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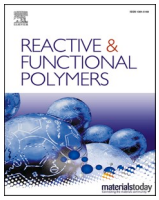

Characterisation and biocompatibility of crosslinked hyaluronic acid with BDDE and PEGDE for clinical applications

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ABSTRACT

Hyaluronic acid (HA) is a versatile biomaterial frequently utilized in regenerative medicine due to its gel-like properties, making it well-suited for clinical applications. However, its linear form is susceptible to rapid enzymatic degradation, limiting its longevity within the body. To address this challenge, extensive research has focused on crosslinking mechanisms to enhance the durability of HA gels. One early approach involved crosslinking HA with 1,4-butanediol diglycidyl ether (BDDE) to create HA-BDDE, a clinically used product since the 1990s. However, the manufacturing process for HA-BDDE, primarily used in industry, lacks comprehensive documentation in the literature. More recently, poly(propylene glycol) diglycidyl ether (PEGDE) has emerged as an alternative to BDDE for crosslinking, offering improved gel elasticity and reduced cytotoxicity. In this study, we present the manufacturing process for producing both crosslinked gels, HA-BDDE and HA-PEGDE, with negligible residual crosslinkers, as confirmed by FTIR and NMR analysis. We characterize the crosslinking kinetics and the resulting formulations, revealing that HA-PEGDE gel exhibits comparable stiffness (G' = 60 Pa vs. 75 Pa) to HA-BDDE, despite a lower effective crosslinking ratio (CrR = 0.12 vs. 0.24). Intriguingly, our cytotoxicity testing demonstrates significantly greater cell viability for HA-PEGDE compared to HA-BDDE (151% versus 105%). Overall, both gels can be readily manufactured using a similar process and demonstrate excellent in vitro biocompatibility. This study elucidates why HA-BDDE is widely utilized in clinical settings and underscores the potential promise of HA-PEGDE as an emerging variant for clinical applications.

1. Introduction

Hyaluronic acid (HA) is a long-chain polysaccharide composed of repeating glucuronic acid and *N*-acetylglucosamine units. HA occurs naturally in the body as part of the extracellular matrix, inspiring its use in medical devices due to its high bioactivity. It has gained significant clinical adoption in medical fields such as ophthalmology, aesthetics (dermal fillers), and orthopaedics (viscosupplementation) [1,2]. Initially, hyaluronic acid was derived from animals, primarily roosters [3], making it expensive and subject to regulatory challenges. However, over the last few decades, bacterial fermentation has become the gold standard for hyaluronic acid production, offering high reproducibility, cost-effectiveness, and regulatory favourability [4].

A notable limitation is that hyaluronic acid is rapidly cleared from the body due to cleavage by the hyaluronidase, typically with a half-life ranging from a few hours to a couple of days [5]. Covalent crosslinking has emerged as a popular strategy to address this issue and enhance the therapeutic effect of hyaluronic acid gel. One approach is to crosslink hyaluronic acid with 1,4-butanediol diglycidyl ether (BDDE), forming covalent bonds through ring-opening esterification via nucleophilic attachment of the reactive hydroxyl groups found in HA [6,7]. Alternatively, HA gels can be created through base-catalysed reactions in an

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organic solvent, where carboxyl groups are activated and subsequently undergo nucleophilic acyl substitution with hydroxyl groups [8]. Similarly, carbodiimides like 1-ethyl-3-(3-dimethylaminopropyl) (EDC) can be used as catalysts to induce crosslinking between HA polymers. This occurs in a two-step process: activation of the HA's carboxylic group by EDC in an acidic environment, followed by ester bond formation between HA's carboxylic acid group and the adjacent HA's hydroxyl group, with subsequent EDC recovery [9]. For example, crosslinking can also be achieved through photopolymerization by functionalizing carboxylic acid groups with methacrylate end-groups [10]. While various strategies for crosslinking hyaluronic acid groups exist, HA crosslinked with BDDE remains the most popular in clinical applications, primarily dermal filling [11]. However, it has also undergone in vivo trials for vitreous substitution [12] and inter-articular injection [13].

The biomedical applications of hyaluronic acid are diverse, and products based on it achieve their therapeutic effects through different mechanisms. Dermal fillers rely on the material to provide volume and maintain volume stability over time. In the case of inter-articular injections, hyaluronic acid aims to alleviate pain by lubricating the rheumatic knee. In ophthalmology, hyaluronic acid can be used both to lubricate and retain water in patients prone to dry eye syndrome [14]. Hyaluronic acid crosslinked with BDDE has been used in dentistry to fill periodontal pockets and stimulate regeneration [15,16]. Regardless of the application and mechanism of action, high biocompatibility is a prerequisite. Hyaluronic acid is a native component of the human extracellular matrix and exhibits a strong affinity to cells through binding to cluster determinant 44 (CD44) [17–20]. Such binding inhibits the expression of the pro-inflammatory cytokine interleukin (IL)-1β and reduces the production of matrix metalloproteinase (MMP) [20], which is responsible for cleaving multiple crucial synovial fluid components and accelerating osteoarthritis. Also, hyaluronic acid exhibits intrinsic anti-inflammatory properties by inhibiting the GRP78/NF-kB [21].

When hyaluronic acid is crosslinked, some carboxylic acid and hydroxide groups are occupied, reducing its ability to bind to the CD44 receptor and thereby diminishing its bioactivity [22]. Consequently, there is a trade-off between increased physiological stability and reduced bioactivity as the degree of crosslinking increases. This trade-off is critical when developing new formulations of crosslinked hyaluronic acid for biomedical applications. Recent research has explored the use of polyethylene diglycidyl ether (PEGDE) as an alternative to BDDE, with results suggesting that, for the same number of moles, PEGDE can yield a stiffer gel with less swelling compared to BDDE [23]. This provides a new avenue for optimizing the properties of crosslinked hyaluronic acid gels, and it is already starting to reach the clinic, for instance, in the dermal fillers marketed by Neauvia (CH) [24]. However, the crosslinking agent is just one of many design choices that needs to be made when designing a hydrogel for clinical applications [25].

The aim of this paper is to compare two crosslinked hyaluronic acid gels prepared under GLP using two different crosslinking agents, BDDE and PEGDE, and understand the production variables that will affect their biocompatibility. For both agents, crosslinking can be induced through a similar process. We aim to understand whether these two agents yield different physiochemical properties using techniques such as rheology, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), and Scanning Electron Microscopy (SEM). Furthermore, the secondary objective was to elucidate the characteristics of individual process steps through in situ observation of crosslinking kinetics via rheological measurements. Lastly, we measured the in vitro biocompatibility of the materials using a cytotoxicity and a viability assay.

2. Materials & method

2.1. Materials

Pharmaceutical grade high molecular weight (M_W = 1.5 MDa, IV = 22.2 m^3/kg) hyaluronic acid was kindly supplied by Fidia Farmaceutici S.p.A. (Abano Terme, Italy). 1,4-butanediol diglycidyl ether (BDDE), poly(ethylene glycol) diglycidyl ether (PEGDE, M_n = 500 Da), anhydrous sodium hydroxide and sodium chloride was purchased from Merck KGaA, Germany. Potassium bromide (99%, IR grade) was purchased from J&K Scientific Gmbh, Germany.

2.2. Gel production

Hyaluronic acid was dissolved at 10 *w/v*% in 0.3 M NaOH solution under manual agitation. 1.6 *v*/v% BDDE or 3.0 v/v% PEGDE was added and stirred in. The solution was incubated for 4 h at 40 °C in a closed container. After that, the gel was transferred to a cellulose membrane (MWC = 14 kDa) and dialysed in distilled water for 18 h. The gel was granulated by extruding it through a 130 μm pored mesh, before the final concentration of 20 mg/mL was obtained by adding distilled water. All manufacturing steps occurred under Good Laboratory Practice (GLP) conditions, in an ISO 13485-2016 certified facility.

2.3. Rheology

2.3.1. Rheology of gel samples

The viscoelastic region of the gel samples was investigated using amplitude sweeps by applying shear strain logarithmic ramp between 0.01 and 1400% at a 10 rad/s frequency (MCR 302, Anton-Paar, Graz, Austria). 25 mm stainless steel parallel plates with 1 mm gap were used, with excess gel removed. The temperature was controlled to 25 °C with an integrated water cooling system. Frequency sweep analysis was applied to understand the crosslinking properties of the gel across a frequency range of 0.1–40 rad/s at a 1% shear strain.

2.3.2. Real-time crosslinking analysis

The kinetics of the gel crosslinking was conducted using rheology. The dissolved hyaluronic acid was mixed with the crosslinker and immediately transferred to the rheometer (MCR 502, Anton-Paar, Graz, Austria), where the measurement was started. 25 mm stainless steel parallel plates with 1 mm gap were used, with excess gel removed. The temperature was controlled to 40 °C with an integrated water cooling system. Over a 5-h time period under constant shear strain of 0.1% and 10 rad/s frequency, the crosslinking kinetics at 40 °C was observed by monitoring the change in viscoelastic properties.

2.4. FTIR

A FTIR spectrometer (Varian 640-IR, Agilent Technologies, Santa Clara, CA, USA) was employed to determine the infrared spectra of pure HA, and the synthesized HA-BDDE and HA-PEGDE hydrogels. Spectra were recorded using KBr pellets in the 400–4000 cm^{-1} range at a 4 cm^{-1} resolution and 50 scans/spectrum. An average of the 50 scan was presented 300 mg KBr and 20 mg of gel were used for the gel samples.

2.5. Morphological characterisation

After crosslinking step, samples were dehydrated in baths of increasing ethanol concentration up to absolute ethanol, then dried overnight at 60 °C and 10% humidity. The samples were cut in two with a sharp scalpel to expose the cross-section. Environmental SEM analysis was performed on gold sputtered samples at 15 kV with Evo 50 EP Instrumentation (Zeiss, Jena, Germany).

2.6. Swelling study

Approximately 0.1 mL of the gel samples were added to inserts with 0.4 μm Polyethylene terephthalate (PET) membranes (Merck), which were subsequently placed into the wells of a 24 well plate. The wells were filled with 1 ml of distilled water and left to swell in ambient

conditions. The mass of the gel was measured at different time points up to 24 h to determine the degree of swelling. The study was stopped after 24 h as the gel had reached the top of the inserts and the PET membrane was no longer in contact with the well's water.

2.7. NMR of gels

NMR spectroscopy was used to investigate the hyaluronic acid functionalization and the degree of crosslinking of the gels. ^{1}H and ^{13}C NMR spectra of the gels swollen in deuterated water (4 mg/mL) were collected on a Bruker NEO 500 console with a 11.74 T Cryomagnet (Bruker Avance Neo 500, Bruker Corporation, Billerica, MA, USA) equipped with a direct observe BBFO (broadband including fluorine) iProbe. ^{1}H spectra were recorded with 8 scans using 32,768 points, while 1D ^{13}C-(^{1}H) spectra were acquired using inverse-gated decoupling sequence with a relaxation delay D1 = 10 s, 32768 points, and 14 K scans.

Integrations of the signals of ^{1}H and ^{13}C-(^{1}H) spectra were employed to measure the degree of modification (MoD), i.e., the moles of crosslinker bound per disaccharide unit (usually expressed as a percentage) and the effective crosslinking ratio (CrR) of both HA-BDDE [26] and HA-PEGDE gels [27]. These values are an average degree of modification as crosslinked PEGDE shows a distribution of molar masses.

2.8. Leaching study

A leaching study was employed to investigate the removal of unreacted crosslinking agents. The gel was produced as described above, and before and after the dialysis gel was removed, the concentration of hyaluronic acid was modified to 20 mg/ml (normal concentration in clinical hyaluronic acid gels [28–30]), and the sample was granulated as described above. Thereafter 2 g of each sample was moved to a cellulose membrane (MWC = 14 kDa), transferred to an enclosed bottle with 50 mL dH_2O. After 7 days, 1.8 mL solution was removed from each sample and transferred to an NMR tube before the liquid phase was removed. The residuals were then dissolved in 1.8 mL deuterated water and analysed using a Bruker Avance 400 NMR spectrometer operating at 400 MHz. ^{1}H experiments were performed at 298 K, with 256 scans and a delay time D1 of 1 s.

2.9. Cytotoxicity testing

Cytotoxicity testing was conducted to assess the potential impact of any leachables on cell viability, following an experimental design established according to the ISO10993-5 reference standard and the guidance of the LDH kit [31]. Gel preparation followed a procedure similar to the one described above, except for using sterile saline water instead of distilled water. The gel samples were then transferred to syringes and sterilized through autoclaving at 121 °C for 15 min, as is standard for hyaluronic acid gels [32–35].

Mouse pre-osteoblastic cells (MC3T3-E1) were seeded in 24-well plates at a concentration of 40,000 cells per well in 1 mL of culture medium. Approximately 0.1 mL of the gel samples were added to inserts with 0.4 μm PET membranes (Merck), which were subsequently placed into the wells. Each experimental group consisted of eight wells, including a positive control group (cells treated with Triton X-100 for 1 h before assessment) and a negative control group (cells only).

Quantitative cytotoxicity evaluation was performed using the gels' LDH activity, as indicated by the formula (eq. 1), where OD represents the absorbance at 490 nm for each group (G: gel; NC: negative control; PC: positive control). A CCK8 assay was also employed to calculate cell viability (eq. 2), with OD representing the absorbance at 450 nm for each group (G: gel; C: control). Cytotoxicity was normalized according to the control given in the equation below.

$$Cytotoxicity\ (\%) = \frac{OD_G - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100\# \quad (1)$$

$$Viability\ (\%) = \frac{OD_G}{OD_C} \times 100\# \quad (2)$$

The LDH assay measures the quantity of LDH that escapes through the plasma membrane of damaged cells, indicating cytotoxicity. According to ISO 10993-5 standards, cytotoxicity levels below 30% and cell viability exceeding 70% are considered acceptable, which we used as a reference for our modified model.

3. Results

Hyaluronic acid gel crosslinked with BDDE (1.6 vol%) and PEGDE (3 vol%) was successfully synthesized according to the process scheme in Fig. 1.

3.1. Rheology analysis

Rheological testing was instrumental in assessing the mechanical characteristics of the gels (Fig. 2A). Notably, both gel types exhibited comparable behaviour, featuring a wide viscoelastic region capable of enduring shear strains exceeding 100%. The storage modulus (G') for HA-BDDE measured approximately 75 Pa, while that for HA-PEGDE was approximately 60 Pa. Around the flow point (G' = G"), where the shear strain approaches 10^{3}% for BDDE and just past 10^{2}% for PEGDE, G' collapses, suggesting that the material transitions from behaving elastic to viscous. Their frequency sweep behaviour also exhibited remarkable similarities (Fig. 2B**)**. The concentration of PEGDE was meticulously chosen to mimic the behaviour of the 1.6 vol% BDDE gel, as this would reassure injectability through a cannula, and our rheological results substantiate this choice. Interestingly, even though the molar equivalence between HA and the crosslinker was lower for the PEGDE gel compared to BDDE, it suggested that PEGDE imparts a stiffer nature to the gel.

We also conducted real-time rheological measurements during the in-situ crosslinking process of the gels (Fig. 2C). It is evident that both gel types followed a similar kinetic pattern, with HA-PEGDE exhibiting a faster kinetic response, reaching the nominal gelation point (G' = G") in approximately 30 min. In contrast, the HA-BDDE gel reached gelation after roughly 1 h. Both gels appeared to converge towards a G' of around 1 kPa for HA-BDDE and 2 kPa for HA-PEGDE. It is worth noting that the G' values obtained in real-time studies were higher than those observed in the final gel, as the concentration of HA was modified from 100 mg/ml to 20 mg/ml after the crosslinking process.

3.2. Chemical characterisation

We employed FTIR analysis to characterize the material properties from a chemical perspective. The FTIR spectra (Fig. 2D) revealed multiple peaks characteristic of crosslinked hyaluronic acid. The peak at 1650 cm^{-1} corresponds to hyaluronic acid's carboxylate group (C=O) [6]. The broad double peaks observed at 1050–1150 cm^{-1} indicate the C–O–C and C—O stretches [6], typical of the ether bonds formed during crosslinking, though absorbance from hyaluronic acid can have partly contributed to the peak. These findings confirm the successful formation of crosslinked hyaluronic acid gels, utilizing both BDDE and PEGDE as crosslinkers. Additionally, minimal traces of peaks at 2900–3000 cm^{-1} were detected, associated with the epoxy end-groups of the crosslinkers that bind to the hydroxyl groups of HA [6], suggesting the successful removal of any unreacted BDDE or PEGDE during the dialysis step.

Furthermore, a broad peak between 3050 and 3300 cm-1 was observed, indicative of intermolecular OH groups and hydrogen bonds [6]. These intermolecular bonds are critical for the gel's cohesiveness after granulation and contribute to its "sticky" properties, which can be

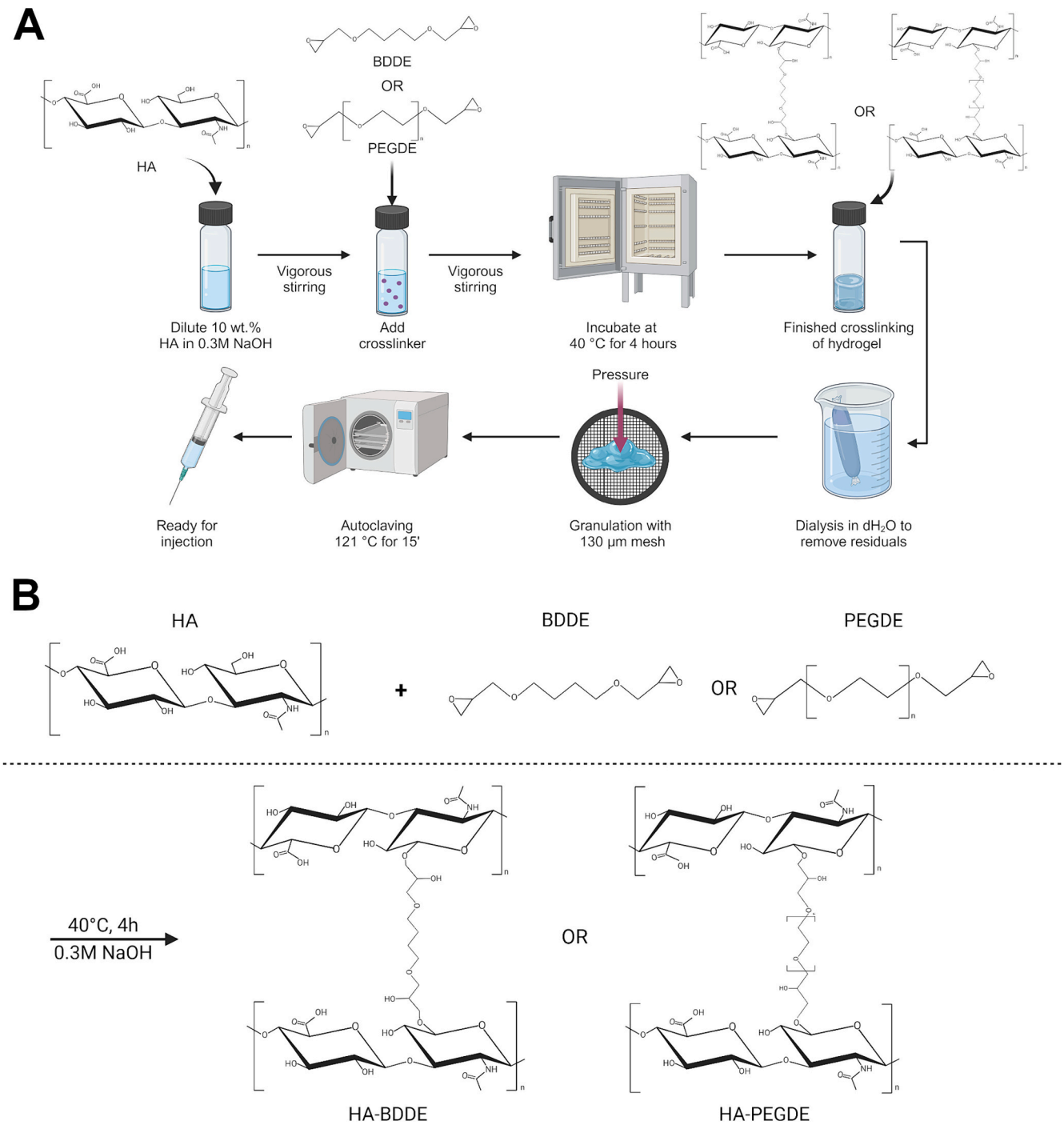

Fig. 1. **A** Schematic representation of the production steps for manufacturing a hyaluronic acid gel crosslinked with either BDDE or PEGDE. **B** Chemical reactions of crosslinking HA-BDDE and HA-PEGDE.

clinically advantageous.

3.3. Swelling study

The swelling study (Fig. 2E) demonstrated significant swelling for both gels, reaching 135% (HA-BDDE) and 172% (HA-PEGDE) after 1 h. The swelling continued but slowly diminished, reaching 596% (HA-BDDE) and 722% (HA-PEGDE) after 24 h. This was a static system, meaning that neither washing out of the gel nor enzymatic degradation from hyaluronidase is considered.

3.4. Morphological characterisation

SEM analysis of the dehydrated gel cross-section (Fig. 2F) revealed variations between the two gels. We observed heterogeneity within each gel's structure, with circular pores in some regions and stretched sheet-like structures in others. This heterogeneity might be attributed to the mixing process of the crosslinker with the highly viscous HA-solution or potential artifacts introduced during the cutting procedure. Notably, the HA-BDDE gel exhibited larger pores compared to the HA-PEGDE gel. Additionally, we identified small square-shaped crystals in the HA-PEGDE gel, which further analysis confirmed to be sodium chloride (EDX analysis, data not included). These observations provide valuable

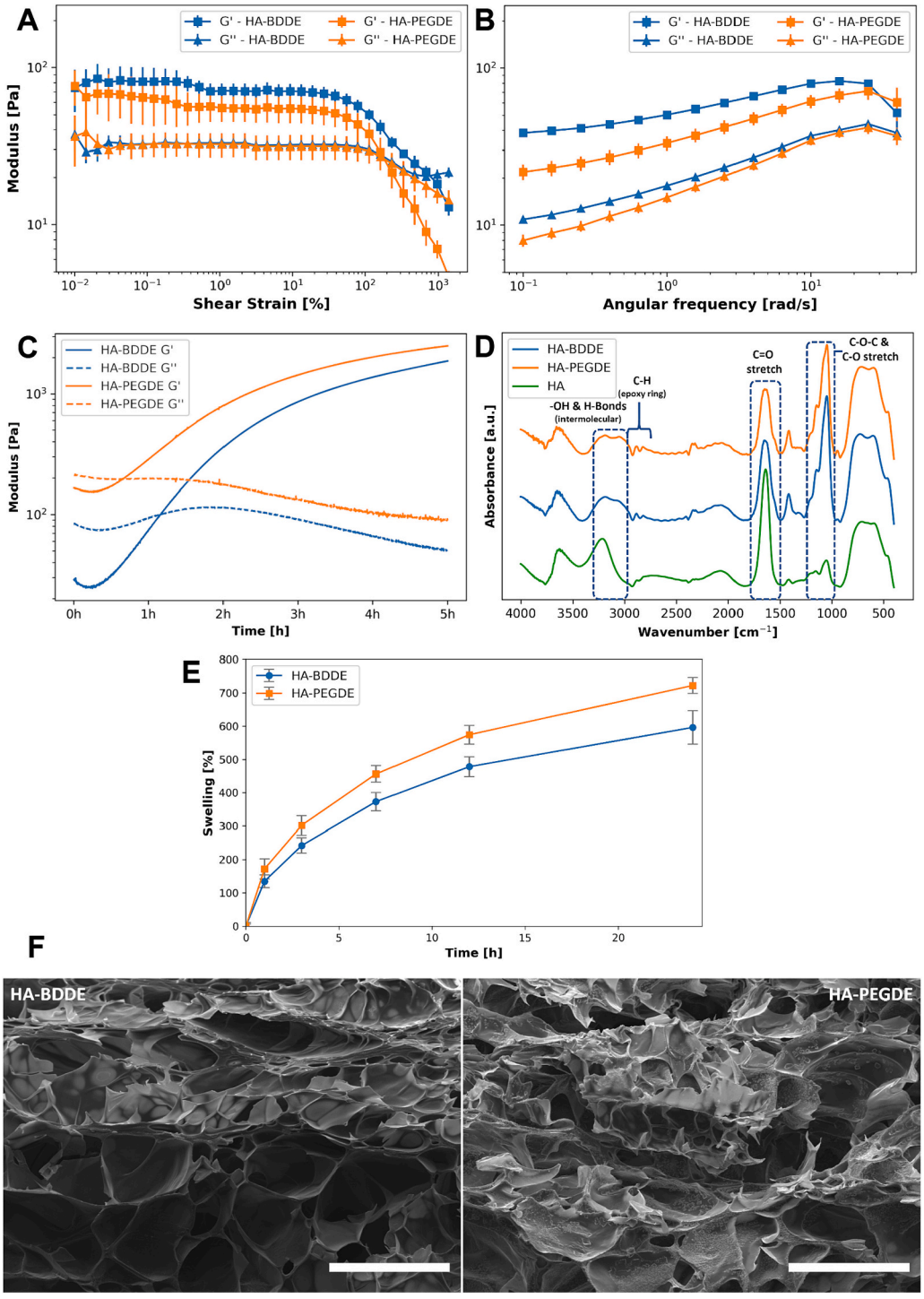

Fig. 2. **A** Rheology: shear strain amplitude and **B** frequency sweeps of the HA-BDDE and HA-PEGDE gel (Mean ± SD, $n = 4$). **C** Representative real-time crosslinking monitoring of the two gels over a 5 h time period at 40 °C. **D** FTIR analysis of the two hyaluronic acid gels and non-crosslinked hyaluronic acid (average of 50 scans). **E** Swelling study of the gels in well-plate inserts submerged in distilled water. **F** SEM analysis of the cross-section of the two gels. The crystals observed for the HA-PEGDE are sodium chloride formed during the neutralization step. Scalebar: 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

insights into the structural characteristics of the gels and potential factors influencing their morphology.

3.5. NMR of gels

We employed NMR analysis to characterize the degree of modification (MoD) and the gels' effective crosslinking (CrR). Both HA-BDDE and HA-PEGDE gels were subjected to ^{13}C-(^{1}H) and ^{1}H NMR spectroscopy, resulting in high-resolution spectra with well-resolved resonances for both gel systems (Fig. 3). MoD and CrR values were determined by calculating the peak integrals of the ^{1}H and ^{13}C resonances, indicated by red asterisks, for both investigated hydrogels. The obtained results are summarized in Table 1.

The ^{1}H and ^{13}C spectra consistently reveal a comparable MoD for

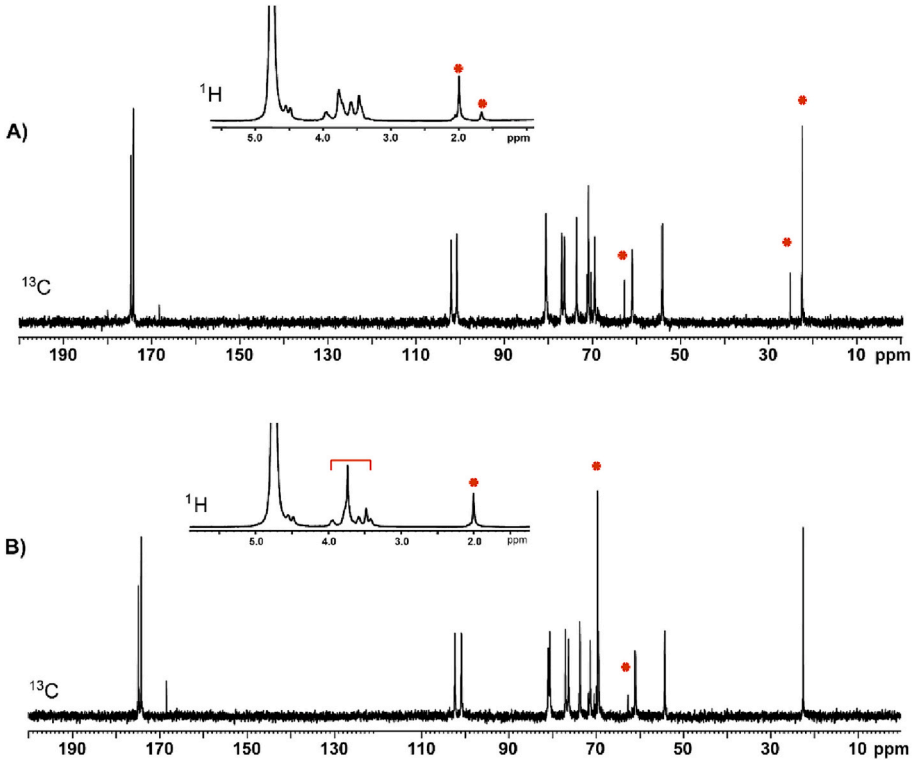

Fig. 3. ^{13}C-(^{1}H) and ^{1}H spectra of **A)** HA- BDDE and **B)** HA-PEGDE hydrogels. Red stars indicate the peaks used for MoD and CrR calculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Average degree of modification (MoD) and effective crosslinking ratio (CrR) for the investigated hydrogels with standard deviation.

Hydrogel	MoD (^{1}H)%	MoD (^{13}C)%	CrR (^{13}C)
HA-BDDE	12.2 ± 0.1	12.1 ± 0.2	0.24 ± 0.02
HA-PEGDE	10.5 ± 0.1	NA	0.13 ± 0.02

HA-BDDE, confirming the MoD calculation equations across both spectroscopic methods. Noteworthy is that despite utilizing 25% less molar quantity of PEGDE than BDDE, the MoD for HA-PEGDE was only marginally lower by approximately 2%. This observation suggests that PEGDE facilitates a more efficient crosslinking process than BDDE, highlighting its effectiveness as a crosslinker for these gels.

3.6. Leaching study

A leaching study was undertaken to assess the presence of residual non-crosslinked crosslinking agents, namely BDDE or PEGDE, following the crosslinking process and to evaluate the effectiveness of the dialysis procedure in their removal. As depicted in Fig. 4, significant peaks in the 4.0–3.4 ppm range were evident for both gels before dialysis (top panel). Following dialysis, these peaks nearly disappeared for BDDE and notably diminished for PEGDE (bottom panel). The repeated ethylene glycol unit is the tall peak of PEGDE at ~3.7 ppm [36]. While this does not quantitatively measure the residual crosslinker concentration, it offers a qualitative demonstration of the impact of the dialysis step.

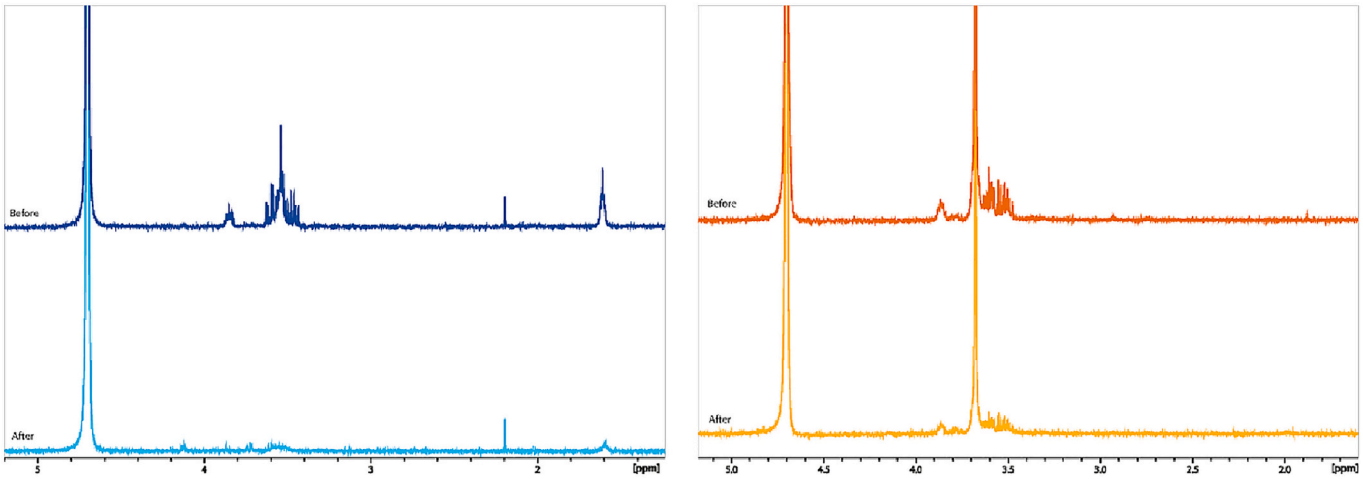

Fig. 4. ^{1}H NMR spectra of leaching medium before (top) and after (bottom) 18 h dialysis in distilled water for HA-BDDE (left) and PEGDE (right).

3.7. Cytotoxicity

Cytotoxicity assessment involved two methods: LDH, which quantifies cell membrane damage (i.e., cytotoxicity), and CCK8, which quantifies the number of viable cells. In both cases, quantification occurred 24 h after exposure, utilizing cell plate inserts to prevent direct contact between the gels and cells. The LDH assay indicated slightly higher cytotoxicity for the hyaluronic acid gels (HA-BDDE = 3 ± 7%, HA-PEGDE = 3 ± 6%) compared to the control baseline (cells alone, 0 ± 8%). However, this difference was not statistically significant and remained below the maximum limit of 30% outlined in ISO 10993-5 for in vitro biocompatibility testing. Regarding cell viability, both hyaluronic acid gel groups outperformed the control baseline (100 ± 5%). Notably, the HA-PEGDE group exhibited significantly higher cell viability (151 ± 12%) than the HA-BDDE group (105 ± 11%) and the control baseline (Fig. 5). ISO 10993-5, the standard for in vitro biological evaluation for medical devices, suggests a maximum allowable cytotoxicity of 30% and a minimum cell viability of 70% [31].

4. Discussion

This work has characterized two different hyaluronic acid hydrogels crosslinked using either BDDE or PEGDE to understand their physiochemical properties and how this would affect their clinical performance. The crosslinking can be induced using a similar methodology in both cases, and we have also conducted a granulation step compatible with needle injection in a clinical setting. The process starts with the dissolving of hyaluronic acid. Since it typically has a solubility limit, dependent on salt and molecular weight, of 4 mg/mL, it is necessary to dissolve it in a basic environment, which we obtained using sodium hydroxide. The basic environment facilitated ring-opening nucleophilic reaction between the epoxy ring of the BDDE/PEGDE and the hydroxide group of hyaluronic acid [37]. We used a 10 wt% concentration of HA as we failed to achieve crosslinking at 5 wt% or lower. Once the hyaluronic acid was dissolved the crosslinkers could be readily mixed in. The crosslinking process is time-dependent but can be accelerated with elevated temperatures [32]. This is beneficial as the high pH accelerates the degradation of hyaluronic acid [38], giving competing reactions between the crosslinking from the BDDE or PEGDE and the degradation from the sodium hydroxide. In our real-time crosslinking study, we could observe a gelation point after approximately 30 min for the PEGDE gel and 60 min for the BDDE gel when incubated at 40 °C. Both gels seemed to follow a sigmoidal crosslinking kinetic, where the increase in crosslinking degree decreases with time, meaning that the engineer must make an educated choice for when to stop the crosslinking, considering the trade-off between HA degradation and increased crosslinking. From an industrial perspective it can be beneficial to limit the crosslinking time out of economic considerations. We chose to stop our crosslinking after 4 h. This can be done by neutralizing the pH with an acid but working on a lab scale we found it easy to do this by shifting directly over to the dialysis step. We used NMR to determine what MoD this gave after 4 h. The MoD for HA-PEGDE is approximately 1.7% lower for HA-PEGDE than for HA-BDDE, which do match with the fact that a 25% lower molar ratio between the PEGDE to HA concentration was used. Interestingly, the difference in MoD is lower than in the molar ratio, but this agrees with the real-time rheology study where the HA-PEGDE seems to gel quicker than the HA-BDDE.

Crosslinking serves to stabilize both hydrogels, enabling them to fill defects over an extended period, which promotes tissue regeneration in voids. Furthermore, from a translational perspective, HA-PEGDE's improved mechanical properties at the same degree of crosslinking, as demonstrated from the rheology analysis, hold promise for different clinical applications, such as intra-articular use in regenerative and sports medicine. This does agree with former investigations [23], and stays true when the gel is granulated for injectability. Moreover, HA-PEGDE has been reported to be more stable against degradation than HA-BDDE [39]. This indeed suggests the potential for an extended therapeutic window.

Maintaining a low MoD is beneficial because research has shown that a higher degree of crosslinking limits HA's ability to bind with the CD44 ligand, thus reducing its bioactivity [22]. Our work demonstrates that HA-PEGDE can be produced similarly to HA-BDDE, but with faster reaction kinetics. Additionally, our rheology results indicate that a given mechanical stiffness (storage modulus) level can be achieved with a lower degree of crosslinking. This has promising clinical implications, as a lower MoD would enhance CD44 ligand binding and inherently improve cell adhesion [40], with a particular potential in soft tissue regeneration.

Jeong and colleagues [41] have demonstrated in vitro that BDDE is toxic above 100 ppm, while PEGDE is toxic above 500 ppm. It has also been demonstrated with BDDE that the toxic byproduct BDPE can be formed during the reaction [32]. It is therefore important to apply a step to effectively remove these residuals and byproducts. In our method, we used the dialysis step to allow the unreacted crosslinking agent to diffuse out of the gel. To assess the effectiveness of our dialysis step, we conducted a leaching study on the gel before and after dialysis, employing NMR to characterize the leached composition. Before dialysis, we observed prominent peaks associated with the crosslinkers. However, after 18 h of dialysis, these peaks had significantly diminished. Furthermore, FTIR analysis of the hydrogels revealed the absence of

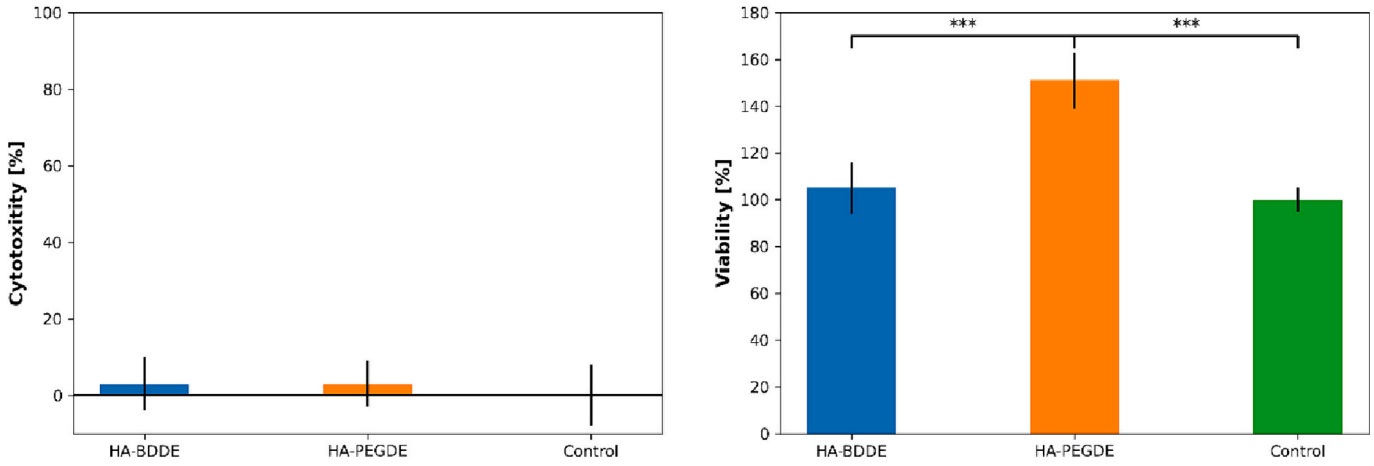

Fig. 5. Cytotoxicity measured using LDH assay (left) and CCK8 assay (right). $n = 8$, mean ± SD, $^{***}p < 0.001$. Control = negative control, meaning seeded wells without any gels introduced.

peaks associated with the epoxy ring of the crosslinkers.

As an alternative approach to our leaching study, Yang and colleagues [7] conducted a leaching study in which the released BDDE was reacted with nicotinamide, resulting in the formation of a strong fluorescent substance that could be excited at a wavelength of 370 nm and emitted at 430 nm. This method enabled them to quantify the residual BDDE released using a fluorescence spectrophotometer. Meanwhile, Vukovic and colleagues employed gas chromatography with a flame ionization detector and benchmarked against a calibration curve [42], and Guarise and colleagues [35] used HPLC-MS. Therefore, multiple methods are available for characterizing residual BDDE content, in addition to the methods used in this paper. In general, the relevant standard (ISO 10993-18) mentions among others NMR, HPLC, MS as methods that can be used to characterize extractables and leachables [43].

There are alternative methods to dialysis. Of particular interest, Vukovic and colleagues [42] introduced a novel method for removing BDDE and its byproduct, 1,4-butanediol di-(propan-2,3-diolyl) ether (BDPE), employing a dynamic crossflow device with rotating semi-permeable ceramic discs. This innovative approach reduced the final residual content to <0.1% of the initial concentration. It is worth noting that although the authors declared no conflicts of interest in their paper, two of the authors are co-inventors of a pending European Patent Application (EP3833695A1) submitted by Merz Pharma Gmbh, with which the authors are affiliated. Consequently, if the patent application is successful, this technology may not be available to HA-gel manufacturers other than Merz before 2039.

Alternatively, researchers at Fidia Farmaceutici S.p.A. [35] replaced the dialysis step with three washes using 80% ethanol before drying and rehydrating the HA-BDDE. They were able to demonstrate significantly lower BDPE residuals compared to products from multiple other HA-BDDE dermal filler manufacturers. However, in our experience, achieving homogeneously hydrated hyaluronic acid during rehydration can be challenging. Moreover, since ethanol was employed, the effective removal of this solvent must be validated before commercialization.

Although a low level of residuals is beneficial, there is a practical limit to the efforts put into removing the residuals, and there will likely be some residuals left. Manufacturers must therefore ensure that the final concentration of residuals does not cause a toxic response. A natural first step is to conduct an in vitro evaluation. We conducted a cytotoxicity test to understand the impact of residual BDDE/PEGDE. For the LDH assay, the hyaluronic acid groups showed a cell cytotoxicity of 3%, significantly lower than the recommended maximum of 30% from the standard for testing in vitro biocompatibility (ISO 10993-5). Similarly, for the CCK8 assay, the gels had a cell viability of 105% (BDDE) and 151% (PEGDE) when the standard's recommended minimum is 70%. Hence, the in vitro results suggest an exceptionally good cytocompatibility for the hyaluronic acid gels crosslinked with BDDE and PEGDE. However, a single cell, 2D environment is not able to replicate the complexity of human physiology; it is therefore recommended to conduct a thorough in vivo investigation to understand the biomaterial's biological performance precisely [44].

When investigating the safety of a new medical device, it is important to explore the adverse events previously reported with the biomaterial used. If identified early, these potential issues can be addressed when the device is still developing, saving cost and time. There are reports of adverse events using hyaluronic acid, particularly for dermal fillers where primarily HA-BDDE is used. Exacerbating events such as foreign body response has been reported on multiple occasions with HA fillers such as late granuloma formation [45–49]. In a recent case by Davis and colleagues [47], they observed a clear foreign body response with multinucleated giant cells and histocytes but highlighted regions with healthy extracellular matrix (ECM) like tissue. Interestingly the patient (76 years old female) had two other HA injections in the facial region that did not exhibit any foreign body response. What causes the foreign body response is not elucidated, but a leading theory is that it is due to a biofilm formed by non-pathogenic bacteria introduced during the injection [48,49], which explains the late onset of the granuloma. This suggests that including an anti-bacterial or bacteriostatic agent can be a favourable component of HA gels used for injection. There has also been theorized that a high degree of modification and crosslinking can cause adverse effects, for instance, for HYACorp's product H1000 (withdrawn from the market) [50], which should be kept in mind when deciding on the crosslinker concentration used and the reaction time. Decades and colleagues [51] elaborated on the issues with two of HYACorp's products specifying that the patients experienced an inflammatory response and theorized that there was an increased risk with increased age.

5. Conclusion

Hyaluronic acid crosslinked with both BDDE and PEGDE demonstrates considerable promise for various clinical applications, underscoring their widespread adoption in the medical field. This study presents a straightforward and scalable manufacturing process that employs heat to expedite crosslinking with either BDDE or PEGDE. This process can be seamlessly integrated into large-scale production, and the resulting gels can be conveniently loaded into pre-filled syringes and sterilized via autoclaving, rendering them readily deployable as medical devices for various applications. We have comprehensively elucidated the crosslinking kinetics of the efficacy of dialysis in removing non-crosslinked reaction agents and have demonstrated outstanding in vitro cytotoxicity profiles, affirming the suitability of both gels for clinical use. Moreover, our comparative analysis suggests that HA-PEGDE may offer advantages, as it exhibits faster crosslinking kinetics and yields a stiffer material at a similar crosslinking ratio. Neither material demonstrated in vitro cytotoxicity, but HA-PEGDE notably achieved significantly higher cell viability than both HA-BDDE and the control. This study not only sheds light on the factors contributing to the clinical success of HA-BDDE but also suggests that HA-PEGDE holds significant potential for widespread clinical adoption when integrated into innovative medical devices.

CRediT authorship contribution statement

Øystein Øvrebø: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zoe Giorgi:** Methodology, Investigation. **Angela De Lauretis:** Investigation. **Valeria Vanoli:** Methodology, Investigation. **Franca Castiglione:** Writing – review & editing, Methodology, Formal analysis. **Francesco Briatico-Vangosa:** Writing – review & editing, Formal analysis. **Qianli Ma:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Giuseppe Perale:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Håvard J. Haugen:** Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Filippo Rossi:** Writing – original draft, Supervision, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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