pH 2 → pH 11), which leads to the formation of free carboxylates in all cases, as can be seen in the black backward titration curves (pH 11 → pH 2, D—F). The pK_a value of the *N*-terminal ammonium ion of the glycine ester shifts to higher values in glycine upon hydrolysis due to a shift in the charge distribution towards the carboxylate. For the di- and tripeptide esters, the influence of the hydrolysis on the pK_a value of the *N*-terminal is diminished due to the increased distance from the ester bond. The pK_a value of the *N*-terminal in the peptides is slightly higher but in a similar range as for Gly-OMe. The susceptibility towards hydrolysis of the ester in Gly-OMe is higher than for e.g. of ethyl acetate due to the inductive —I electron withdrawing effect of the α-ammonium group in the protonated form. However also the peptide esters hydrolyse rapidly during titration towards the alkaline. [19,20]. In the conjugation products (C and G-I) no free amines could be found even in the back-titration (ph 11 → pH 2), indicating, that there are covalent and stable amide bonds present between HA and the peptides/amino acids. In the case of the HA-Gly-OMe higher amounts of carboxylate groups per mass could be detected in the backward titration, which is again an evidence for the ester hydrolysis during titration. For all other products a reduction in the carboxylate amount compared to HA or HA DMT points towards the covalent attachment and intact esters bonds, with the latter appearing slightly more stable than in the case of unbound peptides. For HA-TriGly-OEt the forward titration shows a very high carboxylate amounts which points towards the limitation of the method to fully protonate and therefore quantify acids with relatively low pK_a values before the neutralization of the strong HCl starts. It is also possible that the longer peptide attached has an influence on the pK_a value of the uronic acid of polymer.

3.6. *Biocompatibility test of HA conjugates*

An MTS assay was performed to measure the influence of HA and HA conjugates on the metabolic activity of human umbilical vein endothelial cells (HUVECs) at relative diluted concentrations (Fig. 12). For all tested conjugates and dilutions, the cells produced the same or higher amounts of the formazan dye than a control sample without conjugates, indicating a similar viability of the cells. A positive control with 5 % *v*/v formaldehyde showed the expected reduced metabolic activity due to the toxicity of the added compound.

4. Conclusion

It could be shown that DMT is a valuable coupling reagent for the modification of sodium hyaluronate with the *C*-terminal esters of glycine, glycine di- and tripeptides and L-tryptophan. Conjugation of the peptides' *N*-terminal to the carboxylate of HA in the presence of either methyl or ethyl ester protecting groups on the *C*-terminal has the advantage of a stable, unambiguous bond without an additional "non-natural" spacer as in the case of the most common "click reactions". According to 2D NMR, covalent amide bonds are formed with the C6 carboxylate and the primary amines of the peptide esters. 2D NMR but not IR or UV–vis shows that DMT is present either covalently or non-covalently bound to HA in traces, and care must be taken when using this chemistry for biological purposes [13]. The coupling reaction with the peptides yields water soluble products with a degree of substitution of 0.38 for the case of the glycine tripeptide. In the case of L-tryptophan, fluorescent products with a DS of 0.5 are obtained. The degree of substitution can be calculated conveniently from the proton NMR spectra by the ratio of the ethyl ester CH_3 groups of the tripeptide versus the methyl

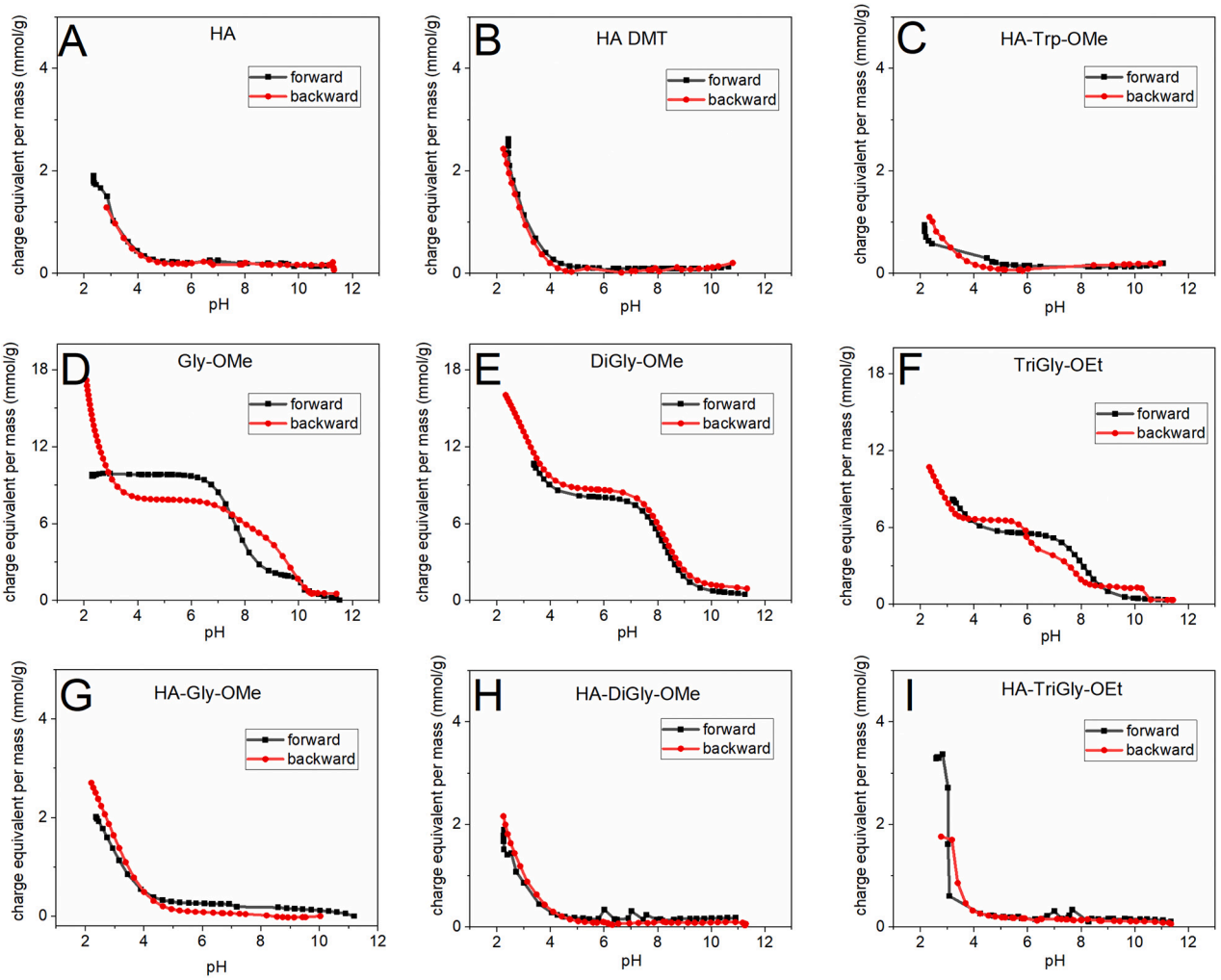

Fig. 11. Charging isotherms of A) unmodified HA, B) HA DMT, C) HA-Trp-OMe, D) Gly-OMe, E) DiGly-OMe, F) TriGly-OEt, G) HA-Gly-OMe, H) HA-DiGly-OMe and I) HA-TriGly-OEt.

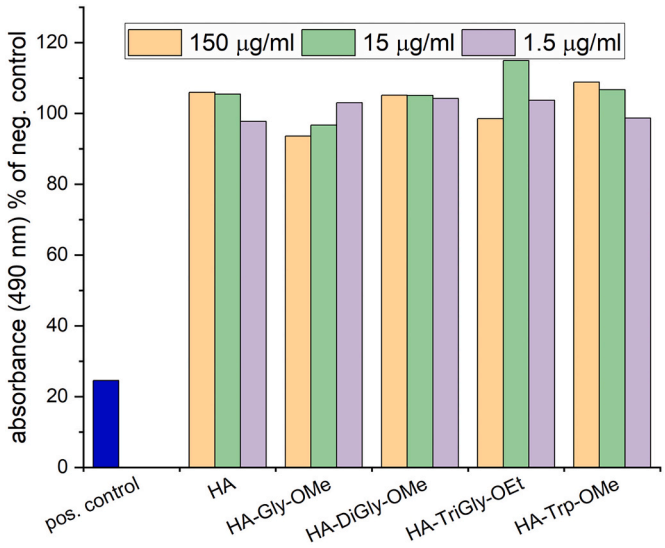

Fig. 12. Absorbance of formazan (λ_{Abs}: 490 nm) produced by human umbilical vein endothelial cells from a tetrazolium compound in the presence of differently concentrated HA conjugates or formaldehyde (positive control). Values are expressed as percent of negative control e.g. without added conjugates.

protons of the *N*-acetyl groups of the polysaccharide. It is therefore recommended to use ethyl or t-butyl esters of peptides, not overlapping with the saccharide proton signals as tracers for this purpose. Potentiometric charge titrations show that the ester groups of the alpha amino acid at the C-terminal of the peptides hydrolyze quickly but probably not fully above a pH value of 8 in water. For the glycine methyl ester this shifts the pK_a value of the respective alpha ammonium ion on the *N*-terminal to higher pH values. This shift dimishes with the length of the peptide [19]. However, in the conjugation products, no free amines could be detected by titration, indicating a covalent, stable bond of the peptide to hyaluronate. Moreover, it seems that the ester bonds are more stable in the conjugate than in the free peptides which is important for later deprotection or derivatization [10]. None of the investigated products show a significant reduction in MTS cell viability assays at lower concentrations, paving the way for further investigations of the products as biomaterials for either tissue growth scaffolds, as protease substrates or as drug delivery vehicles. More complex peptides should be coupled with proper protecting groups or better with more selective chemistry. Even though HA has advantages due to its regioselective carboxylate group, the issue of in-chain control of the substitution pattern still needs to be solved with some clever methods not reported up to now.

CRediT authorship contribution statement

Fazilet Gürer: Writing – original draft, Methodology, Investigation, Data curation. **Tamilselvan Mohan:** Writing – review & editing, Supervision. **Matej Bračič:** Writing – review & editing, Writing – original draft, Investigation. **Ariana Barlič:** Writing – review & editing, Supervision, Project administration. **Damjan Makuc:** Writing – review & editing, Validation, Investigation, Data curation. **Janez Plavec:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Karin Stana Kleinschek:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Rupert Kargl:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] K. Saravanakumar, S. Park, S.S. Santosh, A. Ganeshalingam, G. Thiripuranathar, A. Sathiyaseelan, S. Vijayasarathy, A. Swaminathan, V.V. Priya, M.-H. Wang, Application of hyaluronic acid in tissue engineering, regenerative medicine, and nanomedicine: a review, Int. J. Biol. Macromol. 222 (2022) 2744–2760, https://doi.org/10.1016/j.ijbiomac.2022.10.055.

[2] C.M. Nimmo, S.C. Owen, M.S. Shoichet, Diels–Alder click cross-linked hyaluronic acid hydrogels for, Tissue Eng. 12 (3) (2011) 824–830, https://doi.org/10.1021/bm101446k.

[3] M.N. Collins, C. Birkinshaw, Hyaluronic acid based scaffolds for tissue engineering—a review, Carbohydr. Polym. 92 (2) (2013) 1262–1279, https://doi.org/10.1016/j.carbpol.2012.10.028.

[4] R.V. Iozzo, L. Schaefer, Proteoglycan form and function: a comprehensive nomenclature of proteoglycans, Matrix Biol. 42 (2015) 11–55, https://doi.org/10.1016/j.matbio.2015.02.003.

[5] T. Mohan, K.S. Kleinschek, R. Kargl, Polysaccharide peptide conjugates: chemistry, properties and applications, Carbohydr. Polym. 280 (2022) 118875, https://doi.org/10.1016/j.carbpol.2021.118875.

[6] Y. Zhou, S.P. Petrova, K.J. Edgar, Chemical synthesis of polysaccharide–protein and polysaccharide–peptide conjugates: a review, Carbohydr. Polym. 274 (2021) 118662, https://doi.org/10.1016/j.carbpol.2021.118662.

[7] H.-Q. Song, Y. Fan, Y. Hu, G. Cheng, F.-J. Xu, Polysaccharide–Peptide Conjugates: A Versatile Material Platform for Biomedical Applications, Adv. Funct. Mater. 31 (6) (2021) 2005978, https://doi.org/10.1002/adfm.202005978.

[8] C. Schatz, S. Lecommandoux, Polysaccharide-containing block copolymers: synthesis, Properties and Applications of an Emerging Family of Glycoconjugates 31 (19) (2010) 1664–1684, https://doi.org/10.1002/marc.201000267.

[9] K.K. Upadhyay, J.F.L. Meins, A. Misra, P. Voisin, V. Bouchaud, E. Ibarboure, C. Schatz, S. Lecommandoux, Biomimetic doxorubicin loaded polymersomes from hyaluronan-block-poly(γ-benzyl glutamate), Copolymers 10 (10) (2009) 2802–2808, https://doi.org/10.1021/bm9006419.

[10] F. Gürer, R. Kargl, M. Bračič, D. Makuc, M. Thonhofer, J. Plavec, T. Mohan, K. S. Kleinschek, Water-based carbodiimide mediated synthesis of polysaccharide-amino acid conjugates: Deprotection, charge and structural analysis, Carbohydr. Polym. 267 (2021) 118226, https://doi.org/10.1016/j.carbpol.2021.118226.

[11] R. Kargl, V. Vorraber, V. Ribitsch, S. Köstler, K. Stana-Kleinschek, T. Mohan, Selective immobilization and detection of DNA on biopolymer supports for the design of microarrays, Biosens. Bioelectron. 68 (2015) 437–441, https://doi.org/10.1016/j.bios.2015.01.038.

[12] T. Pouyani, J.W. Kuo, G.S. Harbison, G.D. Prestwich, Solid-state NMR of N-acylureas derived from the reaction of hyaluronic acid with isotopically-labeled carbodiimides, JACS 114 (15) (1992) 5972–5976, https://doi.org/10.1021/ja00041a010.

[13] M. D'Este, D. Eglin, M. Alini, A systematic analysis of DMTMM vs EDC/NHS for ligation of amines to Hyaluronan in water, Carbohydr. Polym. 108 (2014) 239–246, https://doi.org/10.1016/j.carbpol.2014.02.070.

[14] P. Ghaffari-bohlouli, J. Simińska-Stanny, H. Jafari, M. Mirzaei, L. Nie, C. Delporte, A. Shavandi, Printable hyaluronic acid hydrogel functionalized with yeast-derived peptide for skin wound healing, Int. J. Biol. Macromol. 232 (2023) 123348, https://doi.org/10.1016/j.ijbiomac.2023.123348.

[15] L.F. Zemljič, D. Čakara, N. Michaelis, T. Heinze, K. Stana Kleinschek, Protonation behavior of 6-deoxy-6-(2-aminoethyl)amino cellulose: a potentiometric titration study, Cellulose 18 (1) (2011) 33–43, https://doi.org/10.1007/s10570-010-9467-x.

[16] Y. Chen, M.D. Barkley, Toward understanding tryptophan fluorescence in proteins, Biochemistry 37 (28) (1998) 9976–9982, https://doi.org/10.1021/bi980274n.

[17] P. Sindrewicz, X. Li, E.A. Yates, J.E. Turnbull, L.-Y. Lian, L.-G. Yu, Intrinsic tryptophan fluorescence spectroscopy reliably determines galectin-ligand interactions, Sci. Rep. 9 (1) (2019) 11851, https://doi.org/10.1038/s41598-019-47658-8.

[18] A.B.T. Ghisaidoobe, S.J. Chung, Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques, Int. J. Mol. Sci. 15 (12) (2014) 22518–22538, https://doi.org/10.3390/ijms151222518.

[19] R. Hay, L. Porter, P. Morris, The basic hydrolysis of amino acid esters, Austr. J. Chem. 19 (7) (1966) 1197–1205, https://doi.org/10.1071/CH9661197.

[20] R.W. Hay, P.J. Morris, Proton ionisation constants and kinetics of base hydrolysis of some α-amino-acid esters in aqueous solution, Part II, J. Chem. Soc. B: Phys. Org. (1970) 1577–1582, https://doi.org/10.1039/J29700001577.