Engineering and characterization of a hydrogel mimicking subcutaneous interstitial space

Agnes Rodler a,b, Ayan Samanta c, Wen-Jun Goh d, Jöns Hilborn c, Per Hansson a,b,*

* Corresponding author at: Department of Medicinal Chemistry, Pharmaceutical Physical Chemistry, Uppsala University, Box 574, 751 23 Uppsala, Sweden.

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A B S T R A C T

We have synthesized and characterized a collagen-hyaluronic acid hybrid network. The aim was to create a hydrogel mimicking the extracellular matrix of adipose tissue, primarily for use in in vitro studies of protein drug transport in the subcutaneous interstitial space. The network was created by covalently crosslinking methacryloyl-functionalized collagen type I and thiol-functionalized hyaluronic acid by means of thiol-Michael and thiol-ene photo-click reaction. The degree of modification corresponded to 74 % of the lysine and arginine groups on collagen, and 16 to 29 % of the carboxylate groups on hyaluronic acid, as determined with 1H NMR. The crosslinked hydrogels mimicking the extracellular matrix of adipose tissue were used as model systems for investigations of protein drug transport.

1. Introduction

Drug injection into subcutaneous (SC) tissue is a common route of administration for protein drugs and other biopharmaceuticals. After administration, the drug molecules can reach systemic circulation by uptake either by blood capillaries or by lymphatic vessels embedded in the extracellular matrix surrounding the adipose cells. However, the events the drugs face in the SC interstitial space are not well understood, which results in poor prediction of absorption and bio-availabilities of the injected drugs [1–4]. High drug concentrations, large volumes administrated (~2 mL [5]), and drug transport from the injection site to the interstitial fluid can lead to aggregation and slowed transport [6–8]. Some events at the injection site, such as tissue deformation [9,10], backpressure and interstitial volume expansion due to high osmolality [11], depot formation [12–14], and the absorption by the lymphatics and blood capillaries, are quite well understood [2,15]. In contrast, many events taking place in the interstitial space are poorly understood, e.g., how drugs interact with the extracellular matrix constituents and how this affects drug transport and bioavailability. This gap in the available knowledge makes it necessary to develop new strategies such as in vivo and in vitro models [16–18]. Second motivation for developing a new in vitro model is to reduce the number of animal studies, in particular since no consensus could be found when predicting the bioavailability by correlating with human data using various animal species [16,19].

Kinnunen et al. has developed an in vitro model for predicting the fate of subcutaneously injected biopharmaceuticals [20], commercially available as SCISSOR® (Pion Inc.). The system comprises a viscous solution of hyaluronic acid in a buffer, mimicking the fluid filling the voids of the ECM in adipose tissue. The system was recently used to...
establish in vitro to in vivo correlations that could be used to predict the bioavailability of subcutaneously injected monoclonal antibodies [20]. Another in vitro model using hyaluronic acid was recently set up in the lab of Hageman and coworkers using different compartments representing blood and lymphatic circulation [21]. So far, those few in vitro models have in common to result in in vitro in vivo correlations for specific molecules but not serving as platform for general predictions.

In our lab we have taken an alternative, bottom-up approach by developing a hydrogel mimic of the extracellular matrix. The primary goal has been to create an in vitro model system allowing investigations of how interactions with the principal macromolecular constituents of the ECM affect the transport of subcutaneously administered drugs. We have focused on collagen and hyaluronic acid, two of the main components of the interstitial space. Collagen is a large biomolecule consisting of three left-handed helices of polypeptide II-type conformation with characteristic repeating amino acid sequences (Gly-Xaa-Yaa)n [22]. Hyaluronic acid is a linear anionic polysaccharide built from repeating β-1-3 D-N-acetylglucosamine, β-1-4 D-glucuronic acid disaccharide units [23]. Contrary to their examples from nature, we functionalize them to link them to a cross-linked network. This is done using Thiol-Michael Addition Click Reaction chemistry, which is a convenient way to covalently crosslink a precursor with thiol functionalities with methacryloyl-functionalized precursors into a stable gel network [24]. One advantage of creating an extracellular matrix model (ECMM) in the form of a covalently crosslinked hydrogel is that we can adjust the mesh size to mimic the obstruction and sieving effects believed to sterically hinder the transport of large proteins. Other advantages are of practical nature, e.g., it is straightforward to determine the partitioning of drugs between ECMM and liquid solution. Moreover, the ECM can be used as a membrane in mass transport experiments, and the absence of convective flows make it suitable for determination of diffusion coefficients with FRAP (fluorescence recovery after photo-bleaching) and other techniques.

In the present paper, we describe the synthesis of the ECMM and carry out a thorough characterization of its mechanical and rheological properties, as well as the swelling response to different media and protein accessibility to the gel interior. In subsequent papers, we will investigate in detail how the interactions with the ECM components affect the diffusivity and mass transport of biotherapeutics intended for subcutaneous administration.

2. Materials and methods

2.1. Synthesis of methacryloyl-functionalized collagen (Coll-MA)

Coll-MA was synthesized after a slightly modified protocol of Ravichandran et al. [25]. 0.02 M acetic acid solution containing collagen at a concentration of 4 mg/mL, with slight variation depending on the batch used (Corning® Collagen I from rat tail tendon, Discovery Labware Inc., Bedford, MA). The solution containing 100 mg collagen (see Supplementary Information part S1) was diluted 1:1 in 0.02 M acetic acid and gently stirred for at least 30 min. We raised the pH of the solution to 10 by slowly adding aliquots of 1 M NaOH; adequate time was given for precipitates to dissolve before each addition. 30 µL (0.205 mmol) methacrylic anhydride (MA, Sigma Aldrich, SE) were added in 5–7 µL steps approx. every 10 min. To keep the reaction going, we maintained pH > 8 by adding dropwise NaOH (0.5–1 M). The pH was then adjusted to 10 and the reaction mixture stirred at 500 rpm overnight. The next day, to remove unreacted methacrylic anhydride, the solution was filled into a dialysis tube (Spectra/Por® 12–14 kDa cutoff RC-membrane, Spectrum Labs, Rancho Dominguez, CA) and dialyzed against water (pH 10) for 3–4 days, until the Coll-MA solution was completely transparent. The slightly gel-like solution was then frozen in liquid nitrogen and freeze-dried for 4 days. We determined the degree of methacryloyl functionalization by quantifying non-reacted free ε-amino groups by the colorimetric TNBS assay [26], and by 1H NMR. For details, see the Supplementary information part S2.

2.2. Synthesis of thiolated hyaluronic acid (HA-SH)

For synthesis of the dithiobis(hydrazide) (DTP) crosslinker, we followed the protocol of Vercruysse et al. [27]; for HA-SH we followed the protocol of Shu et al. [28]. Briefly, 500 mg of sodium hyaluronate (HA-200 K; 151–300 kDa, Lifecore, Chaska, MN, USA), were dissolved overnight in 60 mL milli-Q H2O at a concentration of 10 mg/mL. After addition of 595 mg (2.5 mmol) DTP, the pH was adjusted to 4.75 by adding 1 M HCl, followed by addition of 479 mg (2.5 mmol) 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98+, Acros Organics, Geel, BE). The pH of the reaction (reaction time: 40 min) was kept constant by addition of small aliquots of 1 M HCl. The reaction was stopped by raising the pH to 7 with NaOH, followed by addition of 2506 mg (16.25 mmol) DL-dithiothreitol (DTT ≥ 99 %, Sigma Aldrich, UK) to break the disulfide bridges. After raising the pH to 8.5, the solution was stirred at room temperature for 24 h at 600 rpm. The pH of the solution was adjusted to 3.5 by adding 1 M HCl, filtered through 5 µm Millex Sterile Syringe Filters (Merck Millipore, Burlington, MA) and dialyzed against dilute HCl (pH 3.5) containing 100 mM NaCl using a Spectra/Por® 6 RC-membrane (3.5 kDa cutoff, Spectrum Labs, Rancho Dominguez, CA). After five buffer changes, the solution was dialyzed against dilute HCl solution (pH 3.5) without NaCl for two more days during which the buffer was changed six times. After dialysis, the solution was frozen in liquid nitrogen and lyophilized for 4 days. A colorimetric assay (Ellman’s test), using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB; Sigma Aldrich, SE), was used to quantify free sulhydryl groups in order to determine the degree of hyaluronic acid thiolation [29] in addition to 1H NMR. For details, see Supplementary information part S3.

2.3. Fabrication of collagen-hyaluronic hydrogels

Coll-MA and HA-SH stock solutions were prepared at concentrations of 1 wt% and 1.5 wt%, respectively, in argon-flushed medium (milli-Q water, PBS, SBF). We will consistently let wt% denote concentrations expressed in weight percent, if nothing else is stated. For the gelling reactions we used freshly prepared HA-SH solutions. Coll-MA solutions were stored at 4°C until they were completely dissolved (several days up to one week). When dissolving at physiological conditions (PBS pH 7.4), the solutions became turbid, indicating fibrillogensis of the collagen triple helices. Before mixing coll-MA and HA-SH, we adjusted the molar ratios between thiol and methacrylate groups to 1:2, 1:3.4 and 1:4, corresponding to Coll-MA:HA-SH mass ratios 5:1, 8:1 and 10:1, respectively. Furthermore, an aliquot of a 10 mg/mL solution of the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; Sigma Aldrich, SE) in water was mixed with the HA-SH solution to a final concentration of 0.1 % (v/v).

For collagen concentrations up to 0.7 % (w/v), Coll-MA and HA-SH solutions were mixed by vortexing in a 1.5 mL vial followed by manually up-and-down mixing with a syringe up to 20 cycles. Gels having collagen fractions higher than 0.7 wt% were mixed by a T-piece system developed by Liu et al. [30] and applied by Hilborn and coworkers, [25] where the precursors were put in separate syringes, connected by the T-piece, as shown in Fig. 1a. A mixing cycle was defined as one whole volume transfer from one syringe to the other; the entire cycle took approx. 1 min. The solution was then spun down for 2–5 s to remove air-bubbles. 150 µL of gel precursor was cast in precut syringe templates with 8 mm diameter (BD Discardit II, Becton, Dickinson and Company, Franklin Lakes, NJ) and crosslinked for 5 min under UV at 365 nm wavelength (UVP CL-1000 crosslinker, Analytik Jena, Upland, CA) at 4 cm distance to the UV source. Qualitative tests (gelling/no gelling) by means of the tube inversion method showed that the gelling process required less than 60 min. However, we always left the gel-molds (sealed with parafilm) to cure overnight at room temperature before use, to ensure that the cross-
linking reaction was completed. Furthermore, we confirmed the reproducibility of the synthesis protocol, by measurements of the storage module. Since this hydrogel is not intended for use as a drug delivery vehicle, a safety profile such as a cytotoxicity assay was not carried out.

2.4. VitroGel™ and collagen I as ECM mimicking reference system

VitroGel™ (TheWell Bioscience, North Brunswick, US) was diluted 4:1 and 3:1, respectively, in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Aldrich, SE) according to the manufacturer’s recommendations.

2.5. Nuclear magnetic resonance (NMR)

$^1$H NMR spectra with samples dissolved in 99.9 % D$_2$O (Sigma Aldrich, SE) at concentrations of 4–5 mg mL$^{-1}$ were recorded on a 400 MHz JEOL JNM-ECP FT NMR spectrometer (JEOL, Oxford NMR Instruments, Oxford, UK) with at least 64 scans. The residual D$_2$O peak was used as an internal standard. Evaluation was done with MestReNova software (Mestrelabs, Santiago de Compostela, ES).

2.6. Circular dichroism

Circular Dichroism was measured in water with a Jasco J-1500 spectrometer (JASCO Corporation, Tokyo, Japan) at 25 °C in a Peltier thermostated cell holder using a 0.1 cm path length quartz cell (QS 100–140, Hellma materials GmbH, Jena, GER). Concentrations were 0.1 and 0.2 mg mL$^{-1}$ and wavelength range 260 to 190 nm with a scan speed of 50 nm min$^{-1}$ at 1 nm bandwidth and 1 s Digital Integration Time. Measurements with a High Tension above 600 V were not included. A mean residue weight of 94.2 Da was calculated for collagen. The samples were corrected for a water baseline and normalized in terms of the mean residue ellipticity (MRE) with the equation

$$\theta = \frac{\theta_10 \times n \times c \times l}{10}$$

$\theta$ denotes the observed ellipticity, $n$ the number of residues, $c$ the concentration and $l$ the path length of the cuvette. It was not possible to measure in PBS because of a too high absorption below 200 nm wavelength. Data are presented as the average of at least 3 scans.

2.7. Rheology

The storage modulus $G'$ and loss modulus $G''$ were measured with two rotational rheometers based on different measuring principles: the strain-controlled ARES-G2 instrument (TA Instruments, Sollentuna, SE) at 34 °C, and the stress-controlled Discovery Hybrid Rheometer 2 (DHR-2) at 25 °C. For the ARES-G2, a stainless steel parallel plate with 8 mm diameter was used as upper geometry, the lower geometry was a 60 mm diameter APS quick-change flat plate from hardened chromium. The Advanced Peltier System (APS) accessory was used to thermostatize the lower plate. Oscillation amplitude sweeps were measured at 1 Hz frequency from 0.01 (or 0.1) – 100 % oscillation strain to estimate the linear viscoelastic (LVE) range. The limit of the LVE range was determined by the strain which exceeded the differential $\gamma_{i+1} - \gamma_{i}/\gamma_{i} > 0.01$, see reference [31]. Frequency sweeps were carried out in the LVE range at 1 % oscillation strain from 0.1 to 10 Hz. 1 % strain was chosen for the oscillation frequency measurements since it allowed us to measure
within the torque limits of the instrument but clearly on the LVE plateau. For both methods, we adjusted the gap between the plates in 100 µm steps until an axial force of 30–35 mN was reached and then the experiment was started. Gaps were lying between 1000 and 2100 µm. The axial force was maintained during acquisition, delay time was 1 s. The hydrogels were freshly prepared two days before the measurements; after letting them reconstitute following UV crosslinking, they underwent a washing step with milli-Q water for 24 h and a second equilibration step in PBS pH 7.4 (or water/SBF) for another 24 h. The hydrogel disks were used for only one single measurement. VitroGel™ samples were measured with a 40 mm cone stainless steel upper geometry (20 disks were used for only one single measurement. VitroGel™ was calibrated. Due to the short measuring time (amplitude sweep: 6.5 µm, frequency sweep: 3 min), the use of a solvent trap was not necessary.

2.8. Equilibrium binding isotherms

Gel disks (diameter: 5 mm; average weight: 14 mg) were pre-equilibrated for 24–48 h in PBS pH 7.4, carefully blotted, weighed into 1.5 mL vials, followed by addition of solutions of human serum albumin (HSA, A9511, ≥ 97 %, Sigma Aldrich, SE) in PBS (pH 7.4) having concentrations from 2 to 20 mg mL\(^{-1}\). The amount of HSA added was adjusted in proportion to the hydrogel weight. The vials were equilibrated for 5 days by gentle shaking at 15 rpm (Note: the timespan for reaching equilibrium is assumed to be less than 48 h; timespans longer than 120 h were avoided to avoid microbial growth). After equilibration, the supernatant was separated from the gel by centrifugation and filtering through a 0.22 µm membrane (Millex-HP syringe filter, Merck Millipore, Burlington, MA). HSA concentrations in the supernatant were determined in quartz cuvettes by UV spectrophotometry at 280 nm with a Cary60 UV/VIS spectrophotometer (Agilent, Santa Clara, US) using a calibration curve for HSA. To exclude possible artefacts due to leaching of hydrogel components or photoinitiator residues, samples were measured against a blank containing hydrogels equilibrated in PBS pH 7.4 under the same conditions. Measurements were conducted in duplicates. The amount of bound HSA was calculated by mass balance.

2.9. Swelling ratio

Following a 24 h washing step to leach out non-reacted components and excess photoinitiator with milli-Q water, the gels were frozen in liquid nitrogen and lyophilized for 1–2 days. After determination of the dry weight (w\(d\)), the gels were equilibrated in wells containing 5 mL PBS pH 7.4 at room temperature and weighed after careful blotting at various time steps (w\(t\)). The percentage of swelling ratio (SR) was calculated as

\[
SR(\%) = \frac{w_t - w_d}{w_d} \times 100
\]

3. Results

3.1. Synthesis and characterisation of functionalized precursor polymers

Collagen functionalization. Fig. 2a shows the \(^1\)H NMR spectra of the methacryloyl-functionalized rat tail collagen I determined in D\(_2\)O. The presence of the proton resonance peak at 1.8 ppm from the methyl protons and at 5.28 and 5.51 ppm from the vinyl group of methacryloyl shows that the functionalization was successful. Furthermore, the absence/decrease of proton resonance peaks at 2.85 ppm from the lysine methylenes of unmodified collagen, shows that the collagen lysines were modified. It was not possible to evaluate the NMR spectrum quantitatively for samples with high degree of modification since a signal from the collagen backbone masked the lysine methylene peak. However, we used the TNBS assay for quantification of the fraction of free ε-amine groups in order to determine the degree of modification for two different collagen-MA preparations, one with high and one with low MA content. According to the analysis (see Supplementary information S2) the former had ca. 75 % degree of modification and the latter 40 % modification of the amines, corresponding to amines:MA molar ratios 1:5 and 1:2, respectively. Here, the amine:MA molar ratio is the total amine content (ε-lysine + arginine+guanidine-group-related amines) divided by methacrylic anhydride content.

Collagen secondary structure. Fig. 2c shows far-UV CD spectra of native and modified collagen I in water with different degrees of methacryloyl-functionalization.

The polyproline-II helix feature with peaks at 222 nm (positive) and 198 nm (negative), present in both the native and modified forms, indicates that the collagen triple helix was maintained after introduction of methacrylate groups. By comparing the R\(\varepsilon\)-values, i.e., intensity ratios of positive (222 nm) to negative (198 nm) bands, the collagens with approximately 75 % and 40 % methacryloyl-functionalization showed nearly the same R\(\varepsilon\)-value (0.121) as the unmodified one (0.119), the latter in close agreement with data in the literature (0.13) [32]. Thus, unlike the decrease of triple helix content reported for the introduction of vinyl benzene groups [33], the attached methacrylate groups do not seem to compromise retention of the tripe helical structure.

Hyaluronic acid functionalization. Fig. 2b shows the \(^1\)H NMR spectra of native and thiol-functionalized hyaluronic acid. The peaks at 2.57 and 2.71 ppm, corresponding to the protons of the methylene groups of the hydrazide crosslinker and the peak at 1.85 ppm, corresponding to the N-acetyl group of HA, confirm the introduction of thiol groups in the sample with HA:DTP:EDC molar ratio of 1:1:0.5 (60 min reaction time). Shu et al. reported a yield ≥ 50 % for the same reaction conditions [28]. The reason for the discrepancy is unclear. However, when we changed the HA:DTP:EDC molar ratio to 1:1:0.5 and 1:0.5:0.3 (see Table S1 and S4), the yield was 15 and 14.5 %, respectively.

3.2. Characterization of collagen-hyaluronic acid hydrogels

Fig. 1b shows pictures of hydrogels obtained by linking together methacryloyl-functionalized collagen I (Coll-MA) and thiol-functionalized hyaluronic acid (HA-SH). For the majority of the hydrogels, we used HA with 25 % degree of thiolation. That gave mechanically rigid gels with a charge density of HA not deviating too much from the native form (see below). The shape of the hydrogels was adapted to fit the characterization technique used; for rheology, they were cast in molds to yield a diameter of 8 mm and a height of approximately 2 mm.

Viscoelastic properties. We applied oscillatory shear rheology to the fabricated hydrogels to investigate how the composition affected the viscoelastic properties. We first determined the limit of the linear viscoelastic (LVE) deformation range to ensure that the gels remained structurally intact during the mechanical characterization. The experimental set up with a typical hydrogel slab subjected to rheological testing in the parallel plate configuration is shown in Fig. 3. Fig. 4a shows the result of an amplitude sweep test at 1 Hz frequency on a hydrogel containing 0.7 wt% collagen and 0.14 wt% hyaluronic acid at 34 °C. Both the storage modulus G’ and the loss modulus G’’ display
Fig. 2. a: $^1$H NMR spectra in D$_2$O of methacryloyl-functionalized collagen I (top) (inset: chemical structure of modified Lysine residue) and native collagen I (bottom). Peaks corresponding to protons from newly introduced groups/replaced groups are highlighted with a grey bar. b: $^1$H NMR spectra in D$_2$O of thiol-modified HA (top), and native HA (bottom). The resonance at 2 ppm corresponds to the methyl group of N-acetyl glucosamine (peak c) and was used for normalization. c: Circular dichroism spectra of native and modified collagen I in water with different degrees of methacryloyl-functionalization at 25 °C expressed as mean residue ellipticities (MRE). Green points: 75 % modification, 0.01 w% collagen; Black points: 40 % modification, 0.02 w% collagen; Blue dots: unmodified collagen.
constant plateau values in a range of strains up to ~ 5 %, characteristic of LVE behavior. Triplicate measurements, described in Supplementary information Figure S3, gave 4 % strain as an estimate of the limit of the LVE range for this gel composition. The end of the LVE range is also marked by the point where the loss modulus $G''$ turns upward, indicating that micro-cracks start to form in the network, which develop into macro-cracks until, at the maximum of the $G'$ curve, dividing the entire shear gap [34]. This takes place at the crossover point of storage and loss modulus at 12.5 % strain, where the viscous properties start to dominate over the elastic and the gel loses its structural integrity and starts to flow. For the oscillation strain amplitude shown in Fig. 4a, the phase angle $\delta$ is lower than 6 $^\circ$, showing that the elastic character of the hydrogel dominates. Increase of $\delta$ at strains higher than 8 %, when approaching the crossover point, leads to a stronger viscous response.

For comparison, we made an oscillatory amplitude sweep test (1 Hz frequency; 34 $^\circ$C) for the commercially available ECM mimic Vitro-gel™3D diluted four times in a cell culture medium. Fig. 4b shows that the material displayed a non-linear viscoelastic behavior, with $G'$ and $G''$ gradually increasing with increasing oscillation strain until passing through maximum at around 1 % strain. The crossover point ($G'=G''$) is located at 100 % strain, meaning that Vitro-gel™ can maintain its elastic properties even under high mechanical deformation. For comparison, the viscoelastic behaviour of Vitrogel without stabilization is shown in Supplementary Figure S1.

With the LVE range established, we conducted frequency sweep measurements at 1 % strain. Fig. 5 shows how $G$ depends on the crosslinking method, collagen-to-hyaluronic acid ratio and collagen concentration. $G'$ is higher for the hydrogels made with than without UV-assisted crosslinking, the value for the latter being closer to that for Matrigel™3D. For both hydrogel types $G$ increases with increasing collagen concentration at fixed Coll:HA ratio of 5:1, but the trend is more pronounced for the gels crosslinked without UV (Fig. 5b). Increasing the Coll:HA ratio at fixed collagen concentration (i.e., decreasing the HA concentration), has no clear effect on $G$ for the gels crosslinked without UV, but increases $G$ for the UV- crosslinked gels (Fig. 5a and Figure S3). The latter effect is notable because one could expect that decreasing the HA concentration would decrease the effective crosslinking density and by that the rigidity of the gels. We will discuss possible reasons behind the effect later.

Fig. 5c shows how the loss factor ($\tan \delta = G'/G''$) depends on the collagen concentration at fixed Coll:HA ratio 5:1. The loss factor is of order of 0.1 for all samples independent of composition and preparation method, a common value for hydrogels, showing that the elastic properties dominate over the viscous properties. There is a slight shift towards a more viscous character for non-UV crosslinked gels with increasing collagen concentration. For UV-assisted crosslinked hydrogels, the one with 0.8 wt% collagen concentration has the highest storage modulus (2366 ± 57 Pa) and the smallest loss factor (0.0924 ± 0.0036).

To conclude, the results show that it is possible to fabricate hydrogels with tunable storage moduli in the range 1–3 kPa at 25 $^\circ$C by varying the collagen and HA composition.

3.3. Effect of freeze drying

To investigate if freeze drying causes damage of the hydrogel network we investigated hydrogels with 0.8 and 1 wt% collagen (Coll: HA 5:1; PBS pH = 7.4, CHX51_08, CHX51_10)) that had undergone a lyophilization step and subsequent rehydration by means of swelling experiments.

Fig. 6 shows that the swelling curved reached a plateau within less than one hour. The uptake of buffer, determined as swelling ratio, was $>$ 3000 % of its initial dry weight. The swelling increased with increasing collagen concentration in the gels prior to lyophilization ($\Delta c = 0.2$ wt %), from a swelling ratio of 3200 % to 3800 % for 0.8 and 1 wt% collagen content, respectively. However, the swelling ratio of all gels was smaller than in the equilibrium-swollen state before freeze drying, showing that the lyophilization – reswelling process is not reversible, or that the reswelling process had not reached equilibrium on the time scale of the experiment.

3.4. Equilibrium binding isotherms of human serum albumin and lysozyme

Fig. 7 presents equilibrium binding isotherms of two proteins having different physico-chemical properties, human serum albumin (HSA) and lysozyme. The data were recorded after incubation of hydrogels in protein solutions for at least 72 h. Fig. 7a shows that HSA is enriched in the hydrogel with Coll:HA ratio 5:1. The partition coefficient, calculated assuming that the density of the hydrogel is 1.0 mg/mL, is about 20 for HSA concentrations in the equilibrium solution up to ca. 7 mg/mL; at higher concentrations, the partition coefficient increases progressively with increasing concentration. Reducing the HA concentration in the gel lowers the partition coefficient, as shown by the data for the 10:1 hydrogel. This indicates that the partitioning to the hydrogel is driven by attractive interactions between HA and HSA. However, the magnitude of the reduction of the partition coefficient suggests that other interactions are also involved, as will be discussed later. Fig. 7b shows that lysozyme is also heavily enriched in the 5:1 hydrogel. In the lower concentration range, the partition coefficient is ca. 30, but the isotherm appears to saturate at higher concentrations. Interestingly, lysozyme binding to pure hyaluronic acid gels was weaker than binding to the mixed hydrogels.

4. Discussion

Our approach. The primary goal has been to develop a model hydrogel that can be used in different in vitro experimental settings to disclose physicochemical factors determining the bioavailability and transport rate of drugs in the ECM of adipose tissue. To this end we have focused on the role of collagen and hyaluronic acid, the two most abundant biopolymers present in the interstitial space. We have also considered that the extracellular matrix has mechanical and viscoelastic
properties in common with hydrogels [6,35]. Conceptually, our synthetic approach is inspired by hydrogels used in tissue engineering as scaffolds for the embedding of cells [36–40].

In the tissue, collagen is predominantly found in the ECM surrounding each adipocyte, called the reinforced basement membrane. It can be described as a closed cellular foam with wall thickness ca. 2 \( \mu m \) [41]. The part directly in contact with the cell membrane is a 100 nm thick inner membrane of small mesh size made up from collagen IV; the outer shell is a network of larger mesh size containing a mixture of fibrillary collagen I, III, V and VI, as well as laminin, proteoglycans and other glycoproteins [41]. Mechanical tests and modelling show that the rigidity of the tissue derives chiefly from the elastic properties of the reinforced basement membrane [10,35,42]. The adipocytes are arranged in lobules interspaced by a 10 \( \mu m \) thick matrix called interlobular septa made up of long bundles of collagen fibers containing mainly collagen I. The septa, which has a comparatively small tensile strength [35], has been shown to be easily torn apart upon liquid injection into adipose tissue [9,43]. This creates cracks of width \( \sim 1 \text{ mm} \) filled by the injected formulation. In order for drug molecules to become absorbed by the circulatory system, they need to be released from the formulation and transported through the ECM to blood or lymphatic vessels embedded in the septa, or to the blood capillaries inside the lobules. Depending on the size, charge and other properties of the drug molecules, the transport is believed to be hindered by the interactions with the collagen network and by the hyaluronic acid chains dissolved in the voids between the collagen fibers [6,44].

A physiologically relevant in vitro model should maintain the key properties of the ECM responsible for interactions with proteins and other biomolecules in the living tissue, but at the same time be convenient to work with. Our strategy to form a hydrogel by crosslinking the collagen and HA into a 3D network is a compromise between these demands. The results above show that the hydrogels produced with the present method are easy to handle (Figs. 1 and 3), and the crosslinks prevent the material from dissolving and the components from leaking out when in contact with aqueous solutions. Furthermore, the covalent crosslinking makes it possible to adjust the (average) mesh size and accurately tune the mechanical properties, e.g., by varying the Coll:HA ratio (Fig. 5). In principle, it should be possible to mimic the retarding effect of the ECM on the mass transport of proteins, and even tune it to match the conditions in different species, individuals or sites in the body.

Modifications. The chemical modification of the components may alter their physicochemical properties. In hyaluronic acid, the carboxylic acid groups were replaced with thiol groups by carbodiimide-mediated hydrazide chemistry which means loss of negative charge equivalent to the degree of modification, but also loss of conformational freedom. Direct crosslinking of the polymers by mixing with carbodiimide reagents was not applied since this type of crosslinking is very fast and would result in a macroscopically heterogeneous hydrogel not suitable for the intended application [45,46]. While 19 % thiolation is considered the lower limit for formation of pure hyaluronic acid gels by oxidation of SH-groups [47], ca. 25 % modification of HA, corresponding to ca. 4 nm between the modifications along the chain, should satisfy the physical requirements for creating a 3D network while not
causing too large deviation from the native charge state. Therefore, we aimed at moderate modification of HA in the range of 15 – 30 %.

For collagen, we used a degree of MA modification of the lysines and arginines of ca. 75 %. From the molecular weight per unit length on the triple helix (1000 Da/nm) [48] and the fraction of those amino acids in the molecule (8 %) [48,49], the average length between modifications along the triple helix can be calculated as ca. 2 nm; here we used an average amino acid molecular weight of 110 Da. Considering the high density of modifications, it is encouraging that the CD measurements (Fig. 2c) show that the MA-modified collagen I retained the triple helix structure in the pre-gelling solutions. We did not investigate the hierarchical assembly of collagen in the gel in this study. However, in the investigated concentration range, it is possible that the triple helices are packed into unordered short fibrils, as observed by Ramanujan et al. for unmodified collagen [50]. In their study, both banded fibrils and filamentous structures for collagen concentrations higher than 0.5 % could be confirmed by electron microscopy [51]. However, in our case the crosslinking with HA should prohibit fibrillogenesis beyond triple helix formation.

Obstruction and adsorption. The collagen network in the ECM is expected to lower the rate of diffusive mass transport by obstruction effects, increased hydrodynamic drag, and by providing binding sites for the solutes [6,52]. The free space available between collagen fibrils and the exposed surface area available for ligand binding are hence important network characteristics to maintain in the model hydrogel. It is interesting therefore to compare these characteristics between the ECM and the hydrogel. The free space between the collagen fibrils is related to the mean effective distance between fibrils. To obtain an order of magnitude estimate, we employed the expression derived by Ogston for randomly dispersed, long straight fibers [53]. A calculation for collagen in the ECM of rat and pig adipose tissue [35,54], described in Supplementary Information S5, shows that the distance between collagen

Fig. 5. Viscoelastic properties of (UV-assisted) crosslinked hydrogels equilibrated in PBS pH 7.4. a: Storage moduli in dependence of the mass ratio Coll:HA obtained by frequency sweeps at 1 % strain. The collagen concentration was 0.8 % (w/w). (Samples CHX51_08, CH51_08) b: Storage moduli as function of collagen concentration at kept Coll:HA mass ratio of 5:1 (CHXS1_06, CHXS1_07, CHXS1_08 (white color) and their non-UV treated counterparts (grey color)). c: Loss factor (tan δ) of hydrogels at 1 Hz frequency at a constant Coll:HA mass ratio of 5:1 in dependence of collagen concentration.
fibrils is 14 – 72 nm and the exposed specific surface area of collagen is 5 – 12 m²/gram. The same calculation for a hydrogel of the type made here with 1 wt% collagen gives 8 – 32 nm and 6 – 21 m²/gram, respectively. Thus, despite much lower fibril radius and collagen weight fraction, both the space between fibrils and the fibril area available for ligand binding in the hydrogels in this study are of the same order of magnitude as in the ECM of adipose tissue.

Since HA is also known to affect the diffusive transport of macromolecules [55,56] it is interesting to compare the space between fibrils in the ECM with the space between HA chains. Reed et al. found that the concentration of HA was 0.36 wt% in the interstitium of rat adipose tissue [54]. This is similar to that in hydrogels in this work, which is 0.12 – 0.16 wt% and for selected samples 0.20 – 0.24 % (w/w). For comparison, Kinnunen Bown et al. used 0.625 % (w/v) HA in the Scissor® system when modelling the bioavailability of subcutaneously administered monoclonal antibodies [20]; in earlier tests of the same in vitro model, the HA concentration was 0.1 – 1 % (w/v) [16]. The HA chain can be described as a cylinder of radius 5 Å occupying a volume of 785 Å³ per disaccharide unit [57–59]. By performing the same calculations for HA in the ECM (0.36 %) [54] and the hydrogel (0.16 %), the distance between the HA strands becomes 7 and 10 nm, respectively. Thus, HA is expected to contribute to the obstruction of diffusion in the ECM as well as in the model hydrogel, and fairly equal in both for molecules that are small enough to penetrate the networks. Furthermore, the values are similar to that calculated for collagen triple helices in our hydrogel, showing that in the hydrogel, collagen and HA may obstruct the molecular transport to similar extents.

**Network architecture.** The hydrogels are prepared by mixing a solution of modified collagen with a solution of modified HA. Even with polymers like collagen and HA, which have an affinity for each other [60], it is difficult to accomplish a homogeneous solution. However, after stopping the mechanical mixing, the initially turbid mixture finally became completely transparent, showing that, if heterogeneities existed, the domain sizes were small. A likely scenario is that HA chains, by means of reptation, gradually penetrated initially collagen rich domains, similar as Pupkaite et al. discuss for collagen-PEG/Maleimide hydrogels [31]. At the point of time when crosslinking was initiated, the HA concentration appeared to be uniform but micro-domains slightly enriched in collagen may still have been present. Thus, we expect collagen triple helices and HA chains to form an interwoven meshwork. Now, the chemistry of the modifications allow the formation of collagen-collagen, collagen-HA and HA-HA crosslinks, and since the average separation between adjacent collagen fibers and adjacent HA chains are about the same (see above), we expect crosslinks of all three kinds. However, since HA chains are much more flexible than collagen triple helices, the possibility to create HA-HA and HA-collagen crosslinks should be larger than for collagen-collagen, but the actual number density of each type should also depend on the molar ratio of collagen and HA modifications (Table S1 and S2 in the Supplementary Information). To conclude, our analysis suggests that the number of HA strands between crosslinks in the hybrid network is larger than the number of collagen strands, and that collagen-collagen crosslinks are relatively few. Both collagen and HA should contribute to the rigidity of the hydrogel network, but the contribution from HA is expected to dominate.

**Shear modulus.** As noted above, the rigidity of the adipose tissue derives mainly from the collagen fiber network in the reinforced basement membrane. The HA chains in the ECM are not connected to the collagen network, and should therefore contribute little to the rigidity, in contrast to HA in the hydrogel. Because of the differences in network architecture, it is interesting to compare the elastic characteristics of the two materials. In the Supplementary Information (part S6), we have calculated the shear modulus of the reinforced basement membrane of adipose tissue from the Young’s modulus measured for pig adipose tissue [49]. The resulting value is 3 kPa, which is very close to the values (2–3 kPa) for the UV-crosslinked hydrogels with 0.8 % (w/w), taken from Fig. 5. (Note, for the hydrogels, the shear modulus is practically equal to the storage modulus because the loss modulus is small in the linear viscoelastic range). The agreement is remarkable considering that the collagen concentration is much lower in the hydrogel than in the ECM (~18 w%). It is likely that the stress bearing function of the HA chains compensates for a smaller contribution from the collagen in the hydrogel. To test this hypothesis we employed a model of networks with semi flexible strands due to Dobrynin and co-workers [61,62]. In a gel...
with 0.14 wt% HA and degree of modification 0.2, the concentration of HA strands between modifications is 0.7 mM. By treating the gel as network of flexible strands, the storage modulus becomes ~ 2 kPa (at 34 °C), in good agreement with the experimental estimates (Figs. 4 and 5). The result supports the above conclusion that the HA strands give the largest contribution to the rigidity of the hybrid network. The slight increase of the storage modulus observed when increasing the Coll:HA ratio from 5:1 to 8:1, as obtained by lowering the HA concentration at fixed collagen concentration (Fig. 5a), may seem to contradict that conclusion. However, the trend may be the result of a competition between different types of crosslinks, e.g., that the number of collagen-collagen crosslinks increases with decreasing HA concentration. The ECM can be described as a network of stiff strands. Calculations described in Supplementary Information S6 show that, for the storage modulus to be 3 kPa, as estimated for ECM, the contour length of the strands needs to be 50 to 100 times larger than the diameter. For fibers 10 nm in diameter this means a strand length of 0.5 – 1 μm, which is physically realistic. Our estimate of the mean effective distance between fibers is much smaller. However, the two measures are not in conflict with each other as long as only a fraction of the fibers in the meshwork making up the ECM is stress bearing. To conclude, our analysis suggests that the architecture of the hydrogel network is quite different from that of the ECM. Whereas the rigidity of the ECM derives from a small number of crosslinks between stiff and long fibers, the rigidity of the hydrogel derives to large extent from a large number of stress bearing, flexible HA chains. However, since the hydrogel and the ECM have similar effective mesh sizes and collagen surface areas available for adsorption, the hydrogel may still be a relevant model system for in vitro studies of the rate of diffusive transport of subcutaneously administered peptide and protein drugs.

**Biosimilarity with native and decellularized subcutaneous tissue.** Fig. 8 compares the storage moduli of our UV-crosslinked hydrogels with those for native and commercially available subcutaneous tissue, as well as the commercially available subcutaneous tissue models VitroGel® and Matrigel. All data are from oscillatory shear measurements at 1 Hz (if not stated otherwise). Because of that, the literature values only provide a limited picture of a large span reported. As expected, the storage modulus of native human subcutaneous tissue (10 – 30 kPa) is almost one order of magnitude higher than for our UV-crosslinked hydrogels. However, for a hydrogel with Coll:HA mass ratio of 10:1 we measured a storage modulus of 4 kPa (Supplementary information Figure S4), which is closer to the values (~10 kPa) reported by Patel *et al.* for human subcutaneous tissue from abdomen at 1 % strain at 37 °C [63]. The values for decellularized tissue are very diverse, covering a range from 15 Pa (milled powder) up to several hundred thousand Pa, probably reflecting differences in the methods of preparation. While the storage modulus for Matrigel is very low, the modulus for VitroGel® is similar to our non-UV crosslinked hydrogel (Fig. 5) but lower than for our UV-crosslinked hydrogels. We conclude that the storage moduli of our hydrogels are smaller than of native human subcutaneous tissues. The high rigidity of the latter has been attributed to the reinforced basement membrane surrounding the fat cells, where it is likely that the contribution from the very dense, collagen IV-rich, basal membrane dominates. Therefore, the storage modulus of other parts of the ECM should be closer in magnitude to that of our hydrogels.

**Binding of HSA and lysozyme.** Human serum albumin (HSA) is present in the interstitial fluid in subcutaneous tissue at high concentration (29 g/L) [70], where one role is to transport other molecules. Because of its ability to bind also drug molecules [71] it is a relevant protein to incorporate in in vitro models of ECM. The results from our binding study show that HSA was spontaneously absorbed by, and enriched, in the hydrogels after equilibration in a solution of protein (Fig. 7a). In the gel with Coll:HA mass ratio 10:1, the highest albumin concentration was of the same order of magnitude as in the subcutaneous interstitial fluid. Interestingly, the data for the gel with mass ratio 5:1 show that doubling the HA concentration at fixed collagen concentration resulted in 10 times higher concentrations or more, suggesting that albumin has an affinity for HA. The result contradicts the results from early studies of albumin partitioning between HA solutions and HA-free aqueous solutions, where the protein was partially excluded from the HA solution [59]. However, those studies were carried out at higher HA concentrations than used by us, where excluded volume interactions between the protein and HA are more important than in our gels. From an electrostatic point of view, the result is counterintuitive because albumin, which is net negatively charged at neutral pH, is expected to be repelled by the negatively charged HA. However, the high ionic strength used in the experiments (0.15 M) has two important effects. First, it essentially removes the difference in (average) electrostatic potential between the hydrogel and the solution, meaning that the partition coefficient is little determined by the protein’s net charge. Second, long-range electrostatic repulsions are effectively screened, which increases the importance of electrostatic attraction between HA and positive patches on HSA. Interactions of the latter type has been discussed by Grymonpré *et al.* [72]. They observed complex formation and phase separation in mixtures of

**Fig. 8.** Storage moduli G’ from hydrogels developed in this study (CHX51_06 “0.6 %” Collagen, CHX51_07 “0.7 %”, CHX51_08 “0.8 %”), and selected values for native (ex-vivo and decellularized) subcutaneous tissue and commercially available mimics thereof at a frequency of 1 Hz obtained by rheological measurements. Empty symbols correspond to rat origin (this work, [64]), filled red symbols to porcine [9,65,66], and filled black symbols to human origin [63,67–69]. Measurement temperatures were lying between 20 and 34 °C. DAT: deep adipose tissue.
HSA and linear HA, but only at pH < 5, where the protein is net positively charged. The same authors observed nearly identical results for bovine serum albumin (BSA). However, they based their conclusions on turbidity measurements and intensity light scattering, methods not very sensitive to complex formation unless accompanied by large conformational changes of the polymer. In contrast, Pigman et al. [73] showed, by means electrophoresis and ultracentrifugation measurements, that BSA and HA formed complexes in various buffered solutions (ionic strength: 0.1 – 0.2 M, pH: 7 – 8.6). The results were supported by a study by Filippov et al. [74] who demonstrated that the NMR self-diffusion coefficient of BSA is markedly reduced by the presence of linear HA in aqueous solutions at pH ≈ 7. A two-state model analysis gave by hand that the fraction bound BSA varied between 0.36 and 0.7 in solutions containing 0.25 – 0.75 wt% HA at temperatures between 30 and 45 °C. The measurements were made in the absence of added salt, showing that salt screening is not necessary for the association to take place.

Lysozyme, having a molecular weight of 14 kDa, radius of gyration of 1.4 nm, and eight net positive charges under the measured conditions (pH = 7.4), is also enriched in the 5:1 hydrogel. As expected, the partition coefficient (ca. 30) is larger than for HSA. The small uptake by the pure HA gel is more surprising, but could be due to aggregation of the protein inside the gel. Previous investigations show that lysozyme aggregates formed in the outer layers of sodium polyacrylate [75] and sodium poly(styrene sulfonate) [76] gels hinder further incorporation of the protein.

5. Conclusion

In this work we have shown that biopolymer hybrid-networks comprising collagen I and hyaluronic acid can be synthesized from thiol and methacryloyl functionalized precursors of the respective biopolymer by means of thiol-Michael addition click reaction chemistry. When equilibrated with buffers of physiological ionic strength and pH, the cross-linked materials display viscoelastic properties characteristic of hydrogels. In the linear viscoelastic range, which extends to strains up to ca. 5 % in oscillatory measurements, the storage modulus is typically one order of magnitude larger than the loss modulus at 1 Hz oscillation frequency. The mechanical properties of the hydrogel can be tuned in a controllable and reproducible way by variation of the collagen concentration, the Coll:HA ratio, and the degree of modification of the precursors, making them interesting as biomimetic materials.

The specific aim has been to develop a model system suitable for in vitro studies of protein drug transport in the ECM of adipose tissue. We have found that hydrogels containing 0.6 – 0.8 wt% collagen and 0.1 – 0.16 wt% HA have shear moduli at low strains similar to that of the ECM. The collagen I precursor retains triple helices prior to crosslinking with HA, suggesting that the hydrogel network contains collagen in the form of (micro) fibrils. The material is optically transparent, indicating that the composition is homogeneous down to colloidal length scales. Furthermore, the HA concentration is similar to that in the ECM, which should be important for capturing the effect of electrostatic interactions. Because of manufacturing difficulties, the collagen content is lower than in the ECM. The consequences of that for the rate of protein transport through the hydrogel is currently under investigation in our lab. However, with support from theoretical calculations of the free space between network strands, we argue that a smaller fibril diameter and the presence of crosslinks between collagen and HA can create diffusion hindrances and collagen surface areas large enough to compensate for the concentration difference. Crude estimates indicate that the “mesh size” of the networks is of order 10 nm, which is similar to that of the ECM in adipose tissue, meaning that the steric hindrance to diffusion of small proteins should be small but substantial for large proteins like antibodies. In agreement with that, our binding study shows that small proteins like albumin and lysozyme can be absorbed by the hydrogels, penetrate the network and interact with the HA chains. This is a promising first indication that the hydrogels can be used in in vitro studies of protein drug transport in the ECM, where one goal is to establish in vitro in vivo correlations between drug properties and their bioavailability and rate of absorption by the circulatory system. As such it would be useful in the drug development process with a potential to reduce the number of animal studies. At the moment, we are investigating the diffusivity of different classes of protein and peptide drugs in the ECM with confocal microscopy by means of the FRAP technique (fluorescence recovery after photo bleaching). The goal is to establish in vivo – in vitro correlations to improve the possibility to predict the absorption of different classes of protein and peptide drugs.

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CRediT authorship contribution statement

Agnes Rodler: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.
Ayan Samanta: Methodology, Validation, Writing – review & editing.
Wen-Jun Goh: Investigation, Validation, Writing – review & editing.
Jons Hilborn: Methodology, Validation, Writing – review & editing.
Per Hansson: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, and Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Per Hansson reports financial support was provided by Sweden’s Innovation Agency.].

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2023.112739.

References
