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Titouan Montheil, Laurine Raimond, Laurine Valot, Marie Maumus, Matthieu Simon, et al.. Controlled Silylation of Polysaccharides: Attractive Building Blocks for Biocompatible Foams and Cell-Laden Hydrogels. *ACS Applied Polymer Materials*, 2022, 4 (6), pp.4087-4097. 10.1021/ac-sapm.1c01823 . hal-03692906

**HAL Id: hal-03692906**

**<https://hal.science/hal-03692906>**

Submitted on 22 Nov 2022

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# Controlled silylation of polysaccharides : attractive building blocks for biocompatible foams and cell-laden hydrogels

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ABSTRACT: We established, for the first time, a general methodology to synthesize and isolate hybrid silylated polysaccharides. Well-defined and characterized, these hybrids building blocks react in aqueous buffer at neutral pH by sol-gel inorganic polymerization, which may allow the preparation of cell-laden hydrogels. Alternatively, these hydrogels can be turned into hybrid xerogels by freeze-drying. Such materials are attractive as biocompatible foams for biological applications. We demonstrated the feasibility of our approach by preparing hybrid and polymerizable counterparts of hyaluronic acid, dextrin, chitosan and pectin, which display a whole range of chemical functions (hydroxyl, amine and carboxylic acid). The biocompatibility of selected hybrid hydrogels and foams was assessed at days 1 and 7.

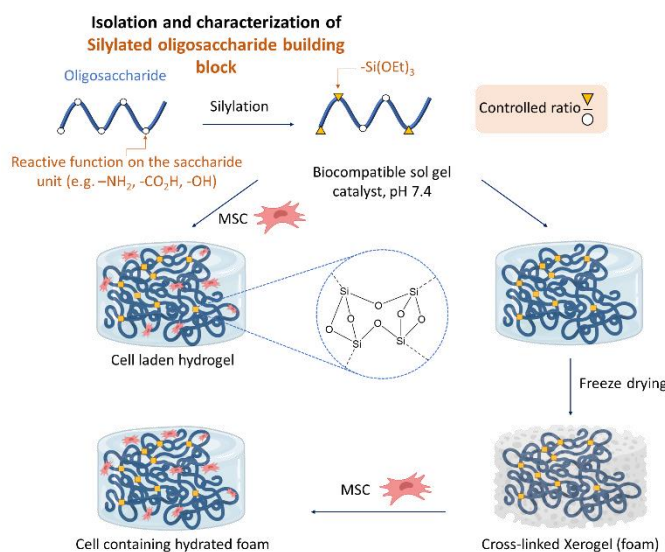
KEYWORDS: silylation · polysaccharides · sol gel · hybrid material · hydrogel · xerogel · foam · cell encapsulation

## 1. INTRODUCTION

Biomolecules are attractive components for designing bioactive materials,<sup>1,2</sup> and in particular for tissue engineering applications.<sup>3,4</sup> These compounds can be added to existing materials to afford specific biological properties<sup>5</sup>, or as starting building blocks for the preparation of novel biomaterials which can be shaped as aerogels, xerogels, membranes, monoliths, scaffolds through casting or 3D printing.<sup>6</sup> For biological applications involving cell culture, hydrogels<sup>7-10</sup> and foams<sup>11</sup> are highly relevant biomaterials. Hydrogels are water-swollen three-dimensional networks,<sup>12</sup> cross-linked either through covalent bonds (chemical hydrogels) or held together via weak interactions (physical hydrogels).<sup>9</sup> They could be used as intermediates to form dry materials by gentle thermal-treatment, freeze or super-critical drying, forming foams.<sup>13,14</sup> Beside hydrogels obtained from synthetic polymers, a large number of them are made of naturally-occurring polysaccharides (alginate, chitosan, agar, etc.),<sup>15-20</sup> or proteins (collagen,<sup>21-23</sup> fibrin,<sup>24,25</sup> etc.). They are biocompatible and some of them display interesting biological properties for biomedical applications like cell adhesion, induction of cell differentiation, biodegradability and resorption.<sup>26</sup> To prepare hydrogels, these biomacromolecules can be used as such, or have to be modified/functionalized (e.g. deacetylation for hyaluronic acid, introduction of acrylate groups). Mostly, these hydrogels are physical ones with low stability and are obtained thanks to weak interactions between biopolymers. They can be prepared in a biocompatible way.<sup>27</sup> Nonetheless, when higher stability over a long period of time and improved mechanical properties are required, covalently cross-linked i.e. chemical

hydrogels have to be chosen. Various reactions could be used to get covalent bonds between the components of the networks,<sup>28,29</sup> but not all of them are biorthogonal (i.e. proceeding chemoselectively and preserving the biomolecule's integrity). Furthermore, toxicity may arise from reagent, catalyst, by-products or reaction conditions (elevated temperature, photo-irradiation, pH, etc.), and even the remaining organic solvent if the reaction does not proceed in water.

To bypass these drawbacks, recently, we proposed an alternative to existing procedures and demonstrated that sol-gel inorganic polymerization could be used as a viable biorthogonal reaction under biocompatible conditions. It first requires the synthesis of well-defined alkoxy silane-modified precursors, which could undergo hydrolysis and condensation to form siloxane Si-O-Si bonds at 37 °C, at pH 7 in a biological buffer. Thanks to non-toxic amounts of sodium fluoride and glycine as catalysts,<sup>30</sup> these hydrogels may form even in the presence of living cells. Thus, we successfully prepared cell-encapsulating hydrogels from three different types of hybrid network precursors : silylated polyethylene glycol,<sup>31,32</sup> collagen-like peptides<sup>33</sup> and more recently hydroxypropyl methyl cellulose (HPMC).



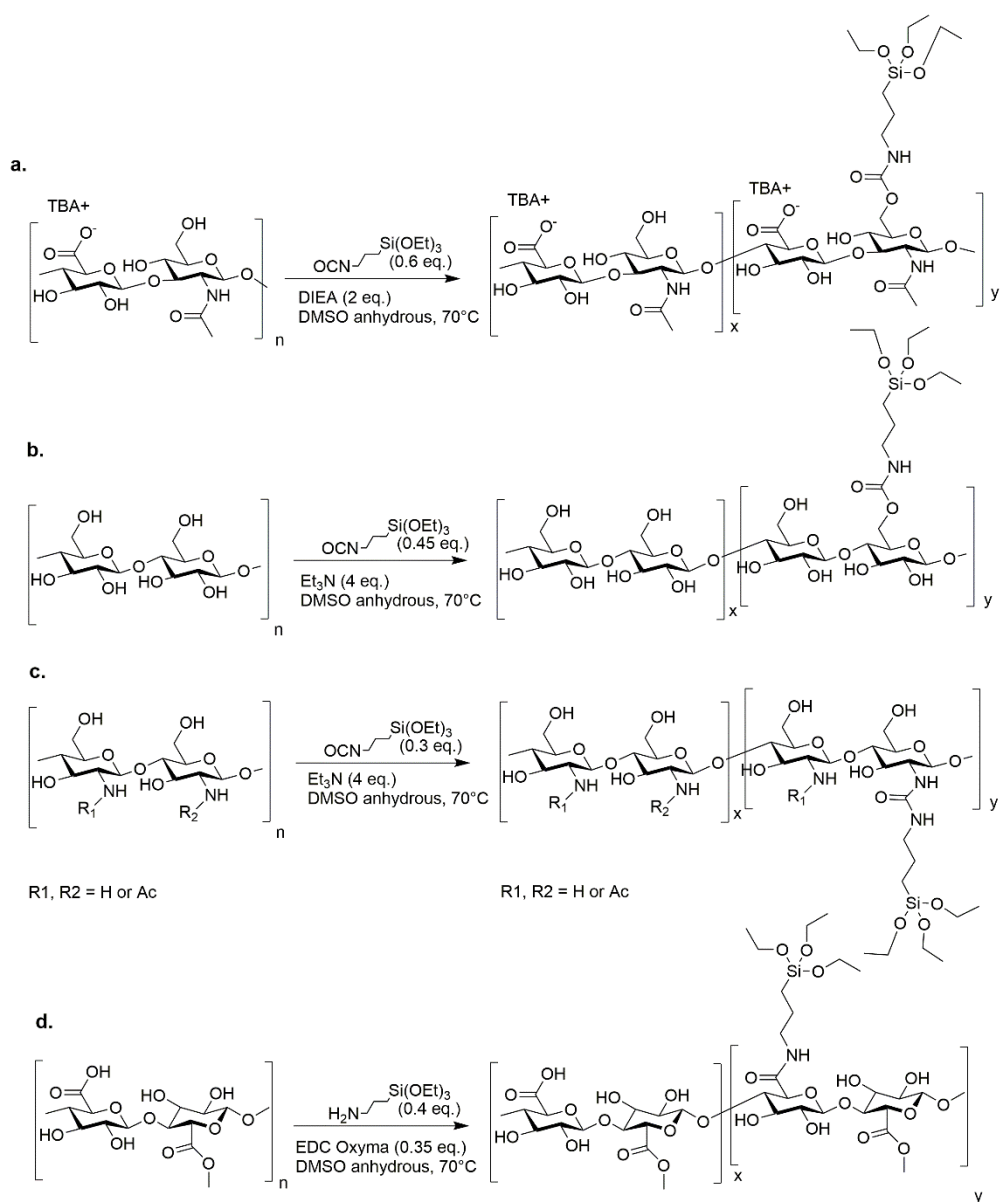
**Figure 1.** Strategies to prepare cell-laden hybrid polysaccharide hydrogels from hybrid precursors or from xerogels.

Noteworthy, the later precursor was used as bioink encapsulating mesenchymal stem cells (MSCs) and 3D-printed by extrusion.<sup>34</sup>

It has to be pointed out that hybrid materials obtained from biomacromolecules were already described including gelatine,<sup>35–37</sup> chitosan,<sup>38–41</sup> hyaluronic acid<sup>42</sup> or alginate<sup>43</sup> as presented in several reviews.<sup>44,45</sup> Nonetheless, the hybrid precursors were never isolated nor purified after their modification. Moreover, the synthesis of the material were done in one pot, with no control of the functionalization degree neither of the unreacted remaining reagents nor of side products.

In the field of polysaccharides, Weiss and co-workers studied extensively the silylation of HPMC to develop hybrid hydrogels for several biomedical applications such as cartilage and bone regeneration.<sup>46–48</sup> The same group also recently report on hydrogels prepared from silylated hyaluronic acid (HA) for cell therapies and tissue engineering.<sup>49</sup>

Nonetheless, the heterogenic conditions used for the introduction of silyl groups on the saccharide backbone did not allow a well-controlled silylation rate and ungrafted silylating reagent<sup>34</sup> was still found in the resulting material because the hybrid precursors were never isolated. In fact, hydrolysis was triggered at very high pH and condensation occurred upon neutralisation with acidic solution. This procedure required a careful pH adjustment before introducing cells to prevent unexpected cell mortality.



**Scheme 1.** Controlled silylation of hyaluronic acid (a), dextrin (b), chitosan (c) and pectin (d).

In our previous work, we set up a different sol-gel approach keeping all along the process, biocompatible conditions. With a bottom up and modular design in mind, we aim at constituting a wide selection of well-defined silylated blocks (e.g. bioactive peptides<sup>31,50-52</sup>, drugs, dyes<sup>50</sup> and (bio)polymers<sup>31,34</sup>) that can be mixed in appropriate ratio to yield ‘à la carte’ and ready-to-use multifunctional materials (Figure 1). In that way, peptide-polymer

hybrid hydrogels were prepared.<sup>53</sup> Besides the synthesis of Si-HPMC which was in-depth discussed and compared with other methods in a previous study,<sup>34</sup> we disclose herein the modification of four common and representative polysaccharides with alkoxy silane moieties: hybrid hyaluronic acid (Si-HA), Si-dextrin, Si-chitosan and Si-pectin (Scheme 1). These biopolymers were chosen regarding their biocompatibility,<sup>4,54,55</sup> but also because of their representative reactive functions displayed by the saccharides units: primary amine, hydroxyl and carboxylic acid groups.

Once synthesized, isolated and fully characterized, the Si-polysaccharides can be used to prepare hydrogels in physiological conditions as we already demonstrated with Si-HPMC<sup>34</sup>. Alternatively, we set up a method to obtain foams by freeze drying hydrogels.

## 2. EXPERIMENTAL SECTION

### 2.1 Material

All reagents and solvents were purchased from Alfa Aesar, Acros, Sigma-Aldrich or Merck and were used without further purification. Chitosan is deacetylated at 72%.

### 2.2 GPC analyses

The molecular weight of the polymer was determined using Gel Permeation Chromatography (GPC). The analysis was performed on an Agilent system consisting of a TSKgel PW5000+PW3000 column, a pump and a UV detector. Acetate buffer (0.15 M; pH 4.6) was used as the eluent at flow rate of 1 mL/min at 25 °C and samples prepared at 5 mg/mL and filtered with RC 0.45 nm filter.

### 2.3 Silylation reactions

The synthesis of hybrid polysaccharides displaying hydroxyl or amine functions was achieved using 3-isocyanatopropyltriethoxysilane (ICPTES), and with 3-aminopropyltriethoxysilane (APTES) for carboxylic acid functionalization, using a procedure inspired from our previous studies on collagen peptides and polyethylene glycol (PEG).<sup>31,56</sup> Hybrid polysaccharide was then stored at 4 °C under argon. Experimental details are given in supporting information (Table S1). Silylation rates were evaluated by <sup>1</sup>H NMR in D<sub>2</sub>O or DMSO-d<sub>6</sub>.

### 2.4 Silylation on OH groups of Hyaluronic acid with TBA<sup>+</sup> as counter-ion (HATBA)

Before silylation, the counter-ion (Na<sup>+</sup>) of HA was exchanged by tetrabutylammonium (TBA<sup>+</sup>) to improve the solubility in organic solvents with previously reported protocols.<sup>57</sup> Briefly, Amberlite IR 120 resin (1.75g) was added to 60 mL of TBAOH solution 0.15 M and stirred at room temperature overnight. The TBA@resin was filtered and washed several times with deionised water until neutrality. HANa (500 mg) was dissolved in 50 mL of deionised water and 1.75 g of TBA@resin were added. After stirring at room temperature overnight, the resin was filtered and the filtrate was frozen in liquid nitrogen and subsequently freeze dried to get HATBA.

HATBA (500 mg, 0.836 mmol of RU) was dried in an oven overnight at 80 °C, and dissolved in DMSO (25 mL) under argon. Then, DIEA (0.276 mL, 2 eq.) and ICPTES (0.132 mL, 0.6 eq.) were added. The mixture was heated at 70 °C for 48h. Then the solvent



was removed under vacuum and reaction mixture was precipitated in THF. After filtration, the solid was washed 3 times with THF and vacuum dried yielding Si-HA as white powder.

#### 2.5 Silylation on OH groups of Dextrin

Dextrin (500 mg, 1.54 mmol of RU) was dried in an oven overnight at 80 °C and dissolved in anhydrous DMSO (5 mL) under argon. Then, Et<sub>3</sub>N (0.858 mL, 4 eq.) and ICPTES (0.183 mL, 0.45 eq.) were added. The mixture was heated at 70 °C for 48h. The solvent was removed under vacuum and the obtained residue was precipitated in THF. After filtration, the solid was washed 3 times with THF and vacuum dried yielding Si-Dex as white powder.

#### 2.6 Silylation on NH<sub>2</sub> groups of Chitosan

Chitosan (500 mg, 2.90 mmol of RU) was dried in an oven overnight at 80 °C and dissolved in anhydrous DMSO (5 mL) under argon. Et<sub>3</sub>N (1.607 mL, 4 eq.) and ICPTES (0.228 mL, 0.3 eq.) were added. The mixture was heated at 70 °C for 48h. Then the solvent was removed under vacuum and the reaction mixture was precipitated in THF. After filtration, the solid was washed 3 times with THF and vacuum dried yielding Si-Chi as an orange powder.

#### 2.7 Silylation on COOH groups of Pectin

Pectin (500 mg, 1.36 mmol of RU) was dried in an oven overnight at 80 °C and dissolved in anhydrous DMSO (50 mL) under argon, along with (EDC) (93 mg, 0.35 eq.) and Oxyma (68 mg, 0.35 eq.). APTES (128 μL, 0.4 eq.) was added. The mixture was heated at 70 °C for 48 h. Then the solvent was removed under vacuum and the reaction mixture was

precipitated in tetrahydrofuran (THF). The solid was washed 3 times with THF and vacuum-dried yielding Si-Pec as a white powder

## 2.8 NMR characterization and determination of the silylation rate

NMR studies ( $^1\text{H}$ ,  $^{13}\text{C}$  and HMBC  $^{29}\text{Si}$ - $^1\text{H}$ ) were performed on silylated polysaccharides in  $\text{D}_2\text{O}$  (hyaluronic acid, dextrin, chitosan and pectin) (see Figures S1 to S17)

## 2.9 Preparation of hybrid hydrogels

Si-polysaccharide (13 wt %, 0.34 - 0.40 M; a volume of 1 mL was commonly used) was poured in Dulbecco's Phosphate Buffered Saline (DPBS) pH 7.4 containing NaF and glycine (respectively 0.1 g/L and 10 g/L) and stirred for 1 hour. When dissolution was complete, solutions were placed at 37 °C.

Controls were made by using unsilylated polysaccharides in the same conditions (solution and concentration).

Gelation times were determined using the 'tilting method', i.e., gelation was observed when the sample could not flow anymore upon turning the vial upside down.

## 2.10 Preparation of hybrid foams

500  $\mu\text{L}$  of hybrid precursor solutions (13 wt %) were dispensed in Eppendorf tubes (2 mL). After gelation, the resulting hybrid hydrogels were aged for 2 days before being frozen in liquid nitrogen and freeze dried in the Eppendorf tube during 24h to get corresponding hybrid Si-polysaccharide foams.

### 2.11 Microscopy analysis

Si-Polysaccharide foams were imaged by a Keyence microscope VHX-7000.

### 2.12 Swelling studies

Si-polysaccharide based foams were cut in three pieces, as triplicates, and weighted after drying ( $m_{\text{dried}}$ ). Then, they were incubated for 1 h or 24 h in 2 mL of deionised water at room temperature in 12-wells plates. Rehydrated hydrogels were quickly blotted with filter paper to remove excess surface water before being weighted ( $m_{\text{wet}}$ ). The mass swelling ratio was calculated as  $m_{\text{wet}}/m_{\text{dried}}$ . The mesh size of hybrid foams and hydrogels were then calculated from this ratio, following equations described in the supporting information (see equations 1 to 6 and Table S2).<sup>58</sup>

### 2.13 Rheological measurements

Measurements were done on a MCR 301 rheometer from Anton Paar, using a parallel plate geometry (25 mm in diameter). The rheological measurements were carried out in the linear domain. The latter was determined for each sample by recording the evolution of the shear stress as a function of the strain in dynamic mode and at a constant temperature of 20 °C (see example in supporting information, Figure S18). The temperature is the same as that used for the spectro-mechanical analysis carried out consecutively. Based on the determination of the linear domain, spectro-mechanical analysis were carried out at 0.4%, 0.2%, 0.5% and 0.3% of strain for pectin-Si hydrogel, pectin-Si rehydrated foam, chitosan-Si hydrogel and chitosan-Si rehydrated foam respectively. The value of storage modulus ( $G'$ ) is read when the curve is stabilised (around 0.01 rad/s).

Measurements were carried out on formed pectin-Si and chitosan-Si hydrogel disks (2.45 mL, 25 mm diameter) and rehydrated pectin-Si and chitosan-Si foam disks (25 mm diameter). The volume used to rehydrate the hybrid foam was the same as that of the corresponding hydrogel (2.45 mL of deionised water). Hybrid foams were allowed to rehydrate for 2 hours before analysis.

#### 2.14 Biocompatibility studies with hybrid foams and hydrogels

##### **Seeding MSCs on hybrid foams.**

Adipose tissue-derived MSCs were cultured in  $\alpha$ MEM medium containing 10% foetal calf serum, 100  $\mu$ g/mL penicillin/streptomycin and 2 mM glutamine, at 37 °C, overnight. Cells were trypsinized, pelleted by centrifugation at 300 g for 10 min, suspended in culture medium and seeded on top of foams ( $1 \times 10^4$  cells/100  $\mu$ L). A positive control was obtained with a commercially available collagen foam (ACE RCF Resorbable Collagen Foam reference 2194-016).

##### **Embedding MSCs in hydrogels.**

MSCs were pelleted as described above and suspended in the culture medium containing catalyst (0.1 g/L NaF and 10 g/L glycine in  $\alpha$ MEM). This suspension was then added to the silylated polysaccharide solution (130 g/L) to get a final concentration of  $1 \times 10^6$  cells/mL. After vortexing, 50  $\mu$ L of the final cell suspensions containing hybrid precursors were loaded in each well of 96 wells ultra-low attachment plates. As control, MSCs were embedded in 3 mg/mL rat collagen type I hydrogel (Corning). Following 2h gelation at 37 °C, 50  $\mu$ L of culture medium were added on top of hydrogels.

In each case (i.e. seeding or embedding), MSCs were cultured for 7 days with medium change at day 3. At the end of day 1 or day 7, cell viability was analysed by confocal microscopy (Leica) after staining the live cells in green and the dead cells in red using the live/dead assay kit (Invitrogen).

### 3. RESULTS AND DISCUSSION

#### 3.1. Polysaccharide silylation

The introduction of alkoxy silane moieties on the biopolymers may proceed on different organic groups displayed on polysaccharides backbones: hydroxyls, amines and carboxylic acids (Scheme 1). As shown previously,<sup>34</sup> the silylation rate can be tuned, thanks to the number of equivalent of silylation reagent used. The silylation ratio is assessed after isolation of the modified biopolymer by <sup>1</sup>H NMR analysis. In this study, we aimed a silylation rate about one third of the saccharide monomers on the biopolymer which will be subsequently involved in sol-gel reticulation. An important point for the reproducibility of the silylation rate is to work in strictly homogenous phase. For that, appropriate organic solvents must be used for each polysaccharide. In the same way, presence of water is not desirable to avoid unwanted premature hydrolysis of alkoxy silane. For that purpose, all biopolymers were dried at elevated temperature before silylation in dry solvents. Last, the recovery of hybrid polymers proceeded through precipitation to avoid solvent evaporation which could generate condensation.

HA was available as a sodium hyaluronate salt which proved to be only soluble in water, on the contrary to dextrin which was soluble in DMSO. To enhance HA solubility in

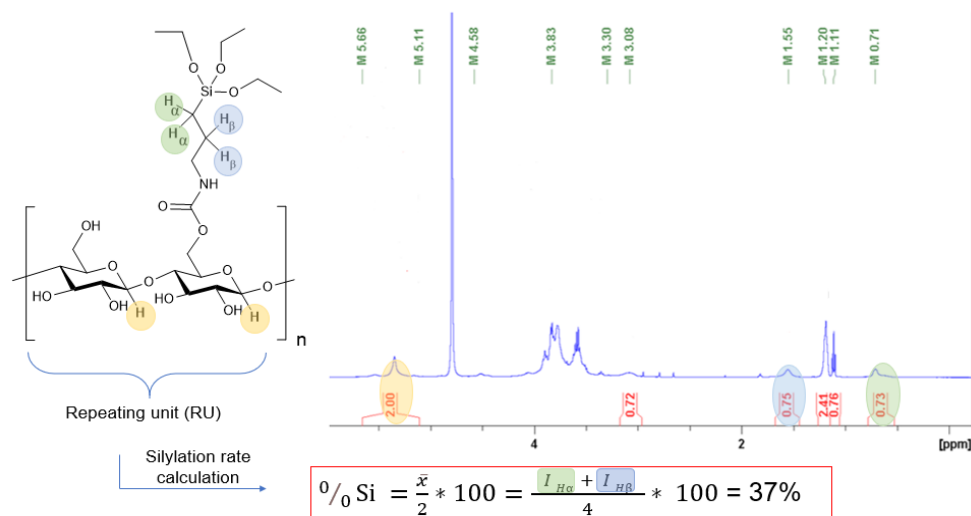
organic solvents, sodium exchange with tetrabutyl ammonium (TBA) was performed quantitatively using a cation exchange resin. HATBA and dextrin displayed respectively one and two primary alcohol function(s) per disaccharide repeating unit (RU). Both biopolymers were solubilized in DMSO and silylated by reacting with respectively 0.6 and 0.45 eq. of ICPTES related to the targeted hydroxyl groups. Silylation occurred in a presence of a tertiary amine, to yield carbamate linkages. The reaction evolved slowly, requiring 2 days at 70 °C to be completed as already observed during the preparation of silylated PEG bearing hydroxyl terminal groups.<sup>53</sup>

Chitosan presents both hydroxyl and amine groups, 38% of them being acetylated and thus, unreactive. We observed that the acetylation rate may vary from batch to batch depending on the chitosan supplier and we determined it by <sup>1</sup>H NMR before performing the silylation. The number of equivalents of ICPTES was voluntary kept at 0.3 eq. related to primary amines (0.72 amino group per RU), to chemoselectively react on amines, leaving the hydroxyl groups unaffected.

Pectin backbone present hydroxyl and carboxylic acid moieties, but the silylation was selectively performed on the later carboxylic acids (a single function per RU) after activation with EDC/Oxyma (0.35 eq.) in DMSO to react with APTES, yielding amide bond.

Polysaccharide	Mn (g/mol) (PDI)	MW of RU (g/mol)	Reactive function	Silylation reagent nbr of eq.	Silylation rate	Hydrogel concentration (g/L)	Gelation time <sup>a</sup>
Hyaluronic acid	2 140 000	379.31	-OH	ICPTES, 0.6 eq	28%	130	< 1 hour
Dextrin	4 600 (4.4)	324.28	-OH	ICPTES, 0.45 eq.	40%	130	~ 15 hours
Chitosan	5 000 (4.6)	322.3	-NH <sub>2</sub>	ICPTES, 0.3 eq.	24%	130	< 1 hour
Pectin	15 600 (2.0)	368.28	-COOH	APTES, 0.4 eq.	22%	130	< 1 hour

**Table 1.** Silylated polysaccharides and their corresponding hybrid hydrogels. <sup>a</sup>gelation time was determined by tilting assay, performed at 37 °C, at pH 7.0-7.8 in DPBS. RU means repeating disaccharide unit



**Figure 2.** Determination of silylation rate by <sup>1</sup>H NMR. Caption:  $\bar{x}$  meaning the average between  $I_{H\alpha}$  and  $I_{H\beta}$

First of all, <sup>29</sup>Si NMR spectroscopy was performed to obtain information about the local environment and the integrity of alkoxy silane moieties. In this respect, the <sup>29</sup>Si NMR spectra exhibit only one resonance peak at -42.0 ppm attributed to T<sup>0</sup> -Si(OR)<sub>3</sub> substructure indicating that no hydrolysis and condensation occurred on the hybrid silylated polymers. Then, silylation rates were determined by <sup>1</sup>H NMR analyses (Figure 2). Briefly, chemical shifts of anomeric protons and  $\alpha/\beta$ -methylene protons CH<sub>2</sub>CH<sub>2</sub>Si were determined by 2D NMR HSQC experiments (Figures S2, S6, S10 and S14). These signals were commonly used for quantification as no other proton signal was detected in this range of chemical shifts. The silylation rate is defined as the number of alkoxy silane grafted per RU.

However, for the sake of homogeneity with HA and pectin which are composed of disaccharide units repeats, chitosan and dextrin (which are composed of a single monosaccharide) were considered as having also a disaccharide RU.



**Figure 3.** Gelation of Si-HA (a), Si-dextrin (b), Si-chitosan (c), and Si-pectin (d). Within each panel, the tube on the left contains non-silylated polysaccharide as control at the same concentration (13% weight).

Results are reported in Table 1. Using the optimized conditions presented below, we obtained silylation rates in the expected range (i.e. one third of RU being modified) ranging from 22% for pectin, to 40% for dextrin.

These silylated polysaccharides are stable for month when stored under argon in the fridge, avoiding degradation or hydrolysis of triethoxysilyl moieties.

### 3.2 Hybrid hydrogels

After several assays, weight concentration of hydrogels was set to 130 g/L. This amount allowed the gelation of hybrid hydrogels for all the studied hybrid polysaccharides within 24 hours after dissolution of hybrid biopolymers in buffer at pH 7.4 containing sodium



fluoride and Glycine.<sup>30</sup> Interestingly, no physical gel formation was observed with non-silylated polysaccharide solutions at the same concentration (Figure 3, left samples of each panel). This comparison highlighted the chemical nature of the hydrogels.

Gelation time slightly varies, depending on the type of biopolymer (Table 1). However, gelation time cannot be related to the silylation rate. Indeed, Si-dextrin, silylated at 40%, gelled after 15 hours compared to less than one hour for the others (less than 30 min for HA and pectin). This may be explained by the nature of biopolymer and its length. Dextrin was the shortest biopolymer with an average molecular weight of 4600 g/mol. HA and pectin displayed much higher molecular weight, thus facilitating the formation of a hydrogel network with the contribution of non-covalent interactions.

Nonetheless, it is clear that molecular weight could not explain itself the variation in the gelation time. Indeed, chitosan and dextrin display close molecular weight, but the gelation time is different. Thereby, for the same silylation rate, others parameters may affect the gelation time such as electrostatic interaction, hydrophobic effect, or Van der Waals forces,...

### 3.3 Hybrid foams

Foams are attractive supports for tissue engineering applications as cells can be seeded on them before being implanted. Alternatively, endogenous cells from the patient can colonize them after implantation. Hybrid hydrogels were turned into hybrid xerogel by freeze-drying. This process is known to increase the pore size, thus facilitating cell migration.

Directed freezing (i.e from the bottom to the top of the hydrogel sample) can be also applied to create columns of ice within the hydrogel, which after freeze drying leave orientated

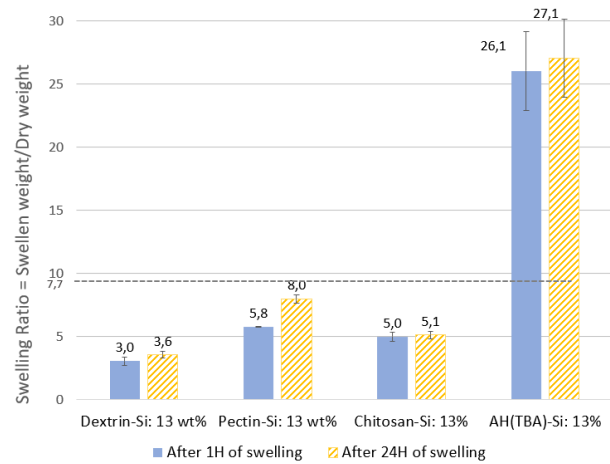
pores crossing the material. Compared to hydrogels, foams are much easier to handle. First, they can be washed to remove reagents used during their preparation, they can be stored in a handier fashion, are less prone to microbial contaminations and can be sterilised before implantation and/or cell seeding.

Surprisingly, only few hybrid polysaccharide xerogels have been described so far. One may cite 3-Glycidyloxypropyltrimethoxysilane (GPTMS)-modified chitosan xerogel,<sup>39,59</sup> or a xerogel obtained from silylated alginate.<sup>43</sup> The carboxylic acid functions of the polymer were activated by EDC/N-hydroxysuccinimide and reacted with APTES.<sup>43</sup> However, as already pointed out, these materials were prepared using a one-pot procedure without isolation of the modified biopolymer before reticulation and freeze-drying, thus implying the presence of unreacted silylation reagents and non-controlled silylation rate.

All the hybrid foams (Figure 4) presented an observable macro-porosity. To calculate the mesh size of network, swelling experiments were performed. It will also assess the propensity of these materials to recapture water. For that, foams pieces were immersed in water and placed in a large 250 mL beakers allowing the foams to swell freely for several days. Swelling ratio was calculated by dividing swollen foam weight over dry foam weight. It means that if a foam absorbs the same amount of water that was contained in its precursor hydrogel, its swelling ratio would be  $1/0.13 = 7.7$ . Results are gathered in Figure 5. Whatever the polysaccharide, no significant difference was observed between 1 hour and 24 hours meaning that the swollen material probably reached equilibrium after one hour.



**Figure 4.** Hybrid foams obtained from HA-Si (a), Dextrin-Si (b), Chitosan-Si (c) and Pectin-Si (d).



**Figure 5.** Swelling ratio of hybrid foams after 1h (blue) and 24 h (yellow dashes) of swelling into deionized water.

More importantly, and on the contrary to non covalent hydrogels, the foams did not dissolve in the buffer, keeping the same shape and size after 7 days. This indicated that the network was cross-linked enough to ensure cohesion of the swollen hydrogel which reached its maximum size. The most absorbent foam appeared to be the one obtained from hybrid HA

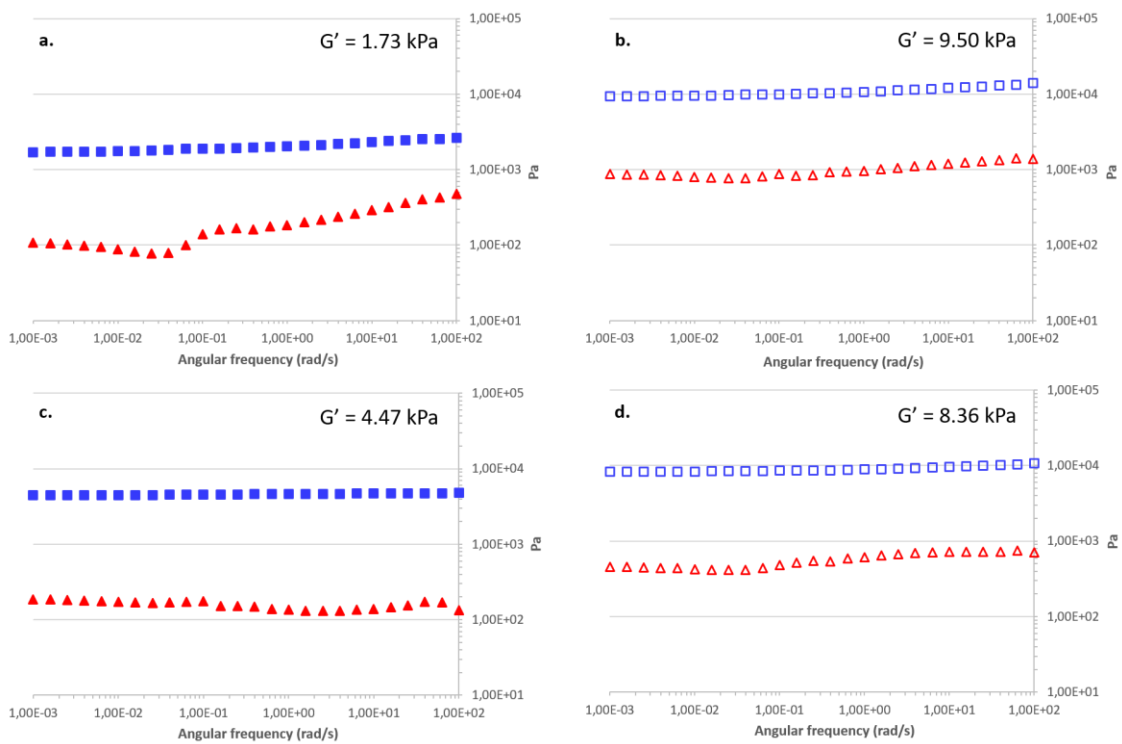
which was able to capture more water than the starting hydrogel (swelling ratio of 27.1). On the contrary, hybrid dextrin foam presented the lowest swelling ratio (3.6). Hybrid pectin and chitosan foams were able to trap almost the same amount of water present in their original hydrogels, having swelling ratio of 8.0 and 5.1, respectively.

Mesh sizes calculated from the swelling ratio showed results correlated to the molecular mass of polysaccharides, with the higher values of 216 nm and 12.4 nm for HA and pectin, respectively, which are the longest polymers ( $2.14 \times 10^6$  g/mol and 15600 g/mol). The shortest polymers of 4600 g/mol and 5000 g/mol, dextrin and chitosan obtained mesh sizes of 5.28 and 6.21 nm, respectively.

### 3.3 Mechanical properties of hydrogel and hybrid foams

Mechanical properties are an important factor for future use such as biomaterials for regenerative medicine. In order to compare hybrid hydrogel and rehydrated foams properties, rheological studies were performed. Pectin-Si and chitosan-Si hydrogel and foam were chosen for these experiments. To representatively compare hydrogel and foam materials, hybrid foams were rehydrated with the same volume as that used for hydrogel preparation. The storage modulus of hybrid hydrogel and rehydrated hybrid foam were measured (Figure 6). For both hybrid polysaccharides materials, it appears that rehydrated foams displays higher rigidity than corresponding hybrid hydrogel (5.5 times higher for pectin-Si, and 1.9 times higher for chitosan-Si). Visually, we further notice that hybrid hydrogels retains water better than corresponding rehydrated foams.

Considering these results, two complementary hypothesis could explain these variations. First, hybrid foams are obtained from hybrid hydrogel by freeze-drying. During this process, the sol-gel crosslinking is pushed to its maximum, resulting in a stiffer material. The second hypothesis relies on the water conformation within the material. Indeed, inside the hydrogel, water is known to be in at least two conformation: free or bound to the network. Considering hybrid hydrogels, there is both these two states, but for rehydrated foams, only free water allows to re-inflate the network, like a sponge. Considering that bound water act as a plasticizer, it could explain why hybrid hydrogels are less rigid. With regard to their specific mechanical properties, various application can then be considered for these hybrid hydrogels and foams.



**Figure 6.** Storage modulus (square) and loss modulus (triangle) as a function of angular frequency of Pectine-Si hydrogel (a), Pectine-Si rehydrated foam (b), Chitosan-Si hydrogel (c) and Chitosan-Si rehydrated foam (d).

### 3.5 Cell seeding on hybrid foams

In a first instance, we assessed dextrin, pectin and chitosan hybrid foams which presented a convenient swelling ratio within the same range, for viability assays. A commercially available collagen foam was chosen as control. At day 2 after seeding, MSCs were adherent and viable on the control collagen foam as shown by the high number of fluorescently labelled cells (Figure 7a). As expected with the other hybrid foams, no toxicity was observed. Aggregates of non-adherent viable cells were observed on chitosan foams while few viable poorly adherent cells and some cell aggregates were detected on dextrin and pectin foams. At day 7, similar viability results were obtained but with fewer cells were detected since MSCs did not adhere on the foams and most of them were removed during medium change (data not shown). Although the polysaccharides were biocompatible, functionalisation of the foams with bioactive peptides should be required to improve adhesion and survival of MSCs on the long term.

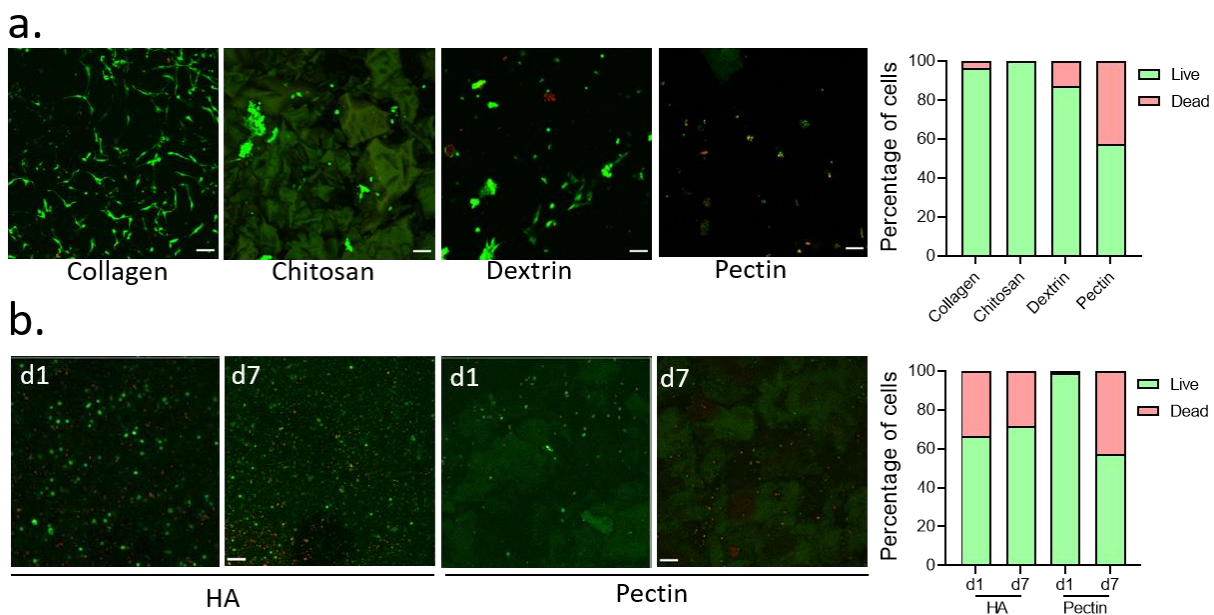
### 3.6 Cell encapsulation in hydrogels

For this study, the two hybrid polysaccharides which yielded hydrogels the quickest were selected for MSC encapsulation: HA and pectin (13% weight/vol). Commercially available type I collagen hydrogel was used as control. As already discussed and in contrast with existing procedures, MSCs were directly mixed with the catalyst-containing hybrid polysaccharide suspension. To limit cell decantation, the tube was placed on a rotator till gelation of the hydrogel, which occurred in less than 5 min for HA and almost instantly for

pectin. As already observed in a previous study<sup>30</sup>, the presence of cells speeded up the gelation.

At day 1 after cell-containing gel deposition, MSCs appeared round and most of the cells were alive in the HA and pectin gels although in less number in pectin gels (Figure 7b). At day 7, cells were still alive in the HA gels but appeared smaller, likely due to the density of the network which entrapped cells within the pores of the gel. In the control collagen hydrogel, MSCs were alive until day 7 (data not shown).

As expected, the sol-gel polymerization of Si-HA and Si-Pectin proceeded in a biorthogonal manner, allowing the survival of MSCs for at least 7 days.



**Figure 7.** (a) MSCs-seeded hybrid foams with collagen foam as control. (b) MSCs-laden hybrid hydrogels of HA and pectin.

These results also show that no deterioration were noticed on hydrogels even after 7 days, meaning that hydrogel are stable in physiological conditions for 7 days at least.

#### 4. CONCLUSIONS

By modifying hyaluronic acid, dextrin, chitosan and pectin, we have shown that silylation can be performed on any type of polysaccharide carrying a hydroxyl, amine or carboxylic acid function. The protocol we set up allows, for the first time, the isolation of the silylated precursors and a good control of the silylation rate, responsible for further reticulation. Interestingly, hybrid hydrogels can be prepared from these precursors via the sol-gel process under biocompatible conditions allowing cell encapsulation. Besides, hybrid foams can be obtained by freeze-drying, demonstrating interesting swelling properties and biocompatibility. Unfortunately, cell adhesion on these foams was not optimal. However, we already demonstrated that additional biological properties can be given to PEG-based materials obtained by sol gel process by adding silylated peptides (RGD) to the hybrid precursors of the network.<sup>53</sup> The same strategy could be applied on the reported polysaccharide materials by adding selected hybrid peptides whose sequences derived from fibronectin or laminin adhesion proteins.

These well-defined hybrid oligosaccharides further enrich the repertoire of functional silylated building blocks (e.g. peptides,<sup>33</sup> drugs, dyes<sup>60</sup>). This diversity allows to rationally conceive multifunctional biomaterials by mixing the appropriate ratio of selected relevant components, covalently linking them by the same biorthogonal reaction: inorganic sol-gel polymerization.



## ASSOCIATED CONTENT

Supporting Information Available.

Supporting information displays NMR analysis ( $^1\text{H}$  NMR,  $^{29}\text{Si}$  NMR,  $^{13}\text{C}$  NMR, 2D HMBC  $^1\text{H}$   $^{29}\text{Si}$ ), mesh size calculations, silylation quantities and reagents table and rheological measurements. NMR analysis allows the characterization of each silylated polysaccharides.

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The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENT

This work was funded by the ANR (Agence Nationale de la Recherche), the French National Research Agency (ANR-16- CE18-0003).

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## TABLE OF CONTENTS

In this study, we propose a generic methodology to functionalize polysaccharide with alcoxysilane groups. These hybrid building blocks are of great interest, especially to be chemically cross-linked by using the sol-gel process, leading to functional hybrid materials. Hyaluronic acid, dextrin, chitosan and pectin were chosen for their biocompatibility, but also for their reactive functions (respectively hydroxyl, amine and carboxylic acid). Once silylated, these hybrid precursors were used to design several polysaccharide-based hydrogels and xerogels.

