Gelatin modified with alkoxysilanes (GelmSi) forms hybrid hydrogels for bioengineering applications

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Abstract: Biopolymers are ideal candidates for the development of hydrogels for tissue engineering applications. However, chemical modifications are required to further improve their mechanical properties, in particular to cross-link them for long-lasting applications or biofabrication. Herein, we developed a novel gelatin-based hydrogel precursor, “GelmSi”. Gelatin was chosen as starting material because of its biocompatibility and bioactivity, favouring cell adhesion and migration. Alkoxysilane moieties were introduced in a controlled manner on the lysine side chains of gelatin to obtain a hybrid precursor which reacts in physiological conditions, forming covalent siloxane bonds and allowing the formation of a three-dimensional chemical network. On the contrary to unmodified gelatin, siloxane covalent network dramatically increases the stiffness and the thermal stability of the resulting gelatin-based hydrogel, making it suitable for cell encapsulation and cell culture. The biorthogonality and versatility of the GelmSi hybrid hydrogel unlock a broad range of gelatin-based bioengineering applications.

Keywords: gelatin, hybrid hydrogel, sol-gel process, cell encapsulation, biorthogonality, tissue engineering

1. Introduction

Tissue engineering aims at producing mature tissue by combining cells with a biomaterial, to restore a damaged or diseased organ.[1] Hydrogels are the preferred scaffolds to tackle these applications thanks to their capability to absorb and retain large amount of water (typically more than 80 %) in their polymeric network. Two types of hydrogels can be defined depending on the forces contributing to the network stability: chemical hydrogels are cross-linked by covalent bonds and are more resistant to environmental changes compared to physical ones, only maintained by weak non-bonded interactions (hydrogen bonds, van der Waals contacts etc.). Some of them are particularly suitable for three-dimensional (3D) layer-by-layer bio-printing to create a 3D construct with a well-defined shape and structure.[2] Network precursors can be derived from natural products or fully synthetic. Naturally-occurring polymers also called biopolymers comprised proteins such as fibrin, collagen, gelatin, fibroin or polysaccharides such as alginate, hyaluronic acid, cellulose or chitosan. In tissue engineering applications, the hydrogel should ideally behave as a substitute of the natural extracellular matrix (ECM) and must therefore be fully biocompatible to allow cells encapsulation. Although it is relatively straightforward for physical hydrogels, it is more demanding for chemical hydrogels where biorthogonal and chemoselective reactions should be applied. Chemical reagents and catalysts used to trigger the gelation must be non-cytotoxic. At last, hydrogels should also display good thermal stability at 37 °C and permeability for continuous access to nutrients. ECM extracts such as Matrigel™ and Geltrex™, but also collagen-based hydrogels are extensively used for tissue engineering thanks to high biocompatibility and bioactivity. However, they are impaired by some limitations including a poor control of their composition (for ECM extracts), a limited range of processing temperature, a low mechanical tunability and they cannot be easily chemically modified. On the other hand, easily processible scaffolds, such as polyethylene glycol and polysaccharide derivatives, natively lack bioactive components necessary for cell adhesion.[3]

Gelatin-based physical hydrogels are probably one of most popular. Gelatin is already used in food industry to improve rheological properties of food without altering taste; in pharmaceutical industry as an encapsulating agent for drug delivery purpose in the form of tablets, capsules, microspheres or nanoparticles;[4] in cosmetic industry[5] and in many other industrial applications like rubbers, adhesives, bioplastics, printing inks, light filter, etc. Because of its inherent biocompatibility and its bioadhesive nature, gelatin has also been widely used in the medical field as a sealant for protheses or absorbent pad for surgical purpose. Gelatin is obtained by the partial hydrolysis of the collagen triple helix through thermal denaturation and alkaline or acidic treatment. These treatments lead to a polydisperse peptide mixture with molecular weight ranging from 50 to 100 kDa.[3] Gelatin keeps similar amino acid composition as collagen,[6] it is non-toxic and does not induce antigenicity.[7] Collagen being the most abundant protein in ECM, gelatin is a very revelant substitute for ECM. Interestingly, gelatin retains the motives of collagen required for cell adhesion such as RGD sequences and the recognition sequences of matrix metalloproteinases (MMPs) that allow the cell-triggered remodelling of the matrix. Gelatin is soluble in water at physiological pH, on the contrary to collagen that displays poor solubility due to an isoelectric point close to 7.5. This solubility facilitates its processing in many applications. Unfortunately, gelatin-based hydrogels display poor mechanical properties and lack thermal stability. Above 30 °C, the hydrogen-bonding network that maintains the partial triple helix organization of gelatin is destabilized and the gel returns to a liquid state. This significant drawback disqualifies gelatin as a good candidate for tissue engineering as cell cultures are typically carried out at 37 °C, where cell growth, division and differentiation are optimal.

To overcome these limitations, different strategies have emerged during the last decade.[8] The first one is to prepare gelatin composites by combination with other biomaterials.[9] Alternatively, it is possible to covalently bridge the peptide chains to reinforce

the network. For that purpose, the modification of gelatin backbone followed by cross-linking strategies have been proposed, including imine formation between lysine side chains and aldehydes,[10] opening of epoxy groups with nucleophilic amino acid side chains or enzymatic cross-linking using the transglutaminase.[11] The most popular gelatin precursor of hydrogels for tissue engineering purposes, is by far the gelatin methacrylate (GelMA).[12] The introduction of methacrylate groups into gelatin proceeds via the reaction of activated acrylic acid with primary amines of lysine side chains. The resulting GelMA polymer can be cross-linked in a very convenient and controlled way. Straightforward UV exposure in the presence of a photoinitiator induces the free-radical polymerisation of the methacrylate group and the subsequent formation of a chemical gel stable at 37 °C. GelMA offers both the desired bioactivity and the tunability of chemical/mechanical properties.[3] However, the free radical polymerisation has disadvantages. First, UV exposure critically damages the DNA, which drives the cells to the apoptosis pathway. Moreover, the free-radical polymerisation induces the generation of reactive oxygen species (ROS) responsible of irreversible oxidation of lipids, proteins and DNA,[13,14] resulting in oxidative stress and cell death. Finally, UV exposure induces a heterogenous polymerisation of the chemical network with a higher degree of reticulation on the edges compared to the core of the hydrogel.

In this study, we developed a new modified gelatin called “GelmSi” with biological and mechanical properties comparable to GelMA, but with an innocuous setup for initiation of the gelation process for bioengineering or medical applications–thanks to the introduction of alkoxysilane moieties on gelatin. Interestingly, alkoxysilane can be placed in controlled positions on a wide range of organic molecules and (bio)polymers[15] including oligosaccharides,[16] peptides,[15,17,18] dyes,[17] drugs[19] to prepare hybrid materials or further functionalize surfaces of materials. Alkoxysilane moieties are the nodes of the covalent assembly linking the different silylated molecules or polymers through sol-gel process. They

undergo hydrolysis and condensation to yield up to three stable siloxane (Si-O-Si) bonds with other silylated precursors. This sol-gel process can be acid- or base-catalysed but interestingly, also proceed at pH 7 at 37 °C, using a nucleophilic catalysis. A combination of low concentration of sodium fluoride and glycine was used as catalyst under physiological conditions.[20] It is worth pointing out that these reactions proceed without affecting the numerous organic functions present in the biomolecules (i.e. alcohol, phenol, carboxylic acid, amine, imidazole, guanidine etc.). Sol-gel chemistry also offers the possibility to integrate other hybrid molecules directly into the siloxane network during the inorganic polymerisation.[15,17,18] Previous works report hybrid foams obtained from gelatin incorporating alkoxysilane moieties.[21–24] Unfortunately, this biopolymer results from a one pot reaction at acidic pH (HCl at 100 or 250 mM), without control of the modification and of the remaining reagents, and does not allow the encapsulation of living cells. To tackle this limitation, we developed the first stable and isolable alkoxysilane-modified gelatin precursor. We also set up a protocol for using GelmSi for tissue engineering application, meeting all the requirements for cell biocompatibility. Rheological and swelling studies were performed to monitor the sol-gel transition during polymerization and to show the significant improvement of the GelmSi hydrogels mechanical properties compared to gelatin.

2. Materials and methods

Reagents and solvents were purchased from Alfa Aesar, Acros, Sigma-Aldrich or Merck and were used without further purification, dimethylsulfoxyde-d6 (DMSO-d6) was obtained from Eurisotop. 3-(Triethoxysilyl)propyl isocyanate (ICPTES) was obtained from TCI Europe.

2.1 GelmSi synthesis

2.1.1 Gelatin solubilization

5 g of gelatin B (GelB) with a bloom of approximately 225 from Sigma Aldrich (CAS: 9000-70-8) were solubilized in 250 mL of dry DMSO at 55 °C during 2 hours. The solution was then dried using sodium sulphate and centrifuged at 3000 g for 10 min in a 250 ml conical centrifuge tube. The supernatant is a solution of gelatin 2 % (w/v) in DMSO, which can be recovered and kept under an inert atmosphere and in the dark during several weeks at room temperature.

2.1.2 GelmSi synthesis and purification

30 mL of the 2 % gelatin solution were then reacted with ICPTES (5 eq.) and N, N-Diisopropylethylamine (DIEA, 10 eq.) for 1 hour at room temperature under an inert atmosphere. Solution was poured dropwise in a 240 ml solution of diethyl ether/tetrahydrofuran (40/60 v/v) under vigorous stirring. Upon addition of 50 ml of diethyl ether, GelmSi precipitated. After centrifugation at 3000 g for 10 min, the supernatant was discarded and the pelleted white powder was washed 3 times using diethyl ether. The powder was then grinded and dried under vacuum overnight (<5 mbar). At the end of the drying process, the powder was aliquoted as 100 mg samples in 2 mL Eppendorf tubes and stored in the dark under inert atmosphere.

2.2 Trinitrobenzene sulfonic acid (TNBS) assays

TNBS assays were performed to quantify the degree of primary amine functionalization (Dof), as previously described.[25] GelmSi and raw gelatin samples were dissolved at 1.6 mg/ml in 0.1 M sodium bicarbonate buffer at 37 °C. Then, 0.5 ml of each sample were mixed with 0.5 ml of a 0.01 % TNBS solution in 0.1 M sodium bicarbonate buffer and then incubated at 37 °C for 2 hours. Next, 0.25 ml of 1 M hydrochloric acid and 0.5 ml of a 10 % (w/v) sodium dodecyl sulphate solution were added to stop the reaction. Absorbance of a 10-fold dilution of each sample was measured at 335 nm. Results were normalized using a negative control sample with no gelatin (0 %) and the commercial GelB sample (100 %).

2.3 ^{1}H NMR

^{1}H NMR spectra were recorded on a 600 MHz Bruker spectrometer equipped with a cryo-probe and pulsed-field gradients. Spectra acquisition and data processing were performed with the TopSpin software (Bruker). The internal reference used for the calibration of proton chemical shifts was trimethylsilyl propionate (TMSP). All spectra were recorded at 298 k using 15 mg of GelB or GelmSi solubilized in DMSO-d6.

2.4 Circular dichroism

Circular dichroism (CD) experiments were performed using a Jasco J815 spectropolarimeter (Jasco, Easton, MD, USA), as already described.[26] Before CD experiments, samples of gelatin and GelmSi at a concentration of 0.2 mg/ml in deionized water were stored at 4 °C and 37 °C for 2 hours. Spectra were then recorded, using a 1 mm pathlength CD cuvette over a wavelength range of 190-260 nm. Continuous scanning mode was used, with a response of 1.0 s with 0.1 nm steps and a bandwidth of 2 nm.

2.5 Energy-dispersive X-ray spectroscopy and scanning electron microscopy

The surface elemental compositions were determined using a scanning electron microscope (Phenom-world ProX) and energy-dispersive X-ray spectroscopy (EDS) using an accelerating voltage of 15 kV on different parts of the sample. Elemental analysis was performed using the dedicated Thermo Scientific Phenom Elemental Mapping Analysis Software.

GelmSi hydrogels were washed with PBS, followed by deionized water and then freeze dried. The obtained foam was then sliced, put on scanning electron microscopy (SEM) plot with carbon conductive tape, sputter coated with a 10 nm thick gold film and finally observed using an accelerating voltage of 10 or 15 kV with a Phenom-world ProX microscope.

2.6 Rheological study

The measurement of viscoelastic properties was performed on a Elastosens™ device.[27] 200 mg of commercial GelB, 200 mg and 260 mg of GelmSi were solubilized in 2 ml of dPBS solution to get, respectively, GelB-10 (Gelatin B 10 % (w/v)), GelmSi-10 (GelmSi 10 % (w/v)) and GelmSi-13 (GelmSi-13 % (w/v)). 200 µl of the catalyst solution were added to obtain a final concentration in the sample holder of 0.1 g/l of sodium fluoride (NaF) and 10 g/l of glycine. The solutions at pH 7.4 (2 ml) were poured into the three ElastoSens™ sample holders, which were then placed in the thermal chamber of the instrument at 25 °C. The measurement of viscoelastic properties was started 10 min after the addition of the catalyst solution. After 10 min samples were put at 4 °C for 30 min and returned in the Elastosens™ at 25 °C for 3 hours of measurement. Measurements on Elastosens™ were then performed at 37 °C on an ad hoc basis until a final measurement after 32 hours. In order to avoid dehydration of the hydrogel, 2 ml of PBS buffer were added on the different samples between each measurement at 37 °C.

2.7 Swelling study

Hydrogels were poured in 20 mL of deionized water at 37 °C for 24 hours. Then, each sample was blotted using a filter paper to remove the excess of water and weighed (wet sample). Samples were then freeze-dried and the obtained foam was weighted (dry sample). Swelling ratio was calculated using this equation:

$$Swelling\ ratio\ = \frac{Weight\ of\ the\ hydrated\ gel - Dry\ weight\ of\ the\ foam}{Dry\ weight\ of\ the\ foam} \qquad (1)$$

2.8 Cell viability assays

Human adipose-derived mesenchymal stromal cells (ASCs) were cultured in α-MEM medium containing 10% fetal calf serum (FCS), 100 µg/ml penicillin/streptomycin (PS), 2 mM

glutamine (Glu), and 1 ng/ml basic fibroblast growth factor (bFGF; CellGenix, Freiburg, Germany) and used at passage 1. Cells were trypsinized and resuspended in the hydrogels at the concentration of 10^6 cells/ml. Hydrogels with embedded ASCs (50 µl) were deposited in the wells of 96-wells culture plates, and stored at 4 °C for 30 min to increase gelation and avoid cell sedimentation, and then at 25 °C for 3 hours. Finally, 100 µl of culture medium were added to each well and plates were incubated at 37 °C for 7 days. Cell viability was evaluated on day 1 (D1) and day 7 (D7) using the LIVE/DEAD™ viability/cytotoxicity kit following manufacturer's instructions (Invitrogen). Cells were imaged by confocal microscopy using a confocal laser microscope (Leica TCS SP8).

3. Results and discussion

3.1 Synthesis of the GelmSi hybrid polymer and hydrogel preparation

Type B gelatin (GelB) was preferred because of its average bloom of 225 and lower endotoxin content than the type A.[14] Endotoxins are cytotoxic complex lipopolysaccharides (LPS) and thus, significant amount are not desired in presence of cells. Gelatin was solubilized in dry DMSO at a concentration of 2 % (w/v) at a temperature of 55 °C for 2 hours (Figure 1, lower panel). Interestingly, a clear solution of GelB was obtained and kept under inert atmosphere and away from light and moisture for several weeks at ambient temperature.

Then, the GelmSi intermediate was prepared using ICPTES along with DIEA to react with the lysine side chains of gelatin (Figure 1, step 1). The excess of ICPTES (5 eq.) and DIEA (10 eq.) reagents was calculated according to the number of mmol of free amino groups afforded by lysine side chains present in gelatin (0.318 mmol/g).[26] In such conditions, urea bonds are preferentially and quickly formed by reacting the primary amines of gelatin with the isocyanate of ICPTES. After one hour at 25 °C, >90 % of lysine side chain reacted as witness by ^{1}H NMR

(Figure 2). Importantly, the absence of water is required to isolate the alkoxysilane biopolymer intermediate, GelmSi. Indeed, water would trigger the premature hydrolysis of triethoxyl groups to corresponding silanol and would initiate the unwanted formation of Si-O-Si bonds and the hydrogel formation.

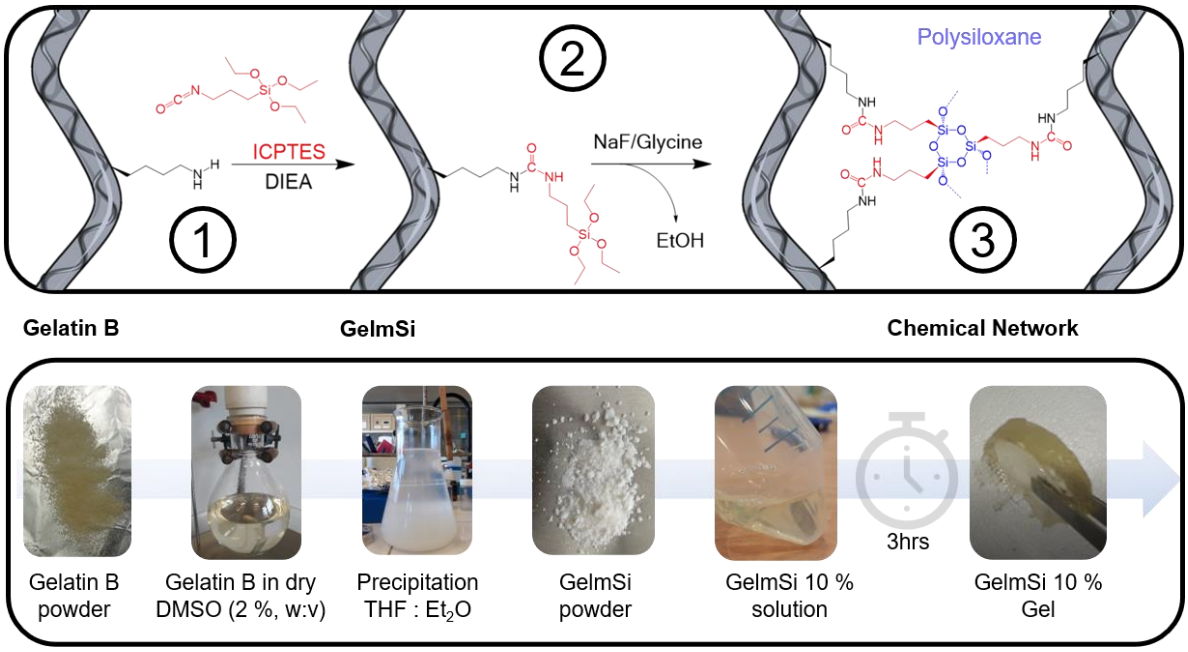

Figure 1: GelmSi synthesis. **Upper panel**: Chemical reactions to obtain a stable GelmSi hydrogel at 37 °C. Step 1: Silylation, reaction of ICPTES with a free amino group of gelatin, step 2: hydrolysis of the alkoxyl group catalysed by NaF/glycine solution and step 3: condensation and formation of siloxane bridges or polysiloxane network after condensation of silanols. **Lower panel**: experimental steps to obtain a 10 % (w/v) hydrogel made of GelmSi starting from the commercial gelatin B.

GelmSi was then purified by precipitation. This step was typically performed by adding a large volume of diethyl ether or THF in the modified gelatin DMSO solution, as previously described for other silylated polymers.[15,16] However, this protocol was not suitable for GelmSi leading to the formation of a sticky paste that trapped a large amount of DMSO, and was no more soluble in water. After testing different combinations of solvents, the DMSO solution

containing GelmSi was slowly added dropwise in a large volume of a THF/diethyl ether mixture (6/4 v/v) under strong stirring, resulting in the formation of a white colloidal solution. Then, dry diethyl ether was added and ten minutes after stopping the stirring, precipitation of modified gelatin occurred. Three cycles of washing/centrifugation were used to remove the excess of DIEA and unreacted ICPTES. A white agglomerate was obtained, crushed with a spatula and dried under vacuum overnight (< 5 mbar) to remove all traces of ether and DMSO. Multigram-scale batches of thin white powder of GelmSi can be obtained within two days and stored under inert atmosphere in the dark at room temperature at least one month. Finally, the last step is the straightforward sol-gel inorganic polymerization of isolated silylated precursor.[18] The GelmSi powder was solubilized in PBS at 37 °C and the catalyst solution (NaF/glycine) was added. Catalyst induces the hydrolysis of triethoxyl groups leading to the corresponding silanols (R-Si-OH) and, the condensation into a polysiloxane network (R-Si-O-Si-R). Importantly, The NaF showed no cytotoxicity at this concentration.[20] After three hours, hydrogels were obtained.

GelmSi intermediate was characterized by UV visible spectroscopy, nuclear magnetic resonance (NMR), circular dichroism (CD) and Energy-dispersive X-ray spectroscopy (EDS) (Figure 2). Then, we carefully studied the sol-gel transition during this polymerization process by testing of the GelmSi hydrogel formation over time. Its mechanical stability and viscoelastic properties were evaluated by following its shear storage modulus (G'). It is of importance to determine the suitable rheological properties for cell encapsulation and 3D extrusion-based printing opportunities.

3.2 Biophysical characterizations of GelmSi

First, trinitrobenzene sulfonic acid (TNBS) assay was performed to quantify the remaining free amino groups of GelmSi (Figure 2a). The absorbance of GelmSi was very close to that of the control sample, which did not contain any protein, witnessing that our protocol

allowed a complete reaction of the primary amines of gelatin with the ICPTES. The degree of functionalization (Dof) was 98 ± 2 %.

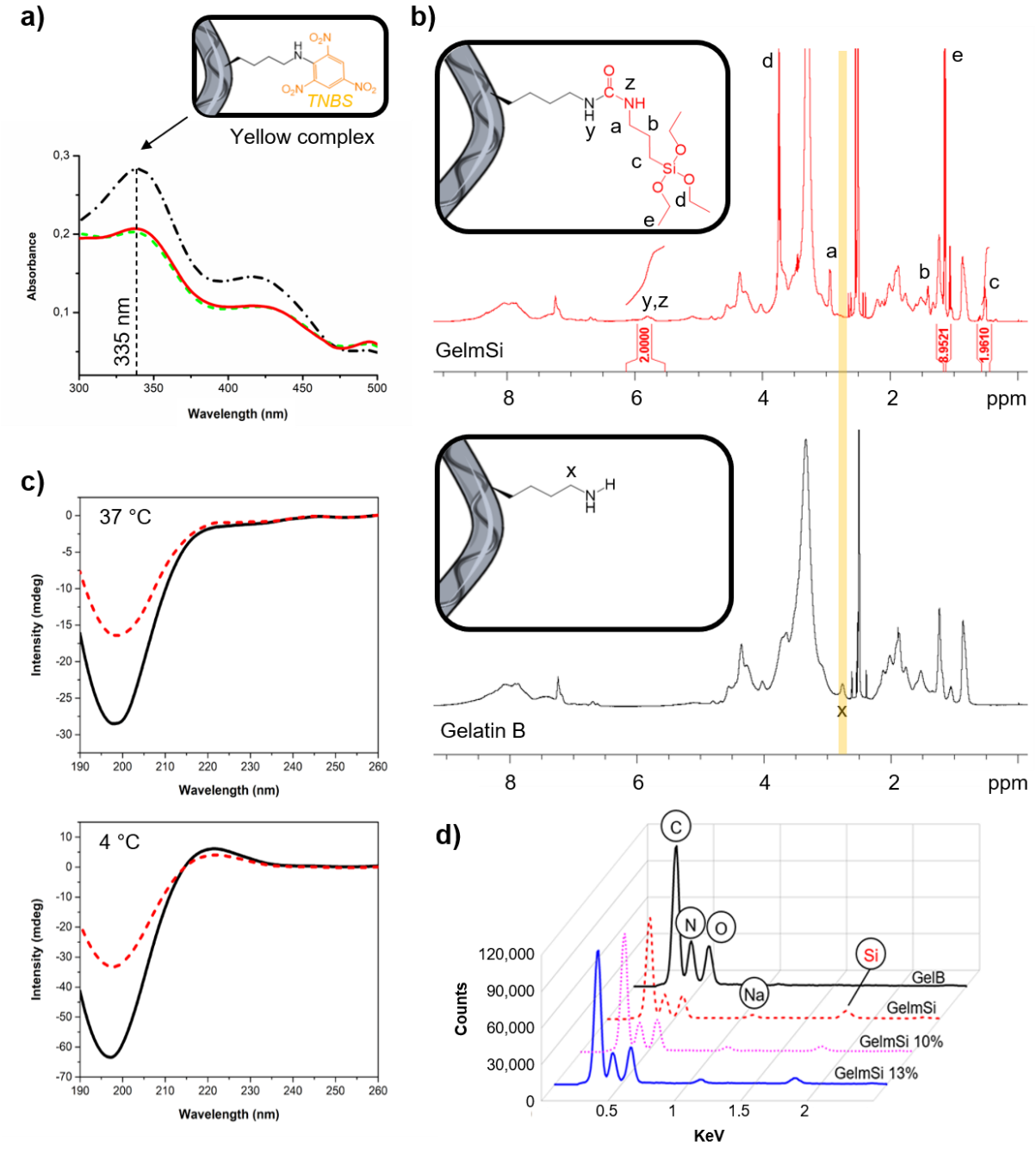

Figure 2: Biophysical characterization of GelmSi. a) TNBS assays performed on GelB (black dashed-dotted), GelmSi sample (red, plain) and control sample without gelatin (green, dashed) using UV-visible spectrometer. Absorbance at 335 nm (vertical dashed line) was used to quantify the amount of ICPTES grafted on the primary amine of the different gelatin samples. b) ^{1}H NMR spectra of 15 mg of GelB (lower panel) and 15 mg of GelmSi (upper panel) in

DMSO-d_6 with the corresponding assignment and integration of ICPTES protons after grafting onto the biopolymers. c) CD spectra of GelB (black, plain) and GelmSi (red, dashed) in deionized water at a concentration of 0.2 mg/mL at 37 °C (upper panel) and 4 °C (lower panel). d) EDS spectra of GelB, 10 and 13 % (w/v) GelmSi powder and GelmSi-foams obtained by freeze-drying of the corresponding hydrogels. Each peak position corresponds to one atom type (C, N, O, Na and Si).

The alkoxysilane group grafting was confirmed by ^{1}H NMR experiments (Figure 2b). The signals corresponding to ICPTES protons were assigned on the spectrum of GelmSi. For integration, we chose isolated protons for straightforward and accurate integrations. First, the signal at 2.7 ppm highlighted in orange was attributed to the methylene proton of the lysin side chain (-CH_2-CH_2-CH_2-C**H**$_2$-NH_2). This signal is commonly used in GelMA synthesis studies to evaluate the degree of functionalization of the lysine side chains with activated acrylic acid[28]. The chemical shift of this signal on the GelmSi spectrum is related to the urea bridge formation that modifies the electronic environment of the neighborhood protons. Thus, complete disappearance of this signal at 2.7 ppm indicated that all lysine side chains were functionalized with ICPTES. The grafting step was also confirmed by the direct comparison of the integration of the two protons (R-N**H**-CO-N**H**-R at 5.8 ppm) of the urea bond formed during the synthesis with the two methylene protons in alpha of the silicon atom (R -C**H**$_2$ -Si-$(OEt)_3$ at 0.5 ppm). Integration of these two resonances is similar on the GelmSi spectrum meaning that all the ICPTES present in the sample have formed an urea bridge with an amino lysine side chain. It also indirectly proved that there was no free ICPTES, showing the efficiency of the washing steps. Finally, integration of the methyl protons of ethoxy group (R-Si-(O-CH2-C**H**$_3$)$_3$) was close to 9H, meaning that little to no hydrolysis has occurred on the grafted ICPTES. NMR analyses performed two weeks after the synthesis showed identical results, witnessing that GelmSi can be stored as a stable powder, meeting here the expected specifications. The residual

solvent concentration of DMSO corresponding to the peak at 2.54 ppm was also evaluated using ^{1}H NMR. This peak was integrated and compared with the peaks corresponding to the traces of DMSO-d_5 (approximately 0.1%, peak at 2.50 ppm) present in the DMSO-d6 (Figure S2).[29] After integration and comparison, it was found that the DMSO residual solvent proportion is negligible in the sample (about 0.007 %).

Then, CD experiments were performed on both raw and modified gelatin, at 4 °C and 37 °C (Figure 2c). The large negative band centered at 198 nm corresponds to the random-coiled conformation of gelatin. At 37 °C, in both samples, this conformation prevailed. At 4 °C, a positive band at 220 nm was also detected in both samples, corresponding to a partial organization of gelatin in the well-known triple helix configuration characteristic of collagen. This means that the chemical modification on the lysine side chain of gelatin did not impact significantly its physical-chemical properties and ability to self-organize in a comparable way as the natural polymer.[26]

Finally, EDS experiments also confirmed the efficiency of the ICPTES grafting on gelatin (Figure 2d). Indeed, characteristic peaks of silicon atoms can be observed on the different samples containing GelmSi contrary to GelB. Interestingly, no trace of sulfur atom was detected in all samples, confirming the NMR results and showing that DMSO solvent was completely removed during the washing and drying steps.

3.3 Rheological and swelling studies of GelmSi hydrogels

GelMA is typically used between 5-15 % (w/v). We decided to work with GelmSi at an initial concentration of 10 and 13 % (w/v) as preliminary assays with GelmSi at 5 % (w/v) was not enough to maintain the hydrogel at 37 °C and solubilization of a GelmSi at 15 % (w/v) led to a very viscous solution difficult to manipulate.

During the first minutes, rheological studies showed that all samples behave the same and display low G' values close to 1 kPa (Figure 3a). At that point, the viscous solutions can easily be mixed with cells to encapsulate them as it was done for cell viability assays (see below). We also took advantage of this similarity of viscosity between the modified and natural polymer at the beginning of the reaction to assay its printability. These first trials were performed with a sample of GelmSi-10 on a Bio-X 3D printer (Figure S1 and video) using printing parameters classically used for GelB or GelMA[30]: pressure of 25kPa, a 27G needle and printing speed of 15 mm/sec. Well-resolved scaffolds were obtained demonstrating the potential of this hybrid material for applications using 3D printing.

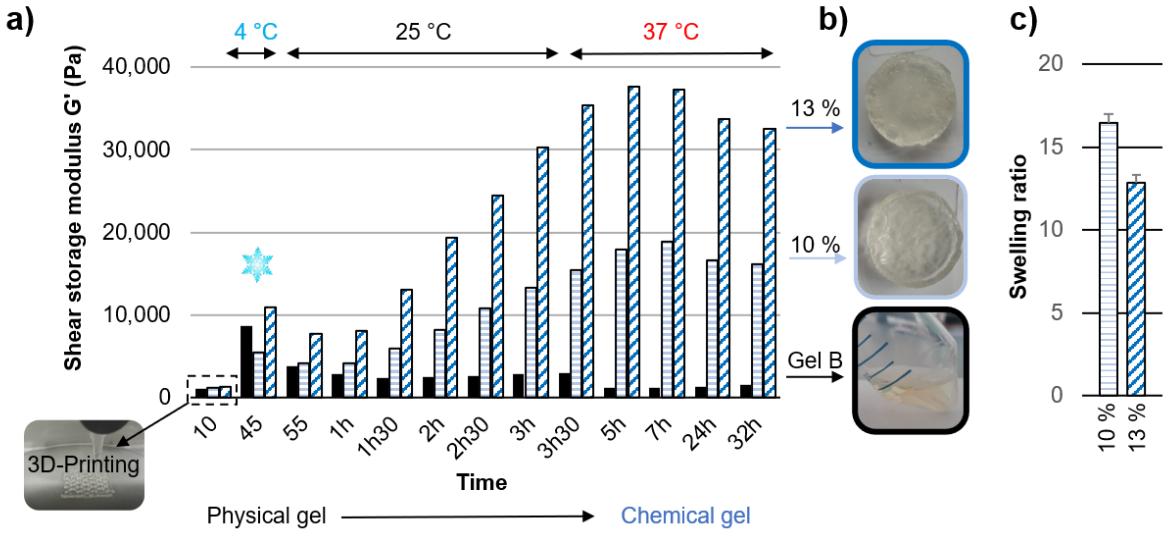

Figure 3: Rheology of GelmSi hydrogels. a) Viscoelasticity properties (G') of GelB-10 (plain black), GelmSi-10 (horizontal, light blue) and GelmSi-13 (slash, blue). B) Physical aspect of each hydrogel after 32 hours. C) Swelling ratio of the GelmSi-10 and GelmSi -13 hydrogels after 24 hours in deionized water.

Then, samples were placed at 4 °C for 30 min to favor the formation of a physical gel as witnessed by the increase of G' to values of about 6-11 kPa. After 45 minutes, the temperature was raised to 25 °C. As expected, the G' value of unmodified GelB-10 hydrogel dropped after

90 min to values around 2.5 kPa. On the contrary, the G' value of the GelmSi-13 and GelmSi-10 hydrogels increased due to the formation of covalent siloxane bonds yielding a stable chemical network. The G' constantly increased for 3 hours at 25 °C, at different rates depending on the initial concentration of the GelmSi hybrid precursors (4.25 and 9.3 kPa/hour for GelmSi-10 and GelmSi-13, respectively). At 32 h, GelmSi-10 and GelmSi-13 samples finally reach a final G' value of 16.1 kPa and 32.6 kPa, respectively, corresponding to their fully hydrated state at 37 °C. The final aspect of the hydrogels is displayed on Figure 3b, and clearly illustrates the successful impact of chemical modification on gelatin leading to a stiff gel for both GelmSi-10 and GelmSi-13 contrary to the GelB-10 sample that became a viscous solution at 37 °C.

Besides the interest of chemically cross-link GelmSi to get a solid gelatin scaffold at 37 °C for a long period of time, it is worth mentioning that the control of hydrogels stiffness through reticulation represents an opportunity to influence the behaviour and the differentiation of encapsulated cells.[31] In this rheological study, G' values obtained for GelmSi hydrogels vary from 16 to 32 kPa. For comparison, a similar range of value was obtained for GelMA concentrations ranging from 10 to 15 % (w/v) with a high degree of acrylate functionalisation,[32] i.e. 82-85 %. Obviously, the control of stiffness can be achieved by varying the concentration of GelmSi or by adjusting the degree of siloxane functionalization. Alternatively, and in agreement with what was already described for unmodified gelatin or GelMA, GelmSi could also be combined with other natural polymers (silylated or not) such as hyaluronic acid, fibrin, alginate or synthetic polymers like polycaprolactone or polyethylene glycol to finely tune their mechanical properties.[33,34]

Swelling studies were performed on GelmSi hybrid hydrogels such an assay was impossible to conduct with the unmodified gelatin which stays liquid at 37 °C (Figure 3b). The swelling ratio were 16.5 et 12.8 (Figure 3c) for GelmSi-10 and GelmSi-13 respectively, confirming the presence of larger pores in the GelmSi-10 hydrogel.

Finally, the ability of the hydrogels to encapsulate cells was evaluated (Figure 4). Briefly, adipose-derived stromal cells (ASCs) were gently mixed with the GelmSi precursor solution at 37 °C follow by the addition of the catalyst. Samples were directly put at 4 °C for 30 min, at this temperature GelmSi viscous solution quickly formed a physical gel, this step was mandatory to avoid cell sedimentation, then to allow the formation of the polysiloxane network samples were put at 25 °C for 3 hours as described in the rheological section. Finally, once the chemical network was established samples were incubated at 37° C for seven days. ASCs viability was investigated at day 1 (D1) and day 7 (D7) by live/dead staining and observed using a confocal microscope (Figure 4a). The living cell density and spatial distribution was the same for the two GelmSi hydrogels. Importantly, most of the ASCs were alive after 1 day. After 7 days approximately 90 % of cells were still alive within both samples which represents a highly satisfactory level of cell viability (Figure 4b).

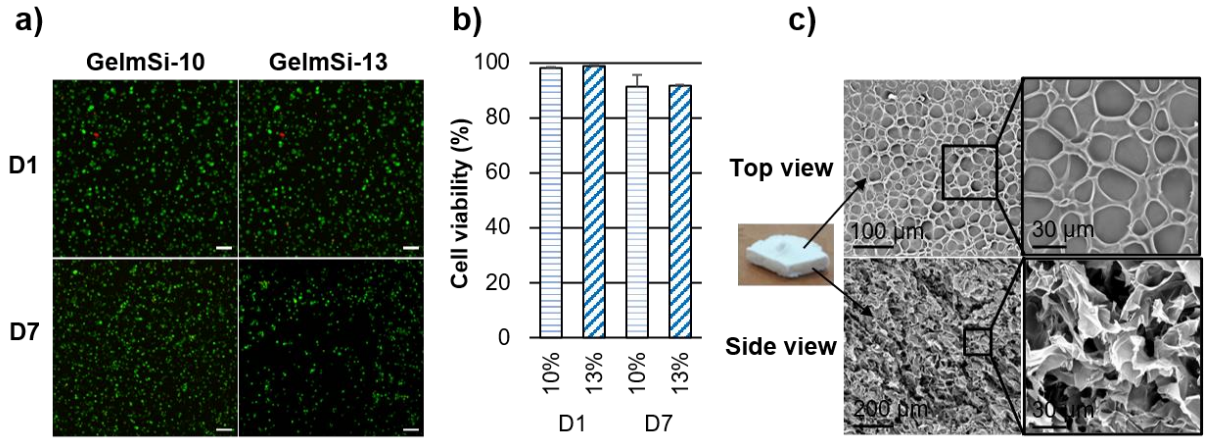

Figure 4: Cell viability after encapsulation in hydrogels. a) Viability of adipose tissue-derived mesenchymal stromal cells cultured in the GelmSi-10 and -13 was evaluated after one (D1) and seven (D7) days. Green: living cells and red: dead cells (scale bar = 100 µm). b) Percentage of viable ASCs in GelmSi-10 (horizontal, light blue) and -13 (slash, blue) at D1 and D7 (n=2 biological replicates). c) Scanning electron microscopy images of a dehydrated GelmSi-10 hydrogel.

GelmSi-10 (Figure 4c) and GelmSi-13 (Figure S3) hydrogels were then freeze dried, sliced and their morphology were observed using scanning electron microscopy. On the sliced view, a structure looking like an accumulation of crumpled sheets of paper can be observed in both foams, this morphology is characteristic of the dry gelatin.[35] On the flat surface of the sample (top view), a clear network can be observed, this heterogenous network has an average pore size of 20,8 µm for GelmSi-10 and 17,4 µm for GelmSi-13 foam. These results are in accordance with the swelling results and confirmed, as expected, the presence of higher pore size in GelmSi-10 compared to GelmSi-13 hydrogels. The presence of smaller pore in GelmSi-13 sample results from the presence of a denser network due to a higher number of silylated gelatin chains explaining also the higher stiffness values (G') observed in GelmSi-13 sample during the rheological study.

4. Conclusion

In this study, we successfully developed a new biocompatible cross-linked gelatin hydrogel based on a silylated gelatin precursor GelmSi. GelmSi can be easily synthesized in large amounts, isolated and characterized. Then, by solubilizing GelmSi in PBS at 10 to 13 % (w/v) using non-cytotoxic concentration of catalysts (NaF/glycine), hydrogels can be obtained in three hours, at 25 °C, through the establishment of a covalent polysiloxane network. Importantly, these GelmSi hydrogels are stable at 37 °C and allow the cells encapsulation with an excellent cell viability after 7 days. As inorganic polymerisation proceeds chemoselectively and bioorthogonally, GelmSi allows to prepare a cell-laden hydrogel by mixing the cells with the precursor solution from the very start of the process.

The versatility of this bioorthogonal approach to biomimetic hydrogels can be further broadened by adding other (silylated) molecules at the beginning of the hydrogel formation, including dyes, drugs, therapeutic proteins, nucleic acids, other biopolymers and relevant

biomolecules. Taken together, these features open broad perspectives for drug release and bioengineering applications like, for example, the design of tuneable bioinks for 3D bioprinting.

Author contribution: DN, GS, AM and CJ supervised the study; DN, GS, MM and MS designed the study; BL, LS, MM, MD and MS performed and analysed the experiments. MS wrote the manuscript and prepared the figures with contribution of all authors.

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Conflict of interest: The authors declare no conflict of interest

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Supplementary Information

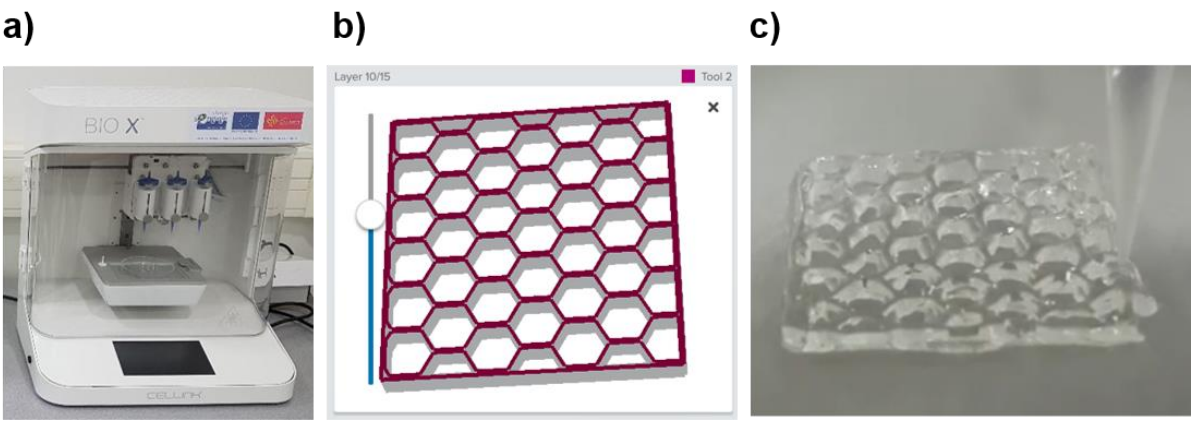

Figure S1: Video and figures illustrating the printability of GelmSi-10. a) BioX extrusion 3D printer from Cellink™ used to performed this assay. b) 3D model used in this study. c) Picture of the corresponding scaffold obtained during the printing step.

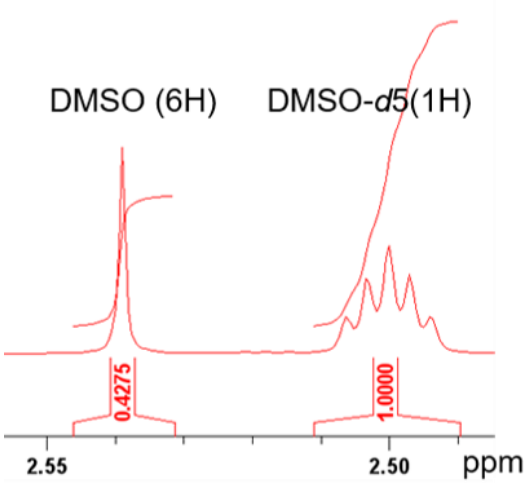

Figure S2: Portion on the ^{1}H NMR spectrum of the GelmSi sample (Figure 2b upper panel) solubilized in DMSO-*d6*. The singlet at 2.54 ppm corresponds to the signal of the six protons of the non-deuterated DMSO and the quintet centred at 2.50 ppm is characteristic of the DMSO-d5 that can be found as traces in the DMSO-d6 solvent.

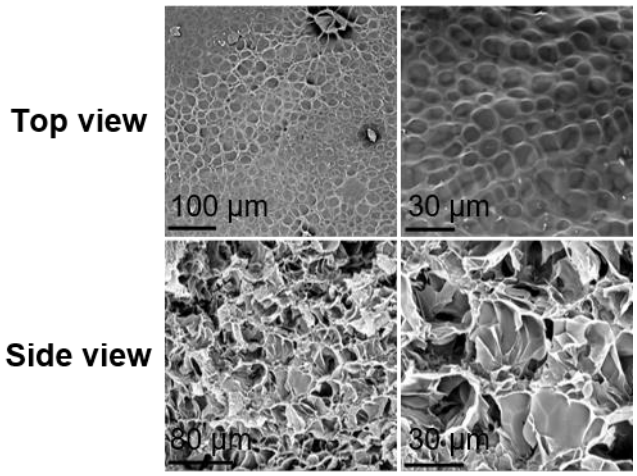

Figure S3: Scanning electron microscopy images of a dehydrated GelmSi-13 hydrogel