Synthesis strategies to extend the variety of alginate-based hybrid hydrogels for cell microencapsulation

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ABSTRACT. The production of hydrogel microspheres (MS) for cell immobilization, maintaining the favorable properties of alginate gels but presenting enhanced performance in terms of *in vivo* durability and physical properties, is desirable to extend the therapeutic potential of cell transplantation. A novel type of hydrogel MS was produced by straightforward functionalization of sodium alginate (Na-alg) with heterocheletic poly(ethylene glycol) (PEG) derivatives equipped with either end thiol or 1,2-dithiolane moieties. Activation of the hydroxyl moieties of the alginate backbone in the form of imidazolide intermediate allowed for fast

conjugation to PEG oligomers through a covalent carbamate linkage. Evaluation of the modified alginates for the preparation of MS combining fast ionic gelation ability of the alginate carboxylate groups and slow covalent crosslinking provided by the PEG-end functionalities highlighted the influence of the chemical composition of the PEG-grafting units on the physical characteristics of the MS. The mechanical properties of the MS (resistance and shape recovery) and durability of PEG-grafted alginates in physiological environment can be adjusted by varying the nature of the end functionalities and the length of the PEG chains. *In vitro* cell microencapsulation studies and preliminary *in vivo* assessment suggested the potential of these hydrogels for cell transplantation applications.

INTRODUCTION

Progress in therapies relying on the allo- or xenotransplantation of immobilized cells and tissue strongly depends on the quality of the immobilizing material. Currently, the translation of related therapies to the clinics, for example to treat end-stage organ failure and end-stage diseases such as cancer, diabetes mellitus and acute liver failure,[1,2] is not at least hindered by the lack of materials having the perfect properties. Hydrogels prepared from the biopolymer sodium alginate (Na-alg) or derivatives of it have been reported in abundant papers as particularly advantageous because they fulfill general requirements such as the spontaneous formations of hydrogels in the presence of divalent cations (e.g. Ca^{2+}, Ba^{2+}) under mild conditions of temperature and pH, and high biocompatibility.[3-6] Maintaining these advantages but overcoming several drawbacks, including the lack of *in vivo* mechanical resistance and stability, and defects in permselectivity,[7,8] are the focus of current research. There are additional limitations caused by the dimension of the targeted final application. This calls in particular for therapies which intend the transplantation of

allo- or xenogeneic cells immobilized in microspheres (MS) in order to allow for mini-invasive surgery.[9,10] The stabilization of alginate gel beads by coating the MS with polycations such as (poly(L-lysine), poly(L-ornithine) and poly(L-guanidine) was investigated, but resulted in worse cell graft function *in vivo*.[11] Another strategy to improve the performance of alginate MS relies on the combination with other polymers such as poly(ethylene glycol) (PEG). Following this approach, the combination of fast ionotropic gelation of Na-alg with slow covalent crosslinking provided by PEG derivatives, either covalently linked to the alginate backbone or in the form of an interpenetrating network, was proposed. This included two-component MS, composed of Ca-alg and covalently cross-linked vinyl sulfone-terminated multiarm PEG[12-14] and one-component MS, prepared from Na-alg derivatives grafted with heterobifunctional derivatives either on the carboxyl groups[15] or the hydroxyl moieties.[16] Despite the improved mechanical properties and permselectivity compared to pure Ca-alg MS, in particular the latter lacked of *in vivo* durability, probably due to the lability of the ester linkage which was introduced as grafting unit. This asked for the development of alternative grafting strategies. A panel of crosslinking strategies based on click reactions have been reported in the literature for the preparation of synthetic-based and biopolymer-based hydrogels to mimic the physiological cellular microenvironment and deliver therapeutic proteins and peptides promoting cell viability and functionality.[17-19] However, these techniques, mainly developed on PEG and hyaluronic acid derivatives, are not favorable for the production of hydrogel MS intended for cell therapies.

The present study intends to enlarge the variety of hybrid alginate materials suitable for cell immobilization in spherical 3D hydrogels, in particular regarding *in vivo* durability. Another key aspect concerns the development of a robust synthetic protocol for the preparation of Na-alg derivatives grafted with heterobifunctional PEG oligomers, amenable to up-scaled production.

First, we produced heterocheletic PEG derivatives presenting α-amine and ω-thiol functionalities to allow grafting to the alginate backbone and covalent crosslinking during MS formation, respectively. Second, a straightforward procedure was developed for the preparation of PEG-grafted Na-alg derivatives using a carbamate linkage to functionalize the hydroxyl moieties of Na-alg. The resulting hydrogels, produced in a one-step process, were characterized in terms of chemical composition and mechanical properties. Finally, the MS were evaluated both *in vitro* and *in vivo,* for their *in vitro* cytocompatibility using mice insulin producing cell lines and for their *in vivo* stability using a mice model.

EXPERIMENTAL SECTION

Materials and Methods. Na-alg Kelton HV (lot no. 61650A, [η] = 813 mL g^{-1} in 0.1 M NaCl, T = 25 °C, G/M = 0.6) was obtained from Kelco (San Diego, USA, CA). Linear PEG (molar mass MM= 1000 $g.mol^{-1}$ and 2000 $g.mol^{-1}$, degree of polymerization DP= 22 and 44, designated as **PEG-a** and **PEG-b**) were obtained from Sigma (Buchs, Switzerland). Other commercial reagents (Fluka, Sigma, Switzerland; TCI Europe, Zwijndrecht, Belgium) were used without further purification. Unless special mention, all reactions were performed under argon atmosphere (1 atm). Anhydrous solvents were obtained by filtration (Puresolv MD 5, Innovative Technology, Oldham, UK). Glassware was dried for 12 h in an oven (T > 100 °C) or under vacuum with a heat gun (T > 200 °C). Reactions were monitored by TLC (Merck silica gel 60F254 plates, Merck, Darmstadt, Germany). Detection was performed by UV light, $KMnO_4$, Ninhydrin or I_2. Purifications were performed by flash chromatography on silica gel (Merck N° 9385 silica gel 60, 240-400 mesh). NMR spectra were recorded on Bruker Avance III-400, Bruker Avance-400 or Bruker DRX-400 spectrometers at room temperature (rt) (400 MHz) (Bruker, Billerica, MA, USA). ^{1}H frequency is at 400.13 MHz, ^{13}C frequency is at 100.62 MHz.

Chemical shifts are expressed in parts per million (ppm) and coupling constants (*J*) in hertz (Hz). Solvents used for NMR spectroscopy were deuterated chloroform ($CDCl_3$, Acros), deuterated methanol (CD_3OD, Acros) and deuterated water (D_2O). Mass spectra were obtained on a Nermag R-10-10C spectrometer with chemical ionization (NH_3) and mode m/z (amu) [% relative base peak (100%)] (Nermag, Santa Clara, CA, USA). IR spectra were recorded on a Jasco FT/IR-4100 spectrometer outfitted with a PIKE technology MIRacleTM ATR accessory as neat films compressed onto a Zinc Selenide window. The spectra are reported in cm^{-1}. Mass spectra were obtained by using a Waters ACQUITY H-class UPLC/MS ACQ-SQD by electron ionization (EI positive and negative) or a Finnigan TSQ7000 by electrospray ionization (ESI+). The accurate masses were measured by ESI-TOF using a QTOF Ultima from Waters.

Formation of one-component microspheres. Solutions of Na-Alg-PEG polymers were prepared according to the following formulation: Na-alg-PEG was dissolved in a solution of 0.4 % NaCl in 100 mM MOPS buffer, pH 7.4 at the desired concentration. After complete dissolution, the polymer solution was directly extruded into a gelation bath (100 mM $CaCl_2$ in 100 mM MOPS buffer, pH 7.4) containing tween 80 (1/10 000). MS were produced employing a coaxial air-flow droplet generator (Encapsulator B-395 Pro, Büchi Labortechnik AG, Flawil, Switzerland). The MS were collected by filtration, washed twice with $CaCl_2$ stock solution, and finally stored in this solution at 4 °C, or in cell culture medium in case of cell microencapsulation.

Physical characterization of microspheres. The average diameter was measured on an Olympus AX70 microscope equipped with an Olympus DP70 color digital camera. The mechanical resistance to 90% compression of the initial MS diameter was analyzed using a texture analyzer (TA-XT2i, software Texture Exponent 32, Stable Micro Systems, Godalming,

UK) equipped with a force transducer (1 mN resolution). A single MS was placed below the probe, for which a constant speed was set as 0.5 mm s^{-1}. Thirty MS of each batch were included in the analysis.

Microencapsulation of MIN6 cells. MIN6 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 1mM Na-Pyruvate, 71 μM β-mercaptoethanol, 15% decomplemented Fetal Calf Serum, 25 mM glucose, Penicillin and streptomycin (DMEM complete medium). $10x10^6$ MIN6 cells were gently mixed in 1 ml of Na-alg-PEG solution. MS were produced using the same procedure as described above for the formation of one-component MS. Encapsulated and free MIN6 cells were cultured up to 15 days in DMEM complete medium and were analyzed for viability and functionality by FDA/PI staining and glucose-stimulated insulin release assay, as previously described.[20]

Transplantation of MS in mice. Animal research was performed according to the Geneva cantonal veterinary authorities (license GE/34/13). 1ml of empty MS was transferred into the peritoneum of anesthetized immunocompetent C57/BL6 mice through a small abdominal incision. After 30 days, mice were sacrificed for a macroscopic evaluation of fibrotic reactions in the peritoneum.

Synthesis protocols. The designation of the compounds refers to the chemical structures presented in Scheme 1 and Scheme 2.

Preparation of compound 2. To a solution of 3-mercaptopropionic acid (1 equiv, 94.2 mmol, 10 g) in H_2O: THF (1:1, 180 mL) were added Boc_2O (1.2 equiv, 113.04 mmol, 24.7 g) and Et_3N (2 equiv, 188.4 mmol, 19.07 g, 25.43 mL) and the reaction mixture was stirred for 12 h at rt. THF was evaporated under vacuo and 1 M HCl (50 mL) was added. The product was extracted

with DCM (3 x 100 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. The product was purified by FCC on silica gel (PE/EtOAc 8:1) to afford **2** as a transparent oil (43.3 mmol, 8.92 g, 46 %). ^{1}H-NMR, ^{13}C NMR,IR (neat), and HRMS-ESI data (supporting information S2).

Preparation of compound 3a. EDCI (3 equiv, 5.64 mmol, 875.6 mg) and Et_3N (3 equiv, 5.64 mmol, 570.7 mg) were added to a solution of **1a** (1 equiv, 1.88 mmol, 2 g) and **2** (2 equiv, 3.77 mmol, 778 mg) in DCM (20 mL). The reaction mixture was stirred 24 h at rt and subsequently washed with sat. NH_4Cl solution (2 x 20 mL) and with sat. $NaHCO_3$ solution (2 x 20 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The product was purified by FCC on silica gel (DCM/MeOH 20:1 to 10:1) to afford **3a** as a yellowish oil (1.74 mmol, 1.94 g, 93 %). ^{1}H-NMR, ^{13}C NMR, IR (neat), and HRMS-ESI data (supporting information S3). Compound **3b** was prepared according to the same protocol.

Preparation of PEG-a I. To a solution of compound **3a** (1 equiv, 1.46 mmol, 1.5 g) in toluene (7.5 mL), PPh_3 (1.8 equiv, 2.70 mmol, 708 mg) was added and the mixture was stirred for 30 min at rt. Then, 1M HCl (19.7 mL) was added and stirring continued for 48 h at rt. The two phases were separated and the organic phase was washed with 1 M HCl (2 x 20 mL). The combined aqueous layers were washed with DCM (10 mL) before being concentrated in vacuo. After co-evaporation with toluene (3 x 10 mL), compound **PEG-a I** was obtained as a yellowish amorphous solid (3.37 µmol, 3.32 g, 95 %). ^{1}H-NMR, ^{13}C NMR, IR (neat) and HRMS-ESI data (supporting information S4). Polymer **PEG-b I** was prepared according to the same protocol.

Preparation of compound 4. EDCI (1.5 equiv, 72 mmol, 11.17 g) and NHS (1.5 equiv, 72 mmol, 8.36 g) were added to a solution of lipoïc acid (1 equiv, 48 mmol, 10 g) in DMF (30 mL).

The reaction mixture was stirred 12 h at rt and concentrated in vacuo. The crude product was dissolved in DCM and washed three times with H_2O and brine. The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The product was purified by FCC on silica gel (EtOAc/PE 1:1) to afford **4** as a yellow solid (22.42 mmol, 6.79 g, 46 %). ^{1}H-NMR, ^{13}C NMR, IR (neat), and HRMS-ESI data (supporting information S5).

Preparation of compound 5a. To a solution of compounds **1a** (1 equiv, 4.72 mmol, 5 g) and **4** (2 equiv, 9.44 mmol, 2.86 g) in DMF (35 mL), Et_3N (1 equiv,4.72 mmol, 477 mg) was added. The reaction mixture was stirred for 48 h at rt before evaporating the solvent in vacuo. The crude product was dissolved in DCM (100 mL) and washed with sat. NH_4Cl (2 x 40 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The product was purified by FCC on silica gel (DCM/MeOH 17:1) to afford **5a** as a yellowish amorphous solid (3.71 mmol, 4.63 g, 78 %). ^{1}H-NMR, ^{13}C NMR, IR (neat) and HRMS-ESI data (supporting information S6). Compound **5b** was prepared according to the same protocol.

Preparation of PEG-a II. Compound **5a** (1 equiv, 866 µmol, 1 g) was dissolved in THF (5 mL). $LiAlH_4$ (2 equiv, 1.73 mmol, 66 mg) was added portion-wise and the reaction was stirred for 40 min at rt. The reaction was quenched by slow addition of EtOAc (2 mL), then H_2O (20 ML) and the product was extracted with DCM (3 x 30 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo to afford **PEG-a II** as a white amorphous solid (791 µmol, 895 mg, 91 %). IR (neat) and HRMS-ESI data (supporting information S7). Polymer **PEG-b II** was prepared according to the same protocol.

Preparation of TBA-alg. Na-alg (Kelton HV, 1.0 g) was suspended in EtOH (50 mL) and the solution was cooled to 0 °C. Formic acid (20 % aqueous solution, 50 mL) was added, and the

mixture was stirred for 12 h at 0 °C. The resulting alginic acid powder was separated by vacuum filtration, washed with a mixture of EtOH/$H_2$0 1:1 (3 x 50 mL) and then acetone (3 x 50 mL). The resulting solid was further dried for 15 min under vacuum at 40 °C, and dispersed in water (50 mL). TBAOH (40 % in water) was added dropwise until reaching pH 7. The solution was directly freeze-dried to afford TBA-Alg as a white solid.

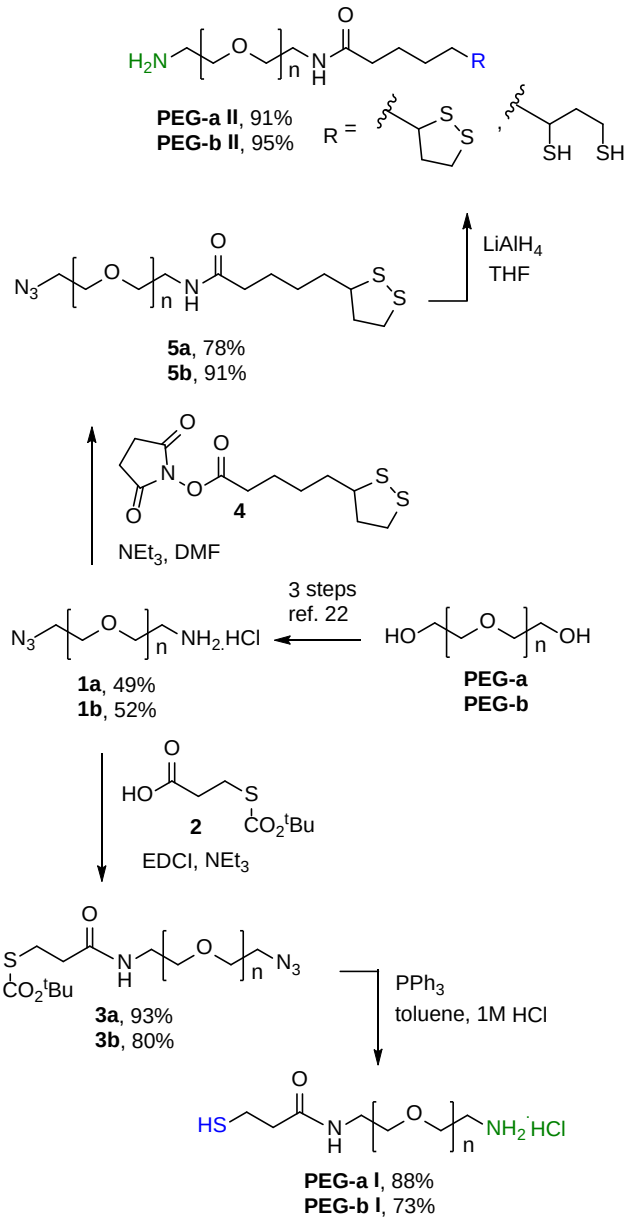
General protocol for the functionalization of TBA-alg. TBA-alg (1 equiv, 0.239 mmol, 100 mg) was dissolved in DMSO (20 mL) and the solution was stirred for 12 h to ensure homogeneity. CDI (1 equiv, 0.239 mmol, 38.7 mg) previously dissolved in a minimum volume of DMSO was added, and the reaction mixture was stirred at rt for 30 min. To enhance precipitation of the imidazolide-alginate intermediate, acetone (40 mL) was added. The resulting precipitate was filtered and washed with acetone (3 x 10 mL). The solid was further dried for 15 min under vacuum at 40 °C, and transferred to a round-bottom flask. Distilled water was added (10 mL), and the mixture was stirred until complete dissolution. Heterobifunctional PEG derivative (0.2 equiv, 47.9 µmol) previously dissolved in a minimum volume of water was added. The solution was stirred at rt for 2 h. The reaction was quenched by addition of 0.05M NaOH solution until reaching pH 11. The solution was directly transferred into a dialysis membrane and dialyzed against distilled water. The first day, the water was changed once. A second water change was performed with prior addition of TCEP (0.1 M, 1 mL) in the dialysis tube. The dialysis was continued for 2 more days, adding TCEP (0.1 M, 1 mL) one more time to the dialysis tube, and followed by dialysis against distilled water the second day (changing water 3 times that day). The last day the dialysis was continued against water, with the addition of $NaHCO_3$ (180 mg in 9 L distilled water) in the dialysis bucket, to reach pH 7. The solution was

filtered (70 µm and 0.22 µm) and freeze-dried to obtain the PEG-grafted alginates as white solids.

RESULTS AND DISCUSSION

Preparation of heterobifunctional PEG derivatives. The development of PEG-grafted alg materials for cell microencapsulation applications requires access to a variety of heterocheletic PEG oligomers. Scheme 1 presents details of the synthesis routes to obtain the heterocheletic derivatives. Following our previous reports on the production of azido- and amino-silanized PEG molecules,[21,22] straightforward synthetic pathways were developed to produce thiol- and 1,2-dithiolane-functionalized PEG derivatives. Starting from the linear **PEG-a** or **PEG-b,** the key intermediates α-amino-ω-azido PEG **1a** and **1b** were obtained in good overall yields (3 steps) according to a previously established protocol.[21] **PEG-a I** and **PEG-b I** oligomers were obtained by a coupling reaction with protected 3-mercaptopropanoïc acid (**2**), followed by simultaneous reduction of the azido group and deprotection of the thiol functionality. Alternatively, conjugation to activated lipoic acid followed by reduction in the presence of $LiAlH_4$ delivered **PEG-a II** and **PEG-b II** as mixtures of opened (reduced) and closed (oxidized) forms of the dithiolane functionality. The end thiol functionalities in **PEG-a I** and **PEG-b I** derivatives are expected to produce disulfide bridge crosslinking during MS formation. The terminal 1,2-dithiolanes in **PEG-a II** and **PEG-b II** compounds were introduced due to their fast and tunable disulfide exchange properties, which can produce disulfide clusters through multiple crosslinking interactions.[23,24]

Scheme 1. Synthesis of heterocheletic PEG derivatives. (for a: n=22, for b: n=44)



Functionalization of Na-alg with PEG derivatives. The alg backbone presents a distribution of carboxyl and hydroxyl functionalities available for chemical functionalization. The chemical modification of the carboxyl groups can be performed by simple esterification[25] or formation of amide linkage[26-29] using carbodiimide chemistry or activation with N-hydroxysuccinimide. However, this technique reduces the number of carboxyl groups for ionic cross-linking. Hydroxyl groups at C2 and C3 positions of the uronic units of alginate can be oxidized[30] for

further derivatization by reductive amination.[31] The oxidation step increases the degradation rate of the resulting alginate backbones and is thus preferred for drug delivery applications.[32] Other strategies, which do not imply the rupture of carbon-carbon bonds in the alginate backbone, include sulfation,[33] activation of the hydroxyl groups as cyanate esters followed by coupling with amine derivatives to produce isourea linkage,[34] or derivatization with succinic anhydride to insert new carboxylic groups on the alginate backbone for subsequent carbodiimide-based coupling.[16] In order to maintain all carboxyl groups of Na-alg available for ionic cross-linking, we focused on the modification of the hydroxyl groups of the alginate backbone.[35] Due to 1,3-diaxial interactions, only the hydroxyl moieties in C3 position of the mannuronic residues and C2 position of the guluronic residues are expected to be derivatized.[36]

Na-alg (Kelton HV) was first converted into tetrabutyl ammonium (TBA)-alg to increase solubility in DMSO for further chemical derivatization (Scheme 2). While heterogeneous acidification of Na-alg with ethanolic solution of HCl led to high variability in terms of viscosity of the solution of final TBA-alg (Table 1, entries 1 and 2), treatment with an ethanolic solution of formic acid (20%), followed by addition of TBAOH, afforded TBA-alg with high reproducibility of its composition and viscosity in 2 wt % solution.[37-39] In this transformation, it was established that the conversion of Na-alg into alginic acid should be performed at 0°C (Table 1, entries 3 and 4) as higher temperature of the reaction mixture led to molar mass reduction of the polymer, indicated by lower viscosity of the solution. In addition, the pH should be strictly adjusted to 7 during the addition of TBAOH to avoid alginate backbone degradation (Table 1, entries 3 and 5). Finally, these optimized conditions allowed scaling-up the protocol to 5 g of starting Na-alg (Table 1, entry 6).

Table 1. Optimization of the production of TBA-alg.

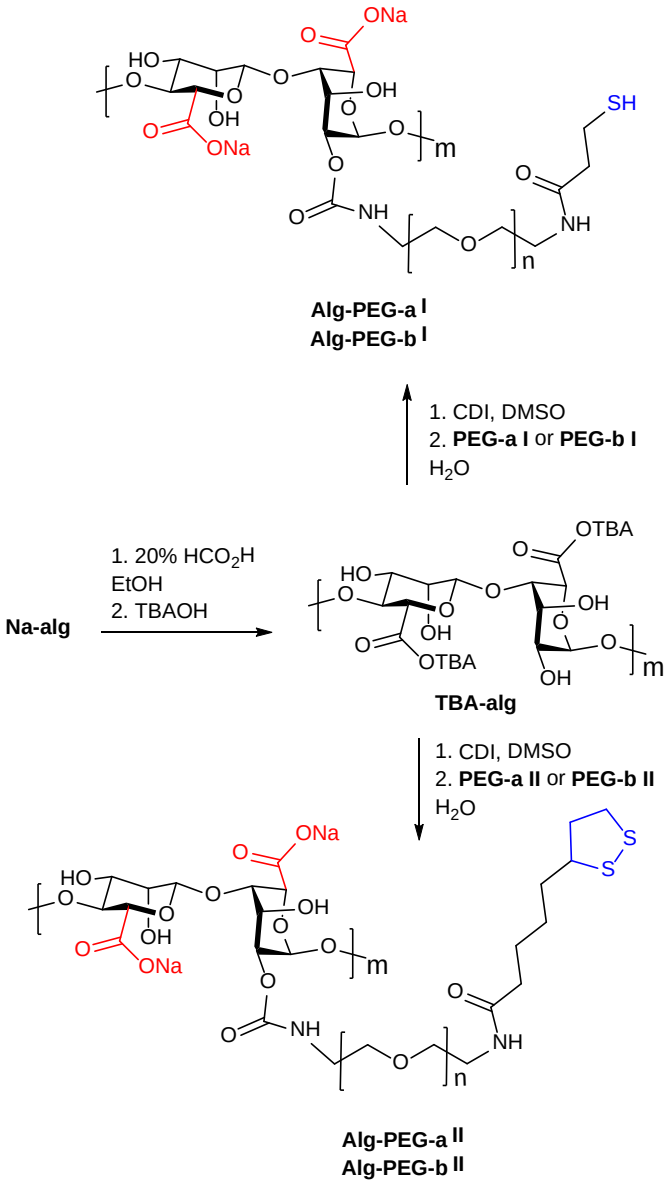
Entry	Acid[b]	T (°C)	pH	Viscosity[c] (mPa.s)
1[a]	HCl	0°C	nd	133
2[a]	HCl	0°C	nd	87
3[a]	HCO_2H	0°C	7	156
4[a]	HCO_2H	24°C	7	77
5[a]	HCO_2H	0°C	9	54
6[d]	HCO_2H	0°C	7	174

[a]Reaction was performed on 1 g of Na-alg. [b]20 % solution in EtOH. [c]Viscosity of the solution containing 2 wt % of TBA-alg (solvent: deionized water; measured at 22°C). [d]Reaction was performed on 5 g of Na-alg.

Conjugation of PEG derivatives to the alginate backbone was achieved by activation of the hydroxyl moieties in the presence of equimolar amount of 1,1'-carbodiimidazole (CDI)[40] followed by coupling with the end amino groups of PEG I and PEG II to afford PEG-functionalized alginates with a carbamate linkage as grafting unit. While running the two steps successively in DMSO was attempted, higher reproducibility was achieved by precipitating the intermediate activated imidazolide from DMSO and performing the second step in aqueous solution. Carbamate formation required a low amount of PEG derivatives (0.2 equiv) and was completed within 2 h at room temperature. Increasing the proportion of heterobifunctionalized PEG compounds did not lead to a higher degree of grafting to the alginate backbone. Other activating agents such as N,N'-disuccinimidyl carbonate, p-nitro-phenylchloroformate and isocyanates were investigated but did not lead to any detectable grafting of PEG derivatives. The isolation and purification of PEG-functionalized Na-alg with suitable physical properties for MS formation involved the addition of 0.05 M NaOH to the reaction mixture to regenerate the sodium salt, followed by successive dialysis cycles against water, in the presence of TCEP to

prevent disulfide bond and disulfide cluster formation, and final neutralization by addition of aqueous $NaHCO_3$.

Scheme 2. Functionalization of Na-alg by formation of carbamate linkage.



The expected chemical structures of PEG-grafted alginates were confirmed by NMR spectroscopy. The ^{1}H-NMR spectrum (supporting information S8) of Alg-PEG-a I displayed

characteristic signals (triplets) at 3.42, 3.23, 2.98 and 2.71 ppm for CH_2 linked to carbamate, amide from the NH and C(O) termini and SH, respectively. A large peak at 3.80-3.63 ppm corresponds to the other PEG CH_2 groups. In addition, PEG grafted alginates were analyzed by Diffusion Ordered Spectroscopy (DOSY), which confirmed that the PEG derivatives were covalently conjugated to the alginate backbone (supporting information S9). The ^{1}H-NMR spectrum (supporting information S10) of Alg-PEG-a II displayed broad peaks at 2.27-2.18 and 1.54 ppm corresponding to CH_2 groups of the 1,2-dithiolane moiety.

We previously established the viscosity of the polymer solution at defined concentration and known degree of grafting as a key parameter, which directly influences the quality and physical properties of the resulting MS.[12,16] Appropriate data is presented for Alg-PEG derivatives in Table 2. While the concentration and the degree of grafting directly correspond to the number of possible ionic and covalent crosslinks, the viscosity is a crucial parameter for the encapsulator settings. It depends on the molar mass and the type of the alginate backbone, the degree of grafting, the length and the type of the grafted PEG chain, the solvent compositing, as well as the concentration and temperature of the solution.

We observed higher degree of grafting upon functionalization with PEG-b but this was not accompanied by higher viscosity of the solution. Different solution behavior at different degrees of branching and length of the side chain branches could be hypothesized causing differences in the viscosity.

Table 2. Characteristics for Alg-PEG derivatives.

Product	Viscosity (mPa.s)[a]		Degree of grafting[b] (%)
	3 wt %	4 wt %	
Alg-PEG-a I	209	-	5.2
Alg-PEG-a II	161	-	6.7
Alg-PEG-b I	-	53	13.0
Alg-PEG-b II	-	161	21.0
Na-alg	245	586	-

[a]Viscosity of the solution containing a defined concentration of Alg-PEG polymer (solvent: deionized water; measured at 22°C). [b]Measured by ^{1}H-NMR

Microsphere formation and characterization. Solutions of the Alg-PEG derivatives in MOPS (10 mM, pH = 7.4), at the concentration indicated in Table 2, were extruded into a gelation bath containing $CaCl_2$ as ionic crosslinker to produce MS which were assessed for their size, mechanical resistance to compression and elasticity/shape recovery. For the derivatives containing end 1,2-dithiolane moieties, dithiothreitol (DTT) (0.5 eq relative to the PEG chains) was added to the initial solution of polymer to favor disulfide clusters formation. For comparison, MS were formed under similar conditions with pure Na-alg at initial concentrations of 3 and 4 wt %. As presented in Table 3, the MS diameter did not show significant change when comparing the data obtained at the day of the production and after storage for 7 days in the gelation bath, For PEG-a and PEG-b, the functionalization with 1,2-dithiolanes (II) led to the formation of smaller beads. When submitted to uniaxial compression 4 days after their formation, all MS presented the same general profile characterized by minimal mechanical resistance until 50% compression of their initial diameter, followed by slight increase until 70% and exponential evolution until 90% compression. When compressed to 90% of their initial

diameter, Alg-PEG-a MS demonstrated, at 3 wt %, higher mechanical resistance than pure Ca-alg MS, while the values for Alg-PEG-b MS were even lower than for pure Ca-alg MS at 4 wt%.

Table 3. Size of MS and mechanical resistance to uniaxial compression of MS

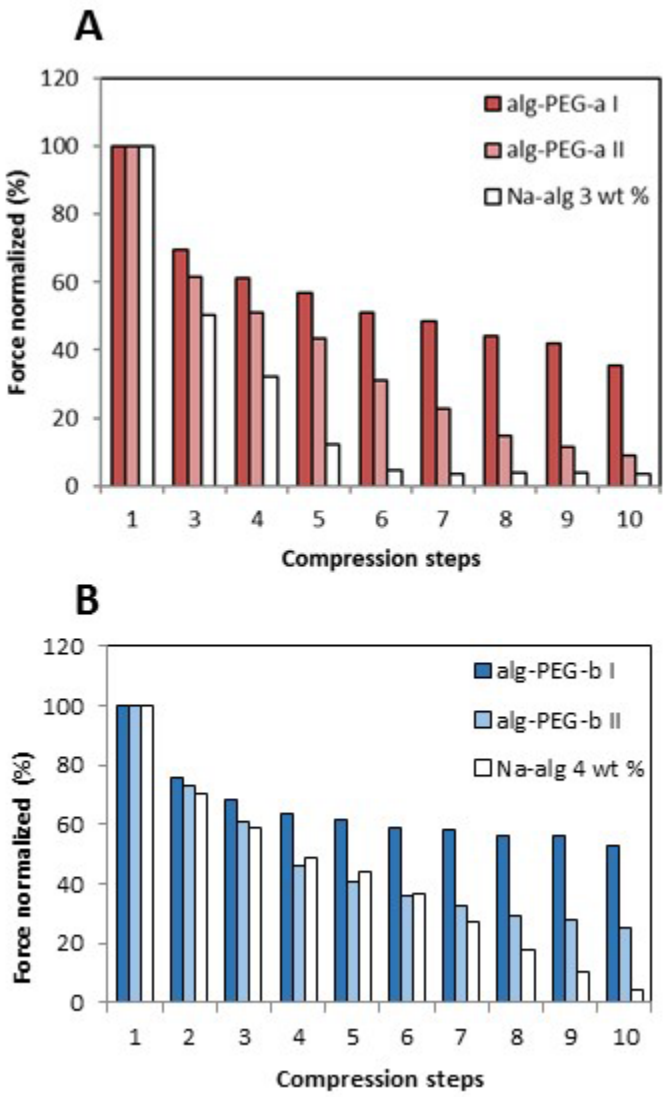
Polymer	Concentration[a] (wt %)	d_1[b] (μm)	d_7[b] (μm)	Mechanical resistance[c] (N/mm^3)
Alg-PEG-a I	3	934 ± 101	913 ± 105	3.0
Alg-PEG-a II	3	667 ± 98	642 ± 84	5.0
Na-alg	3	1048 ± 93	1028 ± 77	2.4
Alg-PEG-b I	4	1335 ± 118	1263 ± 122	1.2
Alg-PEG-b II	4	760 ± 83	715 ± 76	2.0
Na-alg	4	997 ± 127	986 ± 81	2.6

[a] in MOPS (10 mM, pH = 7.4). [b]Measured with an Olympus AX70 microscope equipped with an Olympus DP70 color digital camera. [c]Mechanical resistance to uniaxial compression to 90% of the initial MS diameter, volume-normalized.

The mechanical properties of the MS were further assessed by evaluation of their shape recovery performance upon repeated compression to 90% of the initial MS diameter (Figure 1). Visible become the shortcomings of the physical properties of Ca-alg MS, their limited mechanical resistance and poor shape recovery, as previously reported.[13] The performance of the MS was significantly better for PEG-grafted alginates than for pure Na-alg, which is attributed to covalent crosslinking. At 3 wt % polymer concentration, Alg-PEG-a I MS demonstrated almost 40 % shape recovery after 10 compressions while Ca-alg MS showed complete loss of shape recovery after 5 compressions. For longer PEG chains grafted to the backbone, the recovery performance of alg-PEG-b I MS was higher than 50% even after 10 compressions. The polymers

with end thiol functionalities produce MS with better recovery than the polymers with 1,2-dithiolane moieties (alg-PEG I vs alg-PEG II systems in Figure 1A and 1B).

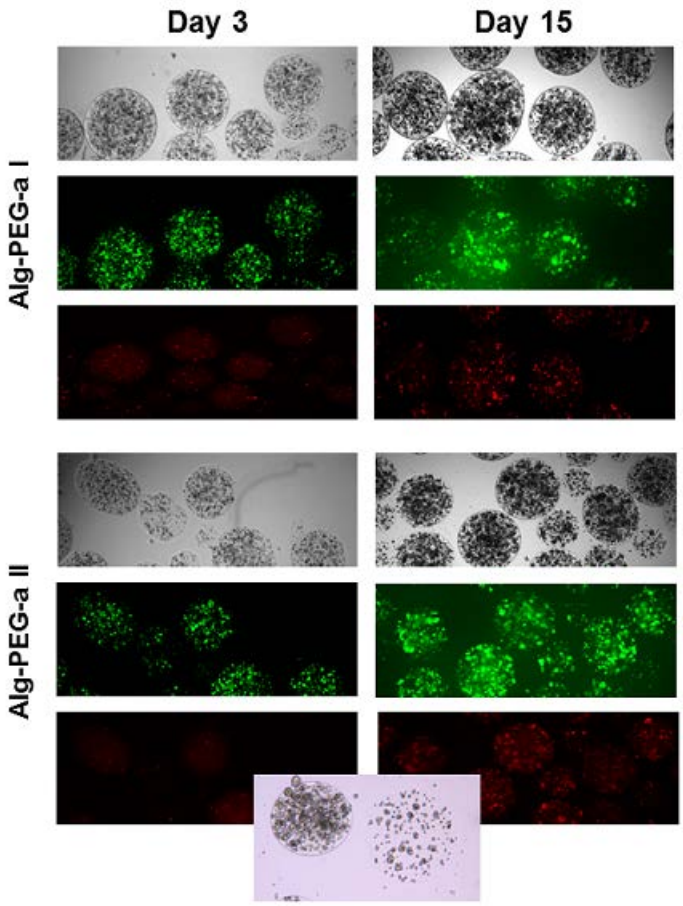
Figure 1. Elasticity/shape recovery upon coaxial compression



(A) Resistance to 10 successive compressions to 90% of Alg-PEG-a I MS (3 wt %), Alg-PEG-a II MS (3 wt %) and Ca-alg MS (3 wt %); (B) resistance to 10 successive compressions of Alg-PEG-b I MS (4 wt %), Alg-PEG-b II MS (4 wt %) and Ca-alg MS (4 wt %).

Cell microencapsulation. Considering their higher mechanical resistance to compression (Table 3), both Alg-PEG-a I and Alg-PEG-a II were considered to evaluate the feasibility of cell microencapsulation, using the mouse insulinoma cell line MIN6 as model cells. Encapsulation of MIN6 cells was performed under physiological conditions by extrusion in a gelation bath containing $CaCl_2$. This one-step process delivered MS of average diameters between 500 and 600 μm. Homogeneous cells distribution within both MS types was observed and no free cells were detected in the surrounding medium. The cell viability, assessed by FDA/PI staining at 3 and 15 days after encapsulation reached almost 80 % with both polymers and was constant over time (Figure 2). However, the nature of the terminal functionality of the PEG chains influenced the integrity of the microspheres network over time. During 15 days, the integrity of Alg-PEG-a I MS was evidenced as no free cells were identified in the culture medium. The MS from Alg-PEG-a II appeared degraded over time and out-diffusion of cells was identified from day 10. By light microscopy, the MS showed a lucent appearance. This observation indicates that covalent cross-linking resulting from 1,2-dithiolane moieties is reversible under the physiological conditions used for cell culture. Changing the functionalities installed on the PEG chain for covalent cross-linking seems to have the potential to modulate the stability of the resulting MS so that the functionalization of the alginate derivative can be selected depending on the type of intended application.

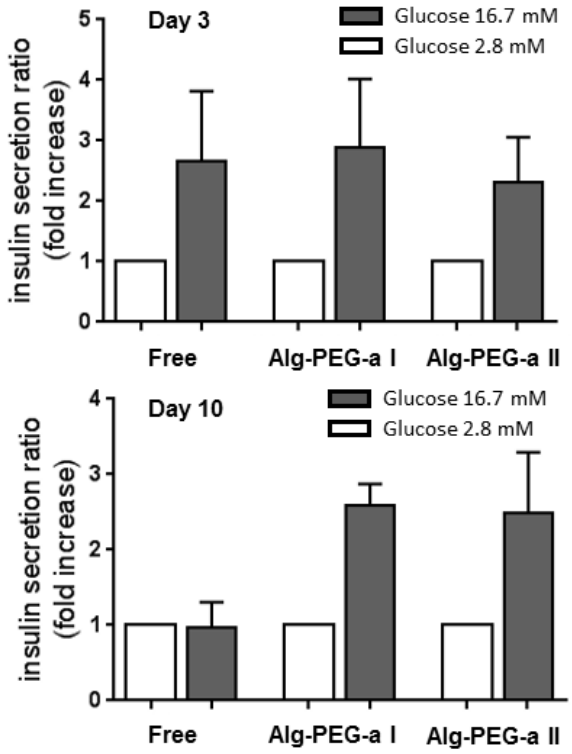
Figure 2. MIN6 cells microencapsulated in Alg-PEG-a I and Alg-PEG-a II MS.



Photographs are from day 3 and day 15 after microencapsulation and culture. For each polymer, upper panel: light microscopy, middle panel: staining of live cells with fluorescein diacetate (FDA, green), lower panel: staining of dead cells with propidium iodide (PI, red). Insert at the bottom: illustrates the loss of integrity of Alg-PEG-a II MS from day 10.

Free non-encapsulated MIN6 cells and microencapsulated MIN6 cells were subjected to a glucose-stimulated insulin release assay under static conditions, for both MS at day 3 and day 10 after microencapsulation (Figure 3). Stimulation was done at a glucose concentration of 16.7 mM, and the fold increase of insulin release was expressed with respect to the insulin release at basal glucose concentration of 2.8 mM, which was set as 1. The assay outcome was the same for free MIN6 cells and microencapsulated MIN6 cells, using either Alg-PEG-a I or Alg-PEG-a II MS, showing that the insulin-secreting capacity is maintained upon microencapsulation.

Figure 3. Glucose-stimulated insulin release for non-encapsulated MIN6 cells (Free) and MIN6 cells microencapsulated in MS of Alg-PEG-a I and Alg-PEG-a II.



Finally, the suitability of Alg-PEG-a I and Alg-PEG-a II systems for cell transplantation was assessed by the transplantation of empty MS formed from both systems in immune-competent mice, with a follow-up period of 30 days. Upon sacrifice of the mice, the MS were macroscopically inspected and retrieved (supporting information S11, Figure S1). At macroscopic inspection MS were visible. There were no signs of inflammation, neither connective tissue formation nor fibrosis, indicating no major host incompatibility for MS from both PEG grafted alginate derivatives.

CONCLUSION

A synthetic route was developed to functionalize the hydroxyl groups of the biopolymer sodium alginate with heterobifunctional PEG derivatives containing end thiol (I) or 1,2-dithiolane (II) functionalities for covalent cross-linking. Alg-PEG derivatives are obtained by activation of hydroxyl moieties in the form of imidazolide, followed by formation of covalent carbamate linkage with heterocheletic PEG derivatives. In comparison to pure Ca-alg MS, the MS obtained from Alg-PEG derivatives presented improved mechanical properties, in particular for shape recovery following multiple compressions. The nature of the end functionality of the grafted PEG chains has a direct impact on the physical properties of the resulting MS, and also influences their stability in physiological environment. For the same encapsulator settings, Alg-PEG I derivatives provided larger MS with superior elasticity performance than Alg-PEG II polymers. The potential of these materials for cell microencapsulation was demonstrated using MIN6 cells which showed good viability and insulin production capacity over two weeks. Interestingly, MS formed by combined ionic gelation and disulfide bride covalent cross-linking present higher durability in culture medium than MS formed by combined ionic gelation and disulfide clusters. The chemical composition of the PEG grafting unit can thus be adjusted to provide long term stability or degradation of the MS over time, depending on the targeted application. Noteworthy, MS are obtained from a single polymeric component, and using a one-step protocol which is not detrimental to cell survival and functionality. Preliminary *in vivo* studies gave evidence for the good bioacceptance of Alg-PEG-a I and Alg-PEG-a II MS, extending the panel of polymeric materials for cell microencapsulation. The chemical pathways disclosed herein provide a new basis for the covalent functionalization of the alginate backbone which can be further applied to the conjugation of bioactive molecules for controlled delivery at the transplantation site.

ASSOCIATED CONTENT

Supporting Information. Analytical data (^{1}H and ^{13}C NMR spectra, MS spectra); transplantation of Alg-PEG-a I and Alg-PEG-a II MS in immunocompetent mice.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Alg, alginate; Boc, *tert*-butyloxycarbonyl; CDI, carbodiimidazole; DCM, dichloromethane; DMEM, Dulbecco Modified Eagle's Medium; DMF, N,N-dimethyl formamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; FCC, flash column chromatography, FDA, fluorescein diacetate; MOPS, 3-(N-morpholino) ethanesulfonic acid; MS, microsphere; PE, petroleum ether; PEG, poly(ethylene glycol); PI, propidium iodide; rt, room temperature; TBA, *tert*-butyl ammonium; TCEP, tris(2-carboxyethyl)phosphine; THF, tetrahydrofuran.

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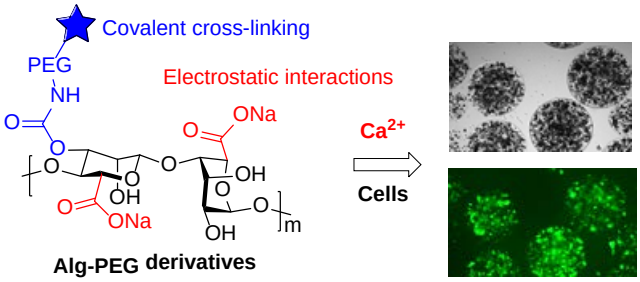
Covalent cross-linking
PEG
NH
Electrostatic interactions
ONa
Ca2+
Cells
Alg-PEG derivatives