

Influence of the Macro- and/or Microstructure of Cross-Linked Hyaluronic Acid Hydrogels on the Release of Two Model Drugs

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Abstract

Injectable hyaluronic acid (HA) hydrogels, crosslinked with 1,4-butanediol diglycidyl ether (BDDE), are widely used in aesthetic medicine. Due to their high clinical tolerance, HA hydrogels are thought to be applicable as injectable drug delivery systems. Here, HA matrix structures of BDDE-crosslinked HA hydrogels were analysed, and the effects of the structures on the release of two model drugs were assessed. Seven crosslinked HA hydrogels were observed by optical microscopy and cryo scanning electron microscopy (cryo-SEM). We observed three specific matrix macrostructures under optical microscopy: two had a "spider web"-like structure, three had a particulate structure, and two had an intermediate structure. These differences were less evident under cryo-SEM, where all hydrogels exhibited fibrous microstructures of different homogeneity levels, with pore sizes between 0.5 and 18 μm . Three cross-linked HA hydrogels with different macrostructures were loaded with bovine serum albumin (BSA) and lidocaine to assess their capacities to release drug over 4 days. No differences in drug release were observed between gels, and BSA was released for up to 4 days, which was four times longer than lidocaine. Thus, BDDE-crosslinked HA hydrogels could be applied as an injectable drug delivery system, particularly for the delivery of high-molecular-weight molecules.

Keywords: Crosslinked HA hydrogel; Cryo-SEM; Matrix structure; Drug release; BSA; Lidocaine

Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide composed of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronate. HA is distributed throughout the human body and occurs in almost all biological tissues and fluids [1,2]. Due to its unique properties, including full biodegradability, biocompatibility, nontoxicity, high water absorption capacity, and nonimmunogenicity [3,4], this biopolymer is widely used for many medical applications. For example, HA is commonly used as an adjuvant for cataract surgery in ophthalmology [5], as a viscosupplement for the treatment of osteoarthritis in rheumatology [6] and for filling wrinkles and/or restoring facial volume in aesthetic medicine [1,7,8].

For many medical applications, HA must be crosslinked in order to improve its biophysical properties and/or to increase its longevity in tissues [9]. The crosslinking process enables to chemically bind linear HA polymer chains together, transforming them into a three-dimensional network [10]. In aesthetic medicine, most of the available HA soft-tissue fillers are manufactured using 1,4-butanediol diglycidyl ether (BDDE) as a crosslinking agent, due to its low toxicity compared to other crosslinkers like 1,8-diepoxyoctane (DEO) or divinylsulfone (DVS). These products, which are injected in the dermis or subcutaneous tissues, are very well tolerated in most individuals as demonstrated by the strong clinical evidence based on several million patients treated worldwide within the last 15 years [11]. Moreover, these products can last from a few months to more than 18 months, depending on the exact characteristics of the formulation [12].

Many recent studies have examined the role of HA in drug delivery reflecting the rise in interest for this biopolymer to target pharmaceutical applications. These studies have shown that HA can either be used as a matrix carrier for drugs or it can be linked directly to the drugs covalently [13-15]. Thus, injectable HA hydrogels crosslinked with BDDE may have applications as drug delivery systems, particularly for injections into the skin and subcutaneous tissues, but also for other areas, as intra-ocular or intra-articular spaces. Continuous efforts are being made to improve the biophysical characteristics of such specific

hydrogels in order to increase clinical usage (e.g. injection techniques, depth of injection and injected quantity), thereby providing patients with a safe, effective aesthetic treatment option [16].

Rheological features and cohesivity are the two main biophysical properties of HA hydrogels, pointing out to the importance of observed differences among the available products on the market [17,18]. Many researchers have studied the biophysical properties of BDDE-crosslinked HA fillers; however, little information is available regarding the matrix structures of these hydrogels, except from that in a recent publication by Flynn et al. [19].

Here, we performed a comparative analysis of the macro- and microstructures of seven injectable crosslinked HA soft-tissue fillers, CE-marked for aesthetic clinical indications and then assessed whether the observed differences in the HA matrix structures affected the drug release kinetics of two model drugs of different molecular weights.

Materials and Methods

Materials

Lidocaine HCl and NaCl were purchased from Cooper (Melun, France). Deionised water and phosphate-buffered saline (PBS) was purchased from B-Braun Melsungen AG (Melsungen, Germany). Potassium phosphate dibasic (K_2HPO_4), high-performance liquid chromatography (HPLC)-grade acetonitrile, bovine serum albumin (BSA), semi-micro PS cuvettes, and toluidine blue were supplied by

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Sigma Aldrich (Buchs, Switzerland). The Spectrapor Float-a lyser G2 dialysis device (CE, 300 kDa molecular weight cut-off, 1 mL) was purchased from Spectrum Labs (DG Breda, the Netherlands). The Pierce Coomassie Plus (Bradford) assay kit was supplied by Thermo Fisher Scientific (Reinach, Switzerland).

The intra-articular viscosupplement Synolis (Aptissen, Geneva, Switzerland) and the HA soft-tissue fillers Restylane, Restylane SubQ (Galderma, Uppsala, Sweden), Juvéderm Voluma, Juvéderm Ultra 3 (Allergan, Pringy, France), Belotero Balance, Belotero Intense, and Belotero Volume (Anteis S.A., Geneva, Switzerland) were all purchased from commercial sources. These HA soft-tissue fillers were selected because they are leading products in aesthetic medicine and their brands are marketed on a worldwide scale, including in USA with FDA approvals. Their corresponding characteristics are given in Table 1.

Methods

Optical microscopy: The hydrogels (BDDE-cross-linked or non-cross-linked) were observed by optical microscopy using a DM750 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a camera (MC 170HD; Leica Microsystems). Briefly, 0.1 g of gel was spread on a 2.5 cm × 2 cm surface of a glass slide. Five drops of a toluidine blue solution (0.1% w/v in water) were added to the gel. After 2.5 min, three more drops were added. The cover slide was placed 2.5 min later under the microscope to allow observations. Samples were visualized without the contrast-phase at 40x magnification. The corresponding images were recorded and processed with Leica Application Suite v 4.5 (Leica Microsystems).

Cryo-scanning electron microscopy (Cryo-SEM): An ESEM XL30 FEG (Philips, Eindhoven, the Netherlands) was used for cryo-SEM observation of the hydrogels. The microscope was equipped with a Gatan Alto2500 cryo transfer and cryo stage system (Gatan, Pleasanton, USA). A droplet of sample was deposited on the sample holder and rapidly immersed in liquid nitrogen cooled at -210°C. Frizzed samples were transferred under the primary vacuum to the cryo transfer system, which was precooled at -185°C and 10⁻⁵ mbar. A cooled cutter was used to fracture the sample. The sublimation process was performed by heating of the fractured, frizzed sample to -95°C under a vacuum of 10⁻⁵ mbar and maintained at this temperature for 5 min. The heating time from -185°C to -95°C was of 3 min. A thin (~ 5 nm) layer of platinum

was deposited by sputtering on the sample. The prepared samples were then imaged by SEM, operated at 10 kV with a chamber pressure below 10⁻⁴ mbar and a cooling stage at less than -135°C.

The cryo-SEM images were processed for pore size measurements using Image J 1.49v software. For each histogram, a minimum of 200 pores were counted to create size distribution histograms.

Preparation of drug-loaded, BDDE-cross-linked HA hydrogels:

Drug-loaded hydrogels were prepared by mixing two syringes using a female-to-female luer lock connector; one syringe contained 20 mg of drug, and the second syringe contained 1g of the BDDE-crosslinked HA hydrogel (CPM-1, CPM-3 or NASHA-1). For each preparation, 30 passages through the connector were performed to ensure complete mixture homogeneity of the drug within the gel.

In vitro drug release studies: *In vitro* drug release studies from the three drug-loaded, BDDE-crosslinked HA hydrogels were performed for 4 days. Briefly, 0.9 g of drug-loaded gel was weighed in a Spectrapor Float-a lyser G2 dialysis device (CE, 300-kDa MWCO, 1 mL). The dialysis bag was then placed in a beaker containing 80 g of a physiological saline solution (9 g/L NaCl). The beaker was incubated at 37°C with gentle stirring for 4 days.

For BSA release experiments, 550 µL of release medium was withdrawn after 20 min, 40 min, 1 h, 2 h, 4 h and 6 h and 100 µL was withdrawn after 24, 48, 72 and 96 h. Each release medium sample was replaced with an equal volume of 0.9% NaCl.

For lidocaine release experiments, 1000 µL of release medium was removed at the same time points as for the BSA release experiments. Each release medium sample was replaced with an equal volume of 0.9% NaCl.

Drug release studies of control solutions for each drug were also performed at a drug concentration of 20 mg/mL in 0.9% NaCl. Additionally, 0.9 mL of the control solution was introduced in the dialysis bag, and the same volumes were withdrawn at the different time points as for the gels. All experiments were carried out in triplicate, and all samples were then assayed to determine drug content and calculate the corresponding percentage of cumulative release, which was plotted against time.

BSA dosage: BSA content was determined using a Pierce

Product reference	HA product name	Manufacturer	Cross-linking technology	HA content (mg/mL)	HA Molecular Weight (MW)	Injection Depth	CE registered indications
CPM-1	BELOTERO Balance	Anteis (Geneva, Switzerland)	CPM HA	22.5	2-4 MDa	Dermis	Indicated to fill moderate facial wrinkles and folds as well as for lip enhancement
CPM-2	BELOTERO Intense	Anteis (Geneva, Switzerland)	CPM HA	25.5	2-4 MDa	Dermis	Indicated to fill deep wrinkles and folds, as well as to restore and enhance soft-tissue volume (e.g., contour of the face, lip volume, etc.)
CPM-3	BELOTERO Volume	Anteis (Geneva, Switzerland)	CPM HA	26	2-4 MDa	Subcutaneous tissues	Indicated to restore facial volume, e.g., to enhance cheeks or chin
HYL-1	JUVÉDERM Ultra 3	Allergan (Pringy, France)	Hylacross	24	2-4 MDa	Dermis	Indicated for the treatment of mid and/or deep depressions of the skin and for lip definition and enhancement
VYC-1	JUVÉDERM Voluma	Allergan (Pringy, France)	Vycross	20	2 HA: 0.1-1 MDa & 2-4 MDa	Subcutaneous tissues	Indicated to restore facial volume
NASHA-1	RESTITLANE	Galderma (Uppsala, Sweden)	NASHA	20	≈ 1 MDa	Dermis	Indicated for correction of wrinkles and lip enhancement
NASHA-2	RESTITLANE SubQ	Galderma (Uppsala, Sweden)	NASHA	20	≈ 1 MDa	Subcutaneous tissues	Indicated to restore facial volume
SYN-1	SYNOLIS	Aptissen (Geneva, Switzerland)	N/A	20	2-4 MDa	Intra-articular	Indicated to reduce pain and improve mobility due to degenerative changes to the joints linked to osteoarthritis

Table 1: Description of the seven CE-marked BDDE-crosslinked HA hydrogels and the one CE-marked non-crosslinked hydrogel, with corresponding manufacturers, crosslinking technologies, HA contents, HA molecular weights, injection depths, and main CE registered indications (N/A=Not Applicable).

Coomassie Plus (Bradford) Assay Kit according to the manufacturer's instructions. Calibration curves with seven standards for BSA concentrations between 0 and 25 µg/mL and between 25 and 500 µg/mL were obtained. Both calibration curves had regression coefficients greater than 0.99.

Lidocaine dosage

The lidocaine contents of the samples were determined at $\lambda=230$ nm using an HPLC-UV system (Ultimate 3000 Dionex PDA; ThermoScientific, USA) with a Hypersil Gold C18 column (100 × 4 mm × 5 µm) mounted with a precolumn Hypersil Gold C18 column (10 × 4 mm; ThermoScientific). The mobile phase (K_2HPO_4 solution [20 mM, pH 9.3]/acetonitrile, 160 40:60) was eluted at 1mL/min at 30°C. Calibration curves between 0.001 and 0.50 mg/mL with nine standards were obtained with a regression coefficient of more than 0.99.

Statistical analysis/data analysis: Data are expressed as the mean ± standard deviation (SD). Outliers determined using the Dixon test was discarded. Results were evaluated statistically using Student's t-tests for unpaired samples. Differences with *p* values of less than 0.05 were considered statistically significant.

Results

In this article, seven CE-marked BDDE-crosslinked HA hydrogels for facial soft-tissue injection and one CE-marked (non-crosslinked) HA hydrogel for intra-articular injection were studied. The general characteristics, especially the crosslinking technology, the HA content and the HA molecular weight, which have all an influence on the HA structure of these products are listed in Table 1.

Optical microscopy

Observation of the seven crosslinked HA hydrogels by optical microscopy resulted in visualization of three different macrostructures (Figure 1). For CPM-1 and CPM-2 (Figures 1a and 1b), a fibrous macrostructure resembling a "spider-web" was observed. In contrast, for NASHA-1, NASHA-2 and VYC-1 (Figures 1e–1g), a particulate macrostructure was observed, whereas an intermediate macrostructure

with a partially fibrous structure and a partially fragmented structure was observed for CPM-3 and HYL-1 (Figures 1c and 1d).

Interestingly, all of the obtained macrostructures of BDDE-crosslinked HA hydrogels were completely different from the non-crosslinked HA hydrogel used as a control (Syn-1; Figure 1h). Syn-1, which possessed the same HA concentration as VYC-1, NASHA-1 and NASHA-2, showed a "film-like" macrostructure.

Cryo-SEM

Cryo-SEM images of the seven cross-linked HA hydrogels are shown in Figure 2. All studied hydrogels showed a highly porous microstructure, previously defined as a fibrous network microstructure [19]. Although there were some differences in the size distributions of the pores between the various crosslinked HA hydrogels, each hydrogel had a pore size distribution within the narrow window of 0.5–18 µm. Nevertheless, two groups of hydrogels could be defined based on structural heterogeneity. Group 1, which included CPM-1, CPM-2, CPM-3 and HYL-1, was characterized by higher homogeneity of the fibrous network microstructure than group 2, which included NASHA-1, NASHA-2 and VYC-1. These results were consistent with observations by Flynn et al. [19], showing that the microstructure of NASHA-1 was more heterogeneous than that of CPM-1.

In vitro drug release

In vitro release profiles of BSA and lidocaine loaded in the BDDE-crosslinked HA hydrogels CPM-1, CPM-2 and NASHA-1 are illustrated in Figure 3. No significant differences were found between the drug release profiles of the three tested hydrogels, regardless of the type of loaded drug; all profiles showed an initial burst, followed by a slower release. As expected, both drugs were released quicker when it was solubilized in 0.9% NaCl rather than when incorporated in hydrogels. For BSA release, the drug was completely released after 48 h for the control and by 96 h (4 days) for all tested gels, following first-order kinetics (Figure 3a). In contrast, a more rapid burst was observed with lidocaine; the release was complete after 6h when lidocaine was solubilized in 0.9% NaCl, but delayed up to 24 h when the drug was loaded in hydrogels.

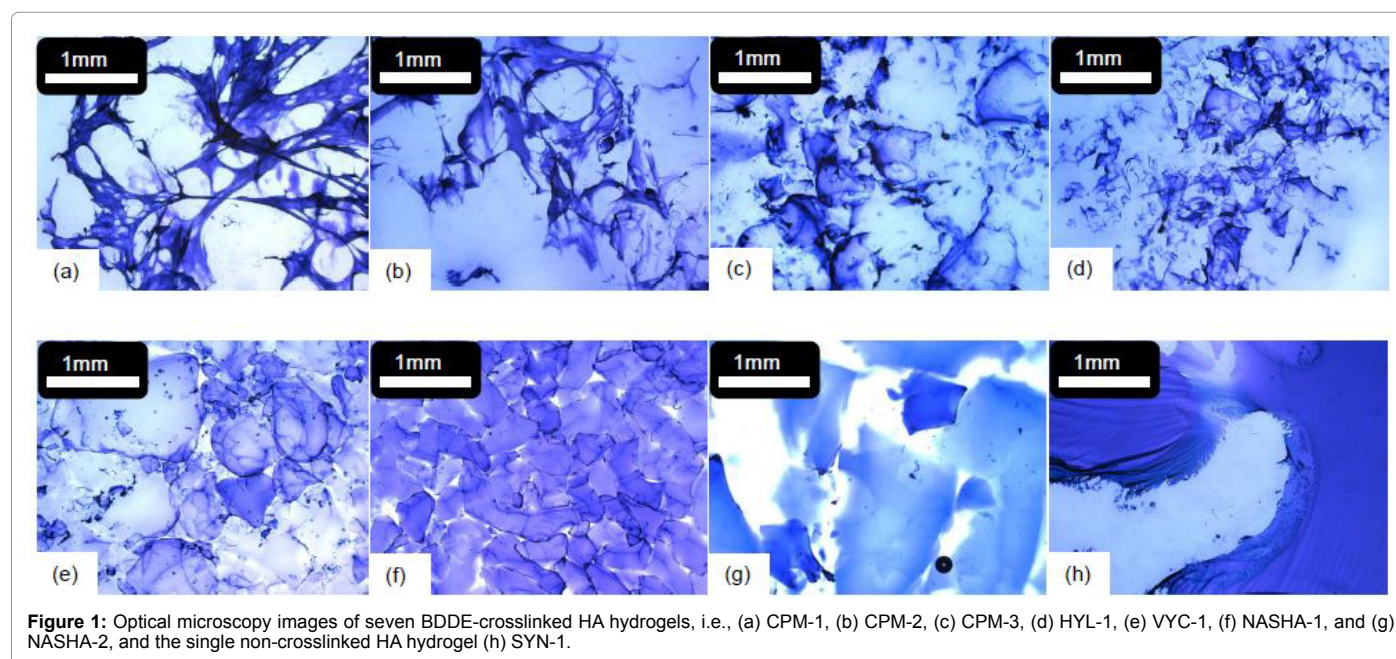


Figure 1: Optical microscopy images of seven BDDE-crosslinked HA hydrogels, i.e., (a) CPM-1, (b) CPM-2, (c) CPM-3, (d) HYL-1, (e) VYC-1, (f) NASHA-1, and (g) NASHA-2, and the single non-crosslinked HA hydrogel (h) SYN-1.

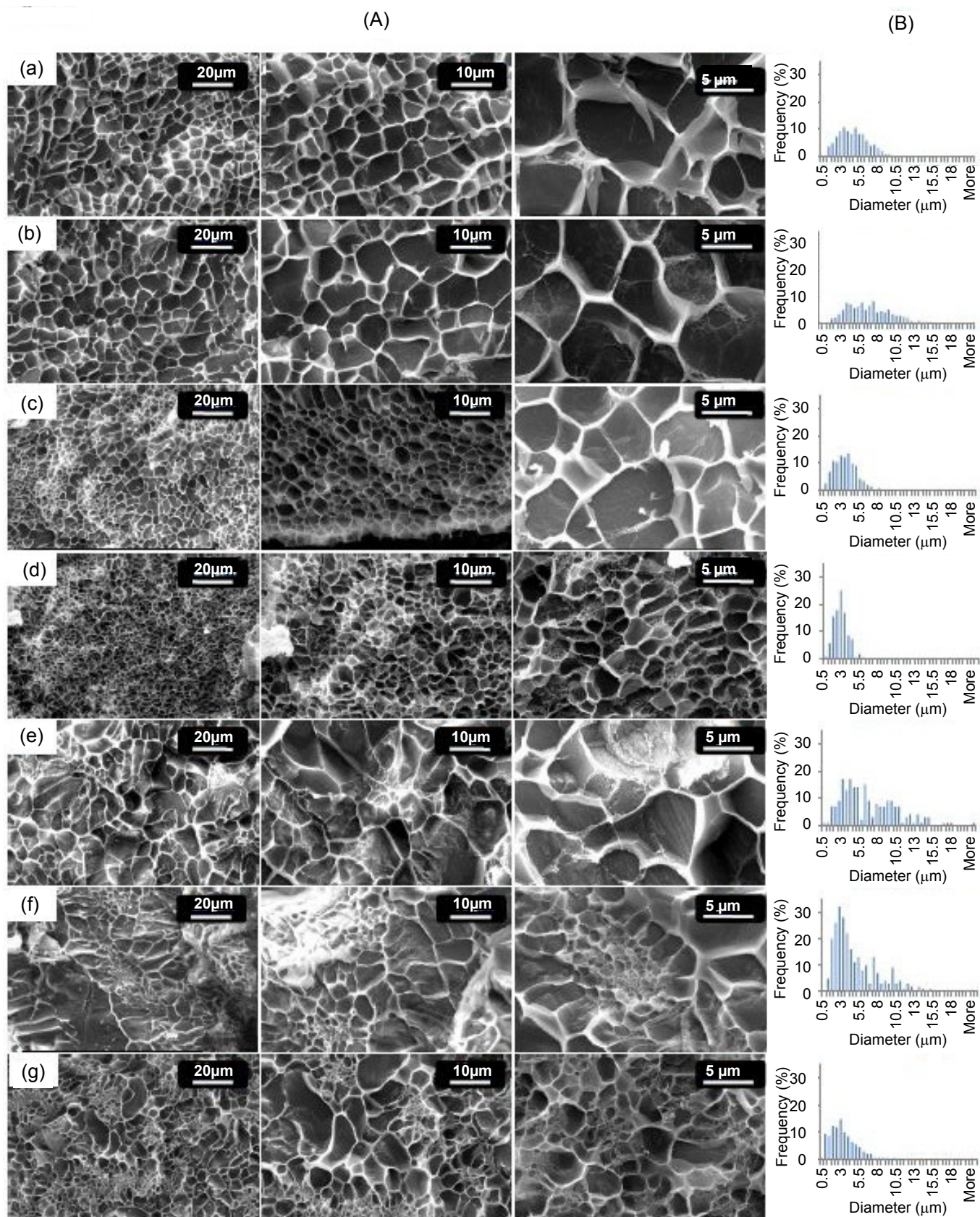


Figure 2: Cryo-SEM (A) and size distribution (B) of seven BDDE crosslinked HA hydrogels: (a) CPM-1, (b) CPM-2, (c) CPM-3, (d) HYL-1, (e) VYC-1, (f) NASHA-1, and (g) NASHA-2.

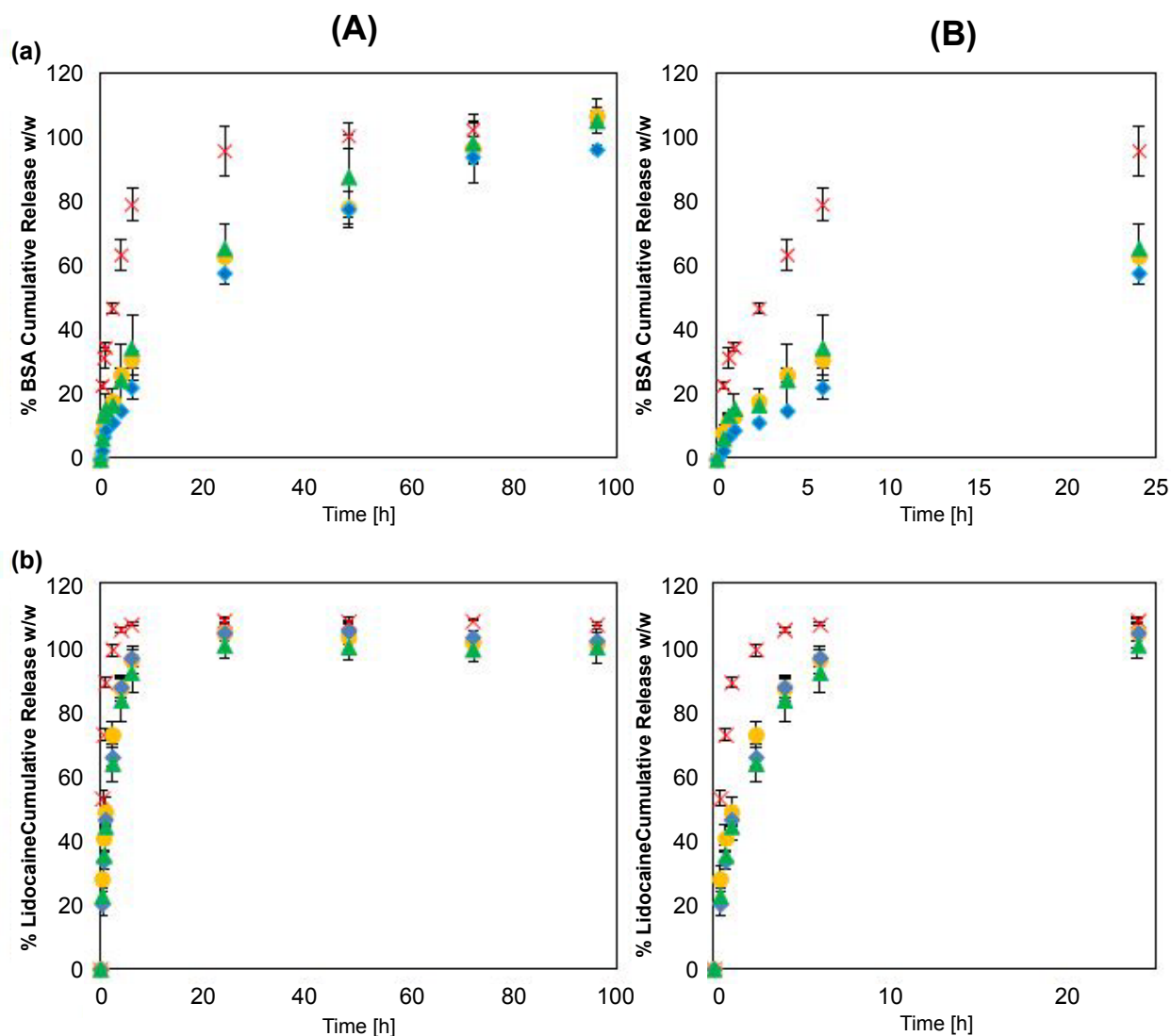


Figure 3: (a) BSA and (b) lidocaine release profiles for 96 (A) and 24h (B) obtained from drug-loaded CPM-1 (●), CPM-2 (◆), and NASHA-1 (Δ) BDDE crosslinked HA hydrogels and from the corresponding control drug solution (×). Mean ± SD, n=3.

Discussion

In aesthetic medicine, crosslinked HA hydrogels with BDDE have been shown to exhibit long-term safety and performance in several million patients after injection in the dermis and subcutaneous tissues. Therefore, it is reasonable to consider BDDE-crosslinked HA hydrogels as a potential drug delivery system for injectable applications in the skin and subcutaneous tissues, as well as other regions, such as the intra-ocular and intra-articular spaces. Accordingly, in this study, we examined the macro- and microstructures of seven injectable crosslinked HA soft-tissue fillers and assessed whether the observed differences in the HA matrix structures affected the drug release kinetics of BSA and lidocaine.

In the literature, the growing interest for a safe and sustained release via drug delivery systems, in particular for subcutaneous administrations, e.g. for the administration of anti-cancer drugs like trastuzumab with HA-tyramine hydrogels, supports the further evaluation of BDDE-crosslinked HA hydrogels for pharmaceutical applications. Indeed, this material was already studied as a matrix for

the release of atorvastatin [20] and for the delivery of growth factors for bone tissue regeneration [21]. BDDE-crosslinked HA hydrogels are generally formulated in PBS and obtained through different crosslinking technologies, which have substantial effects on the matrix network of the gel.

In this study, we found that the macroscopic structures of seven CE-marked BDDE-crosslinked HA hydrogels from different manufacturers could be categorised into three different HA matrix structures: 1) a “spider web”-like structure, 2) a particulate structure, and 3) an intermediate macrostructure. This finding of three groups is consistent with a recent article by Ohrlund and Edsman [22], which highlights that the currently used terminology of monophasic and biphasic (two categories only) for characterising the structures of HA soft-tissue fillers, is not appropriate from a microscopic perspective.

The differences in macrostructures of the seven BDDE-crosslinked HA hydrogels were visually obvious; however, the differences in the microstructures were more subtle. All hydrogels had a fibrous/porous network microstructure with a pore size between 0.5 and 18

μm , but with different homogeneity levels, as was previously observed for three HA soft-tissue fillers [19]. In this study, most homogeneous microstructures were found to have the “spider web”-like and intermediate macrostructures, i.e., CPM-1, CPM-2, CPM-3 and HYL-1, whereas the heterogeneous microstructure had the particulate macrostructure, i.e., NASHA-1, NASHA-2 and VYC-1.

We also assessed the influence of the structure of BDDE-crosslinked HA hydrogels on drug release using two model drugs and three hydrogels. Lidocaine was the first model drug, chosen to represent molecules of low molecular weight (Mw; Mw of 234.34 g/mol). This drug is a local anaesthetic already incorporated in many HA soft-tissue fillers for aesthetic applications in order to improve patient comfort during and after injection. The second drug was BSA, a well-known and highly characterised molecule, which represented a model drug with a higher molecular weight (Mw of 64,000 g/mol) than lidocaine. With a pKa of 7.9, under physiological conditions [23], lidocaine is ionically linked to HA, which is polyanionic at this pH [24]. Therefore, drug release is controlled both by the electrostatic forces and visco-elastic properties of the gel [25,26]. In contrast, BSA, which has an isoelectric point of 5.1–5.5, has an overall negative charge at pH 7.4 [27]. Thus, the release of BSA is controlled only by the diffusion of the drug within the HA matrix owing to its visco-elastic properties. Interestingly, despite its electrostatic interactions with HA, lidocaine was released four times quicker than BSA once incorporated in any tested hydrogels. BSA had a sustained release of 4 days versus 6 h for lidocaine. Thus, even if the charge interaction is one of the reasons for using the polyelectrolyte HA as a delivery vehicle [25], in our case, the large difference in molecular weight masked the influence of the charge interaction, confirming the major effects of molecular weight on drug release from a hydrogel [27]. The rapid *in vitro* release of lidocaine from the studied hydrogels is also consistent with the clinical observations, i.e., reduced pain during and after the administration of HA fillers containing lidocaine compared to fillers without lidocaine [28,29].

Notably, differences in the macrostructures of the three tested hydrogels did not affect drug release of both drugs. Similar release profiles with an initial burst followed by slower release were observed. This mechanism of drug release is typical of biodegradable hydrogels following first-order kinetics. As mentioned in the literature, the drug release of proteins from a hydrogel is mainly based on passive diffusion through the hydrogel network [30,31], the rate being dependent upon the pore size and the hydrogel network homogeneity. To achieve prolonged release, the pore sizes of the network should be smaller than the hydrodynamic radius of the molecule of interest, i.e., $14 \times 4 \times 4 \text{ nm} = 224 \text{ nm}^3$ for BSA [32] and 0.38 nm^3 for lidocaine (estimated from its aqueous density and molar volume [33]). However, hydrogels often have a pore size that is too large, leading to an initial burst effect [34,35]. Interestingly, the release of BSA from cross-linked PEG hydrogels was prolonged from 60% after 1 h to 30% after 67 days owing to modulation of gel degradation and pore size of the network [36]. By playing on several specific parameters, such as the cross-linking ratio/density, HA concentration, and HA molecular weight, and by selecting the most appropriate crosslinking technology, BDDE-crosslinked HA hydrogels can be tuned to target optimised properties for desired drug delivery, from rapid to slow release. BDDE-crosslinked HA hydrogels could thus be a promising platform for drug delivery.

Conclusion

Evaluation of the matrix structures of seven BDDE-crosslinked HA hydrogels showed that three very different macrostructures could be observed, while more subtle differences in the microstructures

were present. All tested hydrogels had a fibrous/porous network microstructure with different levels of homogeneity and with pore sizes ranging from 0.5 to 18 μm . Despite their differences in macrostructure but most probably due to their similar microstructure, the studied BDDE-crosslinked hydrogels exhibited similar drug-release profiles, with sustained release up to 4 days for BSA, i.e., 4 times longer than that of lidocaine, highlighting the importance of the molecular weight of the drug for the development of an appropriate drug delivery system using this specific type of HA. Therefore, in addition to their proven safety in the skin and subcutaneous tissues, BDDE-crosslinked HA hydrogels may have applications as an injectable drug delivery system, particularly for the delivery of high-molecular-weight molecules.

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