DMTMM-mediated amidation of alginate oligosaccharides aimed at modulating their interaction with proteins

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Abstract

Alginate oligosaccharides (AOS) with a weight average molecular weight of 5 kDa were efficiently amidated with amino acids and carbohydrates in aqueous media in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). Here, alanine, leucine, serine, as well as mannose and rhamnose, were amidated at high yields with a good control of the degree of substitution (DS). Amino acid- and carbohydrate-grafted AOS showed improved stability against degradation by alginate lyases having different specificities. This enzyme resistance was correlated with the DS: hydrolysis was reduced by 60 to 70% for low DS (0.1), whereas AOS with DS ranging from 0.4 to 0.6 remained unhydrolyzed. Competitive inhibition assays demonstrated multivalent binding of mannose-amidated AOS to concanavalin A lectin. A 178-fold affinity enhancement was observed for AOS_{Man-0.38} (DS 0.38) over α -methyl-mannoside with an IC₅₀ of 5.6 μ M, lending further evidence for the promising potential of AOS as multivalent scaffolds.

Highlights:

- DMTMM promoted efficient amidation of alginate oligosaccharides (AOS)
- AOS were functionalized with amino acids and carbohydrates
- Grafting conferred improved stability to AOS against alginate lyases
- Mannose-grafted AOS exhibited multivalent inhibition of ConA lectin

Keywords: Alginate oligosaccharides, Amidation, Multivalency, Lectin, Alginate lyases

1. Introduction

Alginates encompass a relatively broad family of linear anionic polysaccharides composed of (1→4)-linked β -D-mannuronic acid (M) and its C-5 epimer α -L-guluronic acid (G), forming homopolymeric (MM- or GG-blocks) and heteropolymeric sequences (MG blocks). The fraction and the distribution of the two monomers vary widely depending on the source. Although some soil bacteria of the genera *Pseudomonas* and *Azotobacter* produce alginates as exopolysaccharides, the main source of alginate is brown seaweeds in which it represents up to 40% of the organisms' dry matter, providing ~30,000 t of alginate for industry annually (Draget, 2009). The ability of alginates to form soft hydrogels in the presence of calcium ions forms the basis for a wide variety of industrial applications, including food and pharmaceuticals. In recent years, alginate biomaterials have received increased attention for use in drug delivery systems or as biocompatible scaffolds for tissue engineering (Lee & Mooney, 2012; Szekalska, Puciłowska, Szymańska, Ciosek & Winnicka, 2016).

Alginate oligosaccharides (AOS) do not form gels, but nevertheless exhibit many important bioactivities in mammals as well as in plants. AOS promote the growth and proliferation of human cells including keratinocytes (Charruyer, Fong, Yue, Arron, Ghadially, 2016; Kawada

et al., 1997; Morvan, Pentecouteau, Grandpierre, Rougier, Vallée, 2009) and endothelial cells (Kawada, Hiura, Tajima & Takahara, 1999). They also potentially protect against oxidative stress in neural cells (Tusi, Khalaj, Ashabi, Kiaei & Khodagholi, 2011). In plants, AOS with an average degree of polymerization (DP) of 6 show elicitor activity in soybean cotyledons, stimulating the accumulation of phytoalexin and inducing phenylalanine ammonia lyase (An, 2009). AOS of DP 2-8 promote plant growth in rice, wheat, lettuce, carrot and tobacco, etc. by enhancing nitrogen assimilation and basal metabolism (González, Castro, Vera & Moenne, 2013 and references cited therein). Finally, AOS also have interesting properties for the fight against microbial pathogens. AOS are able to perturb multidrug-resistant (MDR) bacteria, thereby enhancing the action of selected antibiotics (Khan, 2012). Similarly, they inhibit fungal cell growth and potentiate the activity of antifungals against *Candida* and *Aspergillus* spp. (Tøndervik et al., 2014).

These exciting properties and possible applications in human health and wellness as well as in agronomy encourage research on the chemical modification of AOS to improve their stability and bioactivity. Acylation of the uronic groups is a prominent strategy in this regard. Firstly, the introduction of substituents at C6 can increase the stability of AOS by delaying the action of degrading enzymes, i.e. alginate lyases (Wong, Preston & Schiller, 2000) and by reducing the spontaneous hydrolysis of alginic acid arising from intramolecular catalysis by the carboxylic acid (Smidsrød, Haug & Larsen, 1966). Poly(ϵ -caprolactone)-grafted alginates, whose degradation by *Flavobacterium* sp. alginate lyase can be modulated with respect to the size and grafting density of the polyester chains, have recently been investigated as enzyme-responsive vectors (Benykhlef et al., 2012). Alginate derivatives incorporating either an aliphatic amide or ester groups have been synthesized for the development of hydrolytically stable scaffolds for nerve repair (Vallée et al., 2009). *In vitro* degradation studies have shown that alginamides have better stability than the ester derivatives over a two-month period.

76 Secondly, grafting of cell-specific ligands or signaling molecules at C6 offers the possibility to
77 generate alginates with more targeted actions. Hydrogels made of alginates functionalized with
78 the GRGDY cell adhesion peptide have shown a better capacity to promote the attachment and
79 spreading of myoblast cells over those prepared with unmodified alginates (Rowley,
80 Madlambayan & Mooney, 1999). Alginate-amino acid derivatives have been designed to
81 engineer glycocalyx-like poly(DL-lactic acid) surfaces (Zhu et al., 2002). Alkaline amino acids
82 in particular demonstrate a positive effect on the attachment and growth of chondrocytes.
83 Carbohydrate substituents also have a positive impact on cell adhesion and hydrogels made of
84 galactose-modified alginates show higher recruitment of hepatocytes than those made of native
85 alginates (Donati et al. 2003; Tian, Han, Tan & You, 2014; Yang, Goto, Ise, Cho & Akaike,
86 2002).

87 To the best of our knowledge, amidation reactions on alginates reported in the literature to date
88 are mostly based on aqueous carbodiimide chemistry with *N*-ethyl-*N'*-(3-
89 dimethylaminopropyl)carbodiimide (EDC). This coupling reagent, although effective and
90 popular, presents a major side reaction consisting of the internal rearrangement of the *O*-
91 acylurea activated ester of EDC to an *N*-acylurea that becomes stably incorporated in the
92 substrate (Davidovich-Pinhas, Harari, & Bianco-Peled, 2009). This undesirable and impossible-
93 to-eliminate by-product can be formed in non-negligible amounts, with reports of up to 8% of
94 *N*-acylurea (Tian, Han, Tan, You, 2014). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-
95 methylmorpholinium chloride (DMTMM) is a promising alternative coupling reagent. Since its
96 first report for the synthesis of amides in 1999 (Kunishima et al., 1999), the use of DMTMM
97 has considerably increased with comparable and in some cases greater results than the most
98 popular coupling agents. DMTMM has many advantages, including easy by-product removal,
99 good stability in water, and high reactivity. In addition, DMTMM-activated carboxylic acids
100 show poor reactivity with alcohols. Therefore, this reagent is particularly suitable for generating

amides from hydroxy-acids without requiring protection of the OH group and without ester formation. Furthermore, DMTMM, which is cheaper and less toxic than EDC, does not generate an *N*-acylurea by-product (D'Este, Eglin & Alini, 2014). These features have contributed to the emergence of this reagent for the amidation of polymers and, more specifically, of polysaccharides, including heparin (Reyes-Ortega et al., 2013) and hyaluronic acid (Bergman, Elvingson, Hilborn, Svensk & Bowden, 2007; Loebel, D'Este, Alini, Zenobi-Wong & Eglin, 2015). Nevertheless, to date, there are no reports of alginate amidation with DMTMM.

In this work, we report the C6 amidation of alginate oligosaccharides in aqueous media with amino acids and amine-functionalized glycosides using DMTMM. These modifications were carried out with a view to enhancing the stability of AOS against enzymatic degradation. In addition, carbohydrate grafting was also expected to promote the establishment of multivalent interactions with specific carbohydrate-binding proteins. The synthesis of multivalent glycoarchitectures is the subject of an intense research motivated by major applications in the diagnosis and the treatment of viral or bacterial infections (Bernardi et al., 2013). Synthetic polymers as well as polysaccharides, such as chitosan and cellulose, have been extensively used as multivalent scaffolds. Surprisingly, natural oligosaccharidic platforms, with the exception of cyclodextrins and a few mono- and disaccharides, have never really been investigated for the synthesis of multivalent glycoconjugates. Alginate oligosaccharides may thus offer new interesting scaffolds for the development of multivalent glycoarchitectures.

2. Materials and Methods

2.1 Material and equipment

All amino acids were purchased as methyl esters HCl salt from Sigma–Aldrich except alanine methyl ester HCl which was purchased from TCI. Hydrochloric acid, sodium hydroxide, absolute ethanol, and ammonium carbonate were purchased from Carlo ERBA and used

without purification. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was prepared according to the procedure established by Kunishima et al. (Kunishima et al., 1999). In brief, equimolar quantities of 2-chloro-4,6-dimethoxy-1,3,5-triazine and *N*-methylmorpholine were stirred for 30 min in THF at room temperature, and DMTMM, which precipitates, was then isolated by filtration and dried under vacuum. Alginate lyase from *Pedobacter saltans*, Pedsa-0632 (GenBank accession number ADY54336), was expressed in *E. coli* as recently reported in the literature (Mathieu, Henrissat, Labre, Skjåk-Bræk & Helbert, 2016). Alginate lyase from *Sphingomonas* sp. (2500 U.mL⁻¹ in 3.2 M ammonium sulfate) was purchased from Megazyme. Peroxidase-labeled concanavalin A (ConA-HRP) from *Canavalia ensiformis* was purchased from Sigma.

¹H NMR spectra were recorded on a Bruker spectrometer DPX400 at 400 MHz. Samples (10 mg) were previously freeze-dried in D₂O then solubilized in D₂O (500 µL) and the solvent residual peak was used as an internal standard. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) negative-ion spectra were recorded on a Bruker Daltonics Autoflex apparatus using 2,5-dihydroxybenzoic acid (DHB) as a matrix. GPC-MALLS chromatogram was obtained by injection through three Shodex columns: OHpack SB803 HQ, OHpack SB802.5 HQ and OHpack SB802 HQ placed in series. Detection was performed with a Waters refractometer and a Wyatt multi-angle light scattering detector. UV-visible spectra were recorded on a Varian Cary 50 Bio spectrometer at 235 nm.

2.2 Characterization of native alginate oligosaccharides (AOS)

The molecular weight of AOS was determined by high-performance size-exclusion chromatography (HPSEC) using an eighteen-angle light scattering detector (DAWN™ HELEOS, Wyatt, Santa Barbara, CA USA), coupled with refractive index detector. Elution was performed on three Shodex columns: OHpack SB803 HQ, OHpack SB802.5 HQ and OHpack

SB802 HQ placed in series with 0.1 M NaNO₃ (±10 mM EDTA) solvent. To calculate the molecular mass, the dn/dc value used was 0.165 mL/g (Smidsrød & Haug, 1968; Harding 2005). The molecular weight characteristics of native AOS were the followings: number-average molecular weight M_n = 3.5 kDa, weight-average molecular weight M_w = 5 kDa and polydispersity index PDI = 1.4. The M:G ratio as determined by ¹H NMR according to Grasdalen's method was 0.74 (Grasdalen, Larsen & Smidsrød, 1979). Infrared spectroscopy of AOS displayed characteristic absorptions at 3285 cm⁻¹ for O–H bond stretching; 1607 and 1414 cm⁻¹ for C=O and C–O bond stretching of sodium carboxylate function of the uronate unit; 1136 and 1032 cm⁻¹ for C–O and C–C bond vibrations of carbohydrates. MALDI-TOF mass spectroscopy analysis in negative mode of AOS showed oligomers with a degree of polymerization (DP) ranging from 4 to 22 (see Electronic Supplementary Information for all spectra and chromatogram related to the characterization of AOS).

2.2 Determination of the Degree of Substitution.

The degree of substitution (DS) of amidated AOS was calculated using ¹H NMR according to the following formula:

$$DS = \frac{\frac{I_S}{N_S}}{1.92 \times I_{H1G}}$$

I_S refers to the peak intensity of an isolated signal of the substituent S, and N_S is the number of protons related to this signal. I_{H1G} refers to the signal intensity of the guluronate anomeric protons. The detailed formula for DS determination is reported in the Electronic Supplementary Information.

2.3 Determination of the grafting yield.

The reaction yield was calculated by dividing the number of moles of amidated AOS by the number of moles of native AOS. The molar mass used for native alginate oligosaccharide was 198 g.mol⁻¹. The molar mass of the amidated alginate oligosaccharide was defined using the following equation: $M_{\text{amidated alginate}} = M_{\text{amidated uronate}} \times \text{DS} + 198 \times (1 - \text{DS})$.

2.4 General procedure for the amidation of AOS

To a solution of AOS (0.60 mmol of sodium uronate unit) in ammonium carbonate buffer (4 mL, 0.1 M, pH 9.4) is added the amine-functionalized glycoside or amino acid methyl ester (0.60 mmol), DMTMM (1.20 mmol) and the reaction mixture is stirred for 15 h at 25 °C. For the amidation with amino acids, a solution of sodium hydroxide (6 mL, 1 M, 6.00 mmol) is then added to hydrolyze the methyl esters and the reaction mixture is stirred for 1.5 h at 25 °C before being neutralized using hydrochloric acid (1M).

Isolation and purification of the amidated AOS were achieved according to the following procedure. The reaction mixture is poured into absolute ethanol (90 mL) to precipitate the amidated AOS. After 5 min, the precipitate is centrifuged (4 °C, 8500 rpm, 10 min) then solubilized in 2 mL of water and precipitated again by addition of absolute ethanol (90 mL). The product is centrifuged (4 °C, 8500 rpm, 8 min), dried under high vacuum for 30 min to remove residual ethanol and then freeze-dried.

Characterization of amidated AOS (Distinctive signals used to calculate DS are labeled with * in NMR peak lists):

AOS_{Ala}: ¹H NMR (ppm, D₂O, 298 K) δ 5.30-5.25 (m, H₁G α , H₁M α - reducing end), 5.23-4.97* (m, 1.00 H, H₁G - inner alginate chain), 4.97-4.41 (m, H₁G β , H₁M β - reducing end; H₁M, H₅GM, H₅GG - inner alginate chain), 4.41-3.62 (m, H₂₋₄G, H₂₋₅M - alginate; CH₃-CH(NH)-COONa - alanine), 1.42* (d, J = 7.1 Hz, 3.13 H, CH₃-CH(NH)-COONa - alanine); FTIR

200 (ATER, cm^{-1}): ν 1032 and 1108 (C–O and C–C carbohydrates); 1407 (C–O sodium
201 carboxylate); 1560 (C–NH amide II); 1594 (C=O sodium carboxylate); 1663 (C=O amide);
202 2933 (C–H - alanine); 3285 (O–H - carbohydrate).

203
204 **AOS_{Ser}**: ^1H NMR (ppm, D_2O , 298 K) δ 5.33-5.24 (m, $\text{H}_1\text{G}\alpha$, $\text{H}_1\text{M}\alpha$ - reducing end), 5.24-5.00*
205 (m, 1.00 H, H_1G - inner alginate chain), 5.00-4.44 (m, $\text{H}_1\text{G}\beta$, $\text{H}_1\text{M}\beta$ - reducing end; H_1M ,
206 H_5GM , H_5GG - inner alginate chain), 4.44-4.30* (m, 1.60 H, $\text{HO}-\text{CH}_2-\underline{\text{CH}}(\text{NH})-\text{COONa}$ -
207 serine), 4.30-3.62 (m, H_{2-4}G , H_{2-5}M - alginate; $\text{HO}-\underline{\text{CH}_2}-\text{CH}(\text{NH})-\text{COONa}$ - serine); FTIR
208 (ATER, cm^{-1}): ν 1033 and 1103 (C–O and C–C carbohydrates); 1124 (C–OH - serine); 1409
209 (C–O sodium carboxylate); 1558 (C–NH amide II); 1601 (C=O sodium carboxylate); 1664
210 (C=O amide); 2936 (C–H - serine); 3321 (O–H - carbohydrate).

211
212 **AOS_{Leu}**: ^1H NMR (ppm, D_2O , 298 K) δ 5.32-5.23 (m, $\text{H}_1\text{G}\alpha$, $\text{H}_1\text{M}\alpha$ - reducing end), 5.23-4.99*
213 (m, 1.00 H, H_1G - inner alginate chain), 4.99-4.61 (m, $\text{H}_1\text{G}\beta$, $\text{H}_1\text{M}\beta$ - reducing end; H_1M ,
214 H_5GM , H_5GG - inner alginate chain), 4.41-3.61 (m, H_{2-4}G , H_{2-5}M - alginate; $(\text{CH}_3)_2-\text{CH}-\text{CH}_2-$
215 $\underline{\text{CH}}(\text{NH})-\text{COONa}$ - leucine), 1.80-1.57 (m, 3.867 H, $(\text{CH}_3)_2-\underline{\text{CH}}-\underline{\text{CH}_2}-\text{CH}(\text{NH})-\text{COONa}$ -
216 leucine), 1.04-0.88* (m, 7.636 H, $(\underline{\text{CH}_3})_2-\text{CH}-\text{CH}_2-\text{CH}(\text{NH})-\text{COONa}$ - leucine); FTIR (ATER,
217 cm^{-1}): ν 1035 and 1134 (C–O and C–C carbohydrates); 1402 (C–O sodium carboxylate); 1555
218 (C–NH amide II); 1593 (C=O sodium carboxylate); 1664 (C=O amide); 2957 (C–H - leucine);
219 3318 (O–H - carbohydrate).

220
221 **AOS_{Man}**: ^1H NMR (ppm, D_2O , 298 K) δ 5.28-5.21 (m, $\text{H}_1\text{G}\alpha$, $\text{H}_1\text{M}\alpha$ - reducing end), 5.21-5.00*
222 (m, 1.00 H, H_1G - inner alginate chain), 5.00-4.59 (m, $\text{H}_1\text{G}\beta$, $\text{H}_1\text{M}\beta$ - reducing end; H_1M ,
223 H_5GM , H_5GG - inner alginate chain), 4.90 (s, H_1 - Man), 4.33-3.36 (m, H_{2-4}G , H_{2-5}M - alginate;
224 H_{2-6} - Man, $\text{Man}-\text{O}-\underline{\text{CH}_2}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\underline{\text{CH}_2}-\text{NH}$), 2.88-2.60 (m, 2.602 H, $\text{Man}-\text{O}-\text{CH}_2-\text{CH}_2-$

225 CH₂-S-CH₂-CH₂-NH), 2.04-1.81* (m, 1.309 H, Man-O-CH₂-CH₂-CH₂-S-CH₂-CH₂-NH); FTIR
 226 (ATER, cm⁻¹): ν 1030 and 1126 (C–O and C–C carbohydrates); 1417 (C–O sodium
 227 carboxylate); 1584 (broad, C–NH amide II and C=O sodium carboxylate); 1651 (C=O amide);
 228 2929 (C–H – Man-NH₂); 3348 (O–H - carbohydrate).

229
 230 **AOS_{Rha}**: ¹H NMR (ppm, D₂O, 298 K) δ 5.29-5.19 (m, H₁Gα, H₁Mα - reducing end), 5.19-4.99*
 231 (m, 1.00 H, H₁G - inner alginate chain), 4.99-4.56 (m, H₁Gβ, H₁Mβ - reducing end; H₁M,
 232 H₅GM, H₅GG - inner alginate chain), 4.82 (s, H₁ - Rha), 4.30-3.38 (m, H₂₋₄G, H₂₋₅M - alginate;
 233 H₂₋₅ - Rha, Rha-O-CH₂-CH₂-CH₂-S-CH₂-CH₂-NH), 2.87-2.61 (m, 2.59 H, Rha-O-CH₂-CH₂-
 234 CH₂-S-CH₂-CH₂-NH), 2.04-1.82 (m, 1.262 H, Rha-O-CH₂-CH₂-CH₂-S-CH₂-CH₂-NH), 1.41-
 235 1.22* (m, 2.071 H, CH₃ - Rha); FTIR (ATER, cm⁻¹): ν 1034 and 1127 (C–O and C–C
 236 carbohydrates); 1415 (C–O sodium carboxylate); 1571 (C–NH amide II); 1608 (C=O sodium
 237 carboxylate); 1657 (C=O amide); 2925 (C–H – Rha-NH₂); 3363 (O–H - carbohydrate).

238
 239 2.5 Enzymatic hydrolysis of amidated alginate oligosaccharides
 240 Enzymatic hydrolysis of amidated AOS was carried out with two alginate lyases: Pedsa0632, a
 241 recombinant lyase from *Pedobacter saltans* expressed in *E. coli* (Mathieu, Henrissat, Labre,
 242 Skjåk-Bræk & Helbert, 2016) and a commercial alginate lyase from *Sphingomonas* sp.
 243 (Megazyme, 2500 U.mL⁻¹ in 3.2 M ammonium sulfate). The reaction was performed by adding
 244 5 µl of 10-fold diluted enzymes in Tris-HCl buffer (100 mM, pH 8.0, 2 mM CaCl₂) to a 500 µL
 245 cuvette containing 5 mM of amidated AOS in the same buffer at 30°C. The formation of
 246 unsaturated product was monitored by measuring the absorbance at 235 nm as a function of
 247 time on a Varian Cary 50 Bio spectrometer.

248

2.6 Enzyme-linked lectin assay (ELLA)

The method was adapted from Köhn et al. (Köhn, Benito, Ortiz Mellet, Lindhorst & García Fernández, 2004). Nunc MaxiSorp® plates were coated overnight with yeast mannan purchased from Sigma (100 µL per well at 10 µg.mL⁻¹ in phosphate-buffered saline (PBS prepared by dissolution of tablets from Sigma) containing 0.1 mM Ca²⁺) at room temperature. The wells were then washed twice with washing buffer (200 µL per well, PBS containing 0.05% (v/v) Tween 20 - PBST). The washing procedure was repeated after each incubation throughout the assay. The wells were then blocked with 3% BSA/PBS (150 µL per well) for at least 1 h at 37°C. After washing 3 times, the wells were filled with serial dilutions of peroxidase-labeled concanavalin A (ConA-HRP from *Canavalia ensiformis* (Sigma) previously dissolved at 0.25 mg.mL⁻¹ in PBS; 100 µL) from 10⁻¹ to 10⁻⁵ mg.ml⁻¹ in PBS-BSA 0.3% and incubated 1 h at 37°C. The plates were then washed three times with PBST and one time with PBS and *o*-phenylenediamine dihydrochloride (SIGMAFAST™ OPD) (50 µL per well, 0.4 mg.ml⁻¹) in citrate-phosphate buffer (50 mM, pH 5.0, with 0.4 mg.ml⁻¹ urea hydrogen peroxide) was added. The reaction was stopped after 10 min by adding H₂SO₄ 30% (v/v) (50 µL per well) and the absorbances were measured at 490 nm on a microtiter plate reader (Bio-Rad 680). The concentration of lectin-enzyme conjugate having an absorbance between 0.8 and 1.0 was used for inhibition experiments. The final concentration for ConA-HRP was 3 µg.mL⁻¹.

For inhibition experiments, the microtiter plates were coated overnight at room temperature with yeast mannan (100 µL per well, 10 µg.mL⁻¹). The wells were then washed and blocked with BSA as described above. The mannose-amidated AOS (AOS_{Man}) inhibitors were used as stock solution of 20 mM in water. During the blocking step, in 1.5 mL microtubes, 200 µL of AOS_{Man}, at different concentrations and in a series of three-fold dilutions, was incubated with 200 µl of ConA-HRP (6 µg.mL⁻¹) in 0.3% PBS-BSA for 1 h at 37°C. The above solutions (100 µL per well; 3 different wells for each concentration of inhibitors) were then transferred to the

mannan-coated microplates, which were incubated for another hour at 37°C. The plates were washed and the OPD substrate was added (50 µL per well). Color development was stopped after 10 min and the absorbances were measured. The percentages of inhibition were calculated as follows, where A = absorbance:

$$\% \text{ Inhibition} = (A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}) / A_{(\text{no inhibitor})} \times 100$$

IC₅₀ values were reported as the concentration of inhibitors required to inhibit 50% of the ConA-yeast mannan association.

3. Results and Discussion

Preliminary investigation of DMTMM-mediated amidation of AOS with alanine

Alginate oligosaccharides (AOS) used in this study were prepared from *Laminaria digitata* alginates by controlled enzymatic depolymerization followed by an acid treatment (Gedouin & Vallée, 1998). The weight average molecular weight and the polydispersity index of the AOS as determined by SEC-MALLS were 5 kDa and 1.4 kDa, respectively. A complementary analysis using MALDI-TOF mass spectrometry showed the presence of oligosaccharides with sizes in the range of DP 4 to 22. The M:G ratio as determined using ¹H NMR was 0.74. The DTMMM-mediated amidation of AOS was first investigated with alanine as model amino acid. Preliminary reactions were performed at 25 °C in water in presence of two equivalents of the acylating agent and one equivalent of amino acid per carboxylic acid group. The use of an unprotected amino acid resulted in self-polycondensation reactions exclusively and no amidation of the alginate backbone was observed by proton NMR after precipitation of AOS in ethanol. The following reactions were therefore carried out in similar conditions but with a protected amino acid: L-alanine methyl ester hydrochloride (Scheme 1). Saponification of the methyl ester was achieved *in situ* by adding an aqueous solution of sodium hydroxide and then

neutralizing the reaction media with hydrochloric acid. The alanine-amidated alginate oligosaccharides (AOS_{Ala}) were finally isolated by precipitation in ethanol and freeze-dried.

Scheme 1: DMTMM-mediated amidation of AOS with L-Ala-OMe in water

¹H NMR spectroscopy of the isolated product displayed a broad doublet at 1.41 ppm, which was attributed to the methyl group of the amidated alanine moieties (Fig. 1). Another piece of evidence of the alanine grafting was provided by the broadening of the peaks related to the alginate backbone in comparison with the NMR spectrum of native AOS (See Electronic Supporting Information). The presence of a well-resolved thin doublet at 1.5 ppm attributed to free alanine suggested minor contamination of the product with unreacted amino acids. However, the very low intensity of the signal attested to the satisfactory purity of AOS_{Ala}.

Figure 1: ^1H NMR spectrum of alanine-amidated alginate oligosaccharides AOS_{Ala} recorded at 298K in D_2O

The absence of protons related to the DMTMM or its side products also confirmed the satisfactory purity of the amidated AOS, indicating the efficiency of the precipitation protocol. This simple procedure also eliminated the sodium chloride salts generated by the neutralization reaction after the hydrolysis of the methyl ester. No weight loss was observed after ultrafiltration of the product with a 1 kDa cut-off membrane.

The alanine grafting on AOS was also probed by infrared spectroscopy. IR spectrum of AOS displayed characteristic absorptions peaks at 1607 and 1414 cm^{-1} for $\text{C}=\text{O}$ and $\text{C}-\text{O}$ stretching, respectively, in the sodium carboxylate function of the uronate unit (Fig. 2). On the IR spectrum of AOS_{Ala} , the covalent grafting of alanine was ascertained by both the shifting of the $\text{C}=\text{O}$ band and the presence of additional peaks between 1400 cm^{-1} and 1663 cm^{-1} related to the $\text{C}-\text{N}$

bond and the new carboxylic acid functions. In addition, MALDI-TOF mass spectroscopy confirmed the grafting by the mass gain observed on the spectrum of AOS_{Ala} compared with the AOS spectrum (See Electronic Supplementary Information).

Figure 2: IR spectra of native and alanine-amidated alginate oligosaccharides AOS and AOS_{Ala}

Although alanine grafting proceeded with satisfactory yield (77-90%) in the above-mentioned conditions, there was a high discrepancy on the degree of substitution (DS) determined by ¹H NMR. Under similar reaction conditions and without pH adjustment, the reaction mixture was slightly acidic (pH ≈ 5) and the DS varied from 0.13 to 0.42 (Fig. 3). To circumvent this poor reproducibility, the reaction was evaluated at different pH conditions.

Figure 3: Evolution of the DS of alanine-amidated AOS_{Ala} as determined by ¹H NMR according to the reaction conditions: (A) H₂O (pH ≈ 5), (B) NaOH (pH 7.0), (C) MOPS buffer (0.1 M, pH 7.0), (D) (NH₄)₂CO₃ buffer (0.1 M, pH 9.4)

Adjusting the pH of the reaction mixture to 7 by adding sodium hydroxide just after the addition of all the reactants significantly increased the DS and reproducibility. The addition of sodium hydroxide in the reaction media was beneficial, but it did not keep the pH constant as the reaction progressed. To control the pH more accurately, the reaction was carried out in 3-*N*-(morpholino)-propanesulfonic acid (MOPS) buffer at pH 7 and in ammonium carbonate buffer at pH 9.4, each presenting different advantages. The MOPS buffer, which is soluble in organic solvents, can be efficiently removed by the precipitation in ethanol. Ammonium bicarbonate, on the other hand, is volatile and can be removed by freeze-drying. The DS observed for the reaction conducted in MOPS at pH 7.0 was lower than that observed in presence of sodium hydroxide. A similar result was previously observed in 3-*N*-(morpholino)-ethanesulfonic acid buffer (MES) during DMTMM-mediated acylation of albumin with amino-sugars (Farkaš, Čížová, Bekešová, & Bystrický, 2013). The highest grafting ratio (DS = 0.54) and the best reproducibility were obtained using ammonium carbonate buffer at pH 9.4.

Grafting of amino acids and amine-functionalized glycosides to AOS

After determining the optimal pH conditions for the amidation of AOS with alanine, we evaluated the applicability of this reaction to other amino acids and amine-functionalized glycosides (Scheme 2) as well as the possibility to reach the targeted DS.

i) DMTMM (2 equiv./COOH), amino acid methyl ester, $(\text{NH}_4)_2\text{CO}_3$ (0.1M, pH 9.4) then NaOH; ii) DMTMM (2 equiv./COOH), amino glycoside, $(\text{NH}_4)_2\text{CO}_3$ (0.1M, pH 9.4)

Scheme 2: DMTMM-mediated amidation of AOS with amino acids and amine-functionalized glycosides

First, the number of alanine and DMTMM equivalents was modified and the resulting products were characterized by NMR. The highest DS reached with alanine was 0.54 in presence of 1 amino acid equivalent and 2 DMTMM equivalents (Table 1). Using more equivalents of the acylating reagent did not help to exceed this limit, because above a certain DS (≈ 0.50), the reaction was most likely inhibited by the precipitation of the methyl ester intermediate.

Conversely, using fewer equivalents of alanine (0.35 and 0.15) led to the production of amidated AOS at the targeted DS with extremely satisfactory yields. The degradation of alginates is very slow in the region between pH 5 and 10 (Haug, Larsen, Smirsröd, 1963); however, depolymerization of AOS may occur during the amidation reaction. A SEC-MALLS analysis of AOS_{Ala-0.13} revealed an average molecular weight of 5.2 kDa and a polydispersity index of 1.4, which is in good agreement with the expected theoretical values. Although the chromatogram suggests the presence of low molecular weight compounds in the product, their low amount (about 10%) confirms that no significant degradation occurred (See Electronic Supporting Information). To assess the general applicability of the protocol, hydrophobic leucine and hydrophilic serine were also amidated. Once again, the yields of the reactions were excellent and ranged from 70% to 90%. A good correlation was observed between the number of amino acid equivalents and the DS of the product below 0.5. Beyond 0.5 equivalents, the reaction was again limited by the solubility of the product. Nonetheless, the higher hydrophilicity of the serine made possible to attain a higher DS (0.83) than for alanine or leucine.

Amino acid/ Amine-functionalized glycoside	Number of equivalents per carboxylic function	DS	Product	Yield (%)
L-alanine	1	0.54	AOS _{Ala-0.54}	93
L-alanine	0.35	0.35	AOS _{Ala-0.35}	74
L-alanine	0.15	0.13	AOS _{Ala-0.13}	82
L-serine	1	0.83	AOS _{Ser-0.83}	70
L-serine	0.35	0.33	AOS _{Ser-0.33}	75
L-serine	0.15	0.10	AOS _{Ser-0.10}	87
L-leucine	1	0.65	AOS _{Leu-0.65}	73
L-leucine	0.35	0.34	AOS _{Leu-0.34}	79
L-leucine	0.15	0.14	AOS _{Leu-0.14}	84
Man-NH ₂	2	0.38	AOS _{Man-0.38}	77
Man-NH ₂	1	0.35	AOS _{Man-0.35}	85
Man-NH ₂	0.33	0.24	AOS _{Man-0.24}	87
Man-NH ₂	0.2	0.1	AOS _{Man-0.1}	83
Rha-NH ₂	1	0.35	AOS _{Rha-0.35}	85
Rha-NH ₂	0.5	0.38	AOS _{Rha-0.38}	70

Table 1: Amidation of AOS with alanine, serine, leucine, 3-(2-aminoethylthio)propyl- α -D-mannopyranoside (Man-NH₂) and 3-(2-aminoethylthio)propyl- α -L-rhamnopyranoside (Rha-NH₂).

In light of the versatility and efficiency of amidation with amino acids, we then attempted to introduce carbohydrate ligands onto the alginate backbone. Mannose and rhamnose were selected as model carbohydrates as well as for their attractive biological properties. In particular, both have demonstrated beneficial actions on keratinocytes (Labský et al., 2003, Andrès et al., 2006) and they may have a synergistic effect with alginate oligosaccharides in skin protection or repair. 3-(2-Aminoethylthio)propyl- α -D-mannopyranoside (Roy, Pagé, Figueroa Perez, & Verez Bencomo, 1998) and 3-(2-aminoethylthio)propyl- α -L-rhamnopyranoside (Roy & Tropper, 1988) were prepared according to procedures found in the literature (See Electronic Supplementary Information). Allyl glycosides were synthesized by Fischer glycosylation and they were next engaged in a UV-promoted thiol-ene addition with

cysteamine. In addition to allowing the functionalization of carbohydrates in two steps and without the use of protecting group, this synthetic sequence was carried out in water and the isolation of the product was easily performed on an ionic exchange resin. Amine-functionalized rhamnose and mannose glycosides (Rha-NH₂, Man-NH₂) underwent DMTMM-mediated amidation with AOS (Table 1). The reactions were conducted in ammonium carbonate buffer in the presence of two equivalents of DMTMM and with a variable amount of the carbohydrates. As observed with the amino acids, the yields of amidation for amine-functionalized glycosides were about 80% and were little influenced by the number of glycoside equivalents. No solubility issue was encountered in this reaction, but the DS never exceeded 0.38 for the highest glycoside concentration. The limiting factor is likely due to steric hindrance. For the reactions that were conducted with fewer than five amine-functionalized glycoside equivalents, the DS was fairly well correlated to the amount of monosaccharide introduced but in a relatively less precise way than for amino acids.

Enzymatic hydrolysis of amino acid- and carbohydrate-amidated AOS

A recent report on the enzymatic behavior of alginates grafted with poly(ϵ -caprolactone) (PCL) of various lengths and DS (Benykhlef et al., 2012) shows that degradation by *Flavobacterium* sp. alginate lyase is incomplete and is slowed down by the presence of PCL. However, to the best of our knowledge, no studies have been conducted on amidated alginate oligosaccharides. The stability of amino acid- and carbohydrate-amidated AOS against enzymatic degradation was assessed with two alginate lyases. The first one, having an endo-M specificity, is a commercially available recombinant alginate lyase from *Sphingomonas* sp. (Megazyme, Bray, Ireland). The second one (Pedsa-0632 from *Pedobacter saltans*), has been expressed in *E. coli* (Mathieu et al., 2016), and displays an endo-MG and endo-G specificity. Regardless of their specificity, alginates lyases release unsaturated compounds via a β -elimination mechanism and

their activity can be monitored using UV-visible spectroscopy at 235 nm. Native AOS and AOS amidated with different DS of leucine, serine and mannose thus underwent enzymatic hydrolyses with both alginate lyases under the same conditions and the reactions were monitored using UV spectroscopy (See Electronic Supplementary Information). Figure 4 illustrates the hydrolysis of mannose-amidated AOS by Pedsa-0632 according to their DS.

Figure 4: UV-visible monitoring of mannose-amidated alginate oligosaccharide (AOS_{Man}) hydrolysis by the recombinant alginate lyase Pedsa-0632 from *Pedobacter saltans*

All the amidated AOS, regardless of the nature of their substituent, showed similar behavior in presence of the lyases. Hydrolysis was reduced by 60-70% for the amidated AOS with a DS of only 0.1 and it was completely inhibited for grafting densities above 0.3, except in the leucine series for which no hydrolysis was observed for a DS of 0.6. As expected, enzymatic hydrolysis was hampered by steric hindrance on the alginate backbone. Interestingly, both alginates lyases were able to act on the modified oligosaccharides. This activity highlights the fact that the amidation reaction is likely to occur either on the mannuronic units or on guluronic units and that biodegradation of amidated AOS is still possible.

452

453 ***Multivalent binding of mannose-amidated AOS (AOS_{Man}) with concanavalin A lectin***

454 Alginate oligosaccharides show interesting intrinsic biological properties. Nevertheless,
455 grafting of biomolecules such as amino acids or sugars along the alginate backbone can serve
456 multiple purposes. The ligand can be used for site-specific delivery of the alginate
457 oligosaccharide. Alternatively, the alginate backbone can be seen as a multivalent molecular
458 scaffold. The binding capability of the mannosylated AOS toward concanavalin A (ConA)
459 lectin was thus assessed using a solid-phase competitive enzyme-linked lectin assay (ELLA).
460 ConA is a multimeric protein with a specificity for α -mannosides and its use in several studies
461 makes it a good lectin model (Roy et al., 1998). Yeast mannan was used as ligand for lectin and
462 was immobilized on a microtiter plate. Lectin binding was assessed in the absence and in the
463 presence of the competitive AOS_{Man} inhibitors using a horseradish peroxidase-labeled ConA
464 (ConA-HRP) producing a colorimetric reaction with *o*-phenylenediamine. IC₅₀ values,
465 corresponding to the concentration of inhibitors necessary to inhibit 50% of the interaction of
466 lectin with the surface, was measured for each mannosylated alginate oligosaccharide (AOS_{Man}-
467 0.1, AOS_{Man}-0.24 and AOS_{Man}-0.38) and compared with native AOS and α -Me-mannoside
468 monosaccharide used as control (Fig. 5).

Figure 5: Inhibition of concanavalin A by mannose-amidated alginate oligosaccharides (AOS_{Man})

First, no interaction was observed between AOS and ConA, revealing that the sodium mannuronate residue of alginate does not bind to the lectin. Regarding α -methyl-mannoside and AOS_{Man-0.1}, similar inhibition degrees were observed with IC₅₀ values of the order of 1 mM, meaning that the alginate oligosaccharide with a DS of 0.1 interacts with the lectin as a monosaccharide. These values are in agreement with those found in the literature (Euzen & Reymond, 2011). For mannose-amidated alginate oligosaccharides of higher DS, the inhibition increased drastically. IC₅₀ values down to 56.3 μ M and 5.6 μ M were determined for AOS_{Man-0.24} and AOS_{Man-0.38}, respectively. For AOS_{Man-0.38}, the affinity to ConA was 178-fold higher than for methyl α -mannoside. This result is extremely encouraging because a rapid survey of the literature shows that most inhibitors displaying similar efficiency either present a higher valency or are obtained through a much more complex synthesis process. Low micromolar inhibitors of ConA include glycopolymers carrying about 50 pendant sugars (Gou et al., 2013),

mannose-coated single walled carbon nanotubes (Cid Martin et al., 2016) or “click” high mannose oligosaccharide mimics (Cendret et al., 2012). Considering the ease of our amidation procedure, we believe that alginate oligosaccharides offer interesting perspectives for the development of a wide range multivalent glycoconjugates.

4. Conclusion

Here, we developed an efficient procedure for the amidation of alginate oligosaccharides based on the use of DMTMM. The reaction features easy implementation: it is carried out in a volatile buffered medium and the purification of the product is achieved by precipitation. Amino acids and carbohydrates were introduced in high yield along the alginate backbone at targeted degrees of substitution. The amidated alginate oligosaccharides showed improved resistance to enzymatic hydrolysis by alginate lyases regardless of their specificity. This stability may be related to grafting density. Carbohydrate-amidated alginate oligosaccharides were shown to be efficient multivalent inhibitors of concanavalin A lectin. These findings have exciting perspectives for the design of new anti-infection agents or the improvement of current cosmetic ingredients.

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