Injectable Poly(ethylene glycol) Hydrogels Cross-Linked by Metal−Phenolic Complex and Albumin for Controlled Drug Release

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ABSTRACT: Injectable hydrogel is advantageous as a drug reservoir for controlled drug release since its injectability provides minimally invasive access to internal tissues and irregular-shaped target sites. Herein, we fabricated pH-responsive injectable hydrogels constructed of a supramolecular cross-link network, which contained tannic acid (TA), Fe(III), poly(ethylene glycol) (PEG), and bovine serum albumin (BSA) for controlled drug release. The hydrogel precursors rapidly turned into a gel when co-injected with NaOH in a time scale of seconds. The hydrogel properties and drug release profiles are all tunable by adjusting the concentrations of BSA, NaOH, and doxorubicin (DOX). The Young’s moduli range from 3.19 ± 0.93 to 43.24 ± 1.37 kPa that match internal soft tissues. The hydrogel lasts more than 3 weeks and gradually releases doxorubicin up to 123.6 ± 1.7 μg at pH 6.4. The results of the physical properties and drug release suggest supramolecular interactions that correspond to Fourier transform infrared (FTIR) results. In vitro cytotoxicity was also assessed using L929 cells, and the results demonstrated the material biocompatibility. The tunable properties, controlled release profiles, and biocompatibility of injectable poly(ethylene glycol) hydrogels support that they have great potential as a drug-releasing material for localized treatments.

INTRODUCTION

Injectable hydrogel is a promising biomaterial for drug delivery due to its many advantageous characteristics. Injectable hydrogel contains three-dimensional polymeric networks, which can spontaneously form shortly after hydrogel precursors are injected into a target area. The in situ formed hydrogels are consequently preferable as a biomaterial for noninvasive treatments to avoid patient morbidity from repeated surgeries, while they can concurrently serve as a local treatment to provide sustained drug release.1−4 Injectable hydrogel has been intensively developed in recent decades, especially by modifying polymeric cross-links.5,6 The physical or chemical cross-linking interactions between the polymer chains are the key to manipulating hydrogel formation to have desirable properties.7 Supramolecular interactions have recently emerged as a candidate for generating cross-links of injectable hydrogels.8 The complex supramolecular system is essentially composed of at least two molecules that are chemically linked by noncovalent bonds, such as hydrogen bonds, metal−ligand coordination, host−guest recognition, electrostatic interaction, and hydrophobic interaction.9 Such noncovalent binding forces can be reversible upon environmental stimuli, such as pH,10 temperature,11 and light.12 Due to this spontaneous environmental responsiveness, supramolecular interactions have been increasingly used to generate cross-linking systems that govern the formation, degradation, and properties of injectable hydrogels.13−15 Tannic acid (TA) and Fe(III) create one of the supramolecular systems that can be used to cross-link polymer chains to construct a pH-responsive injectable hydrogel, as previously reported by Fan et al.16 TA is a polyphenolic compound possessing the ability to bind with other molecules—including metals and proteins—via both coordinate covalent bonding and noncovalent interactions.17,18 Various types of polymers, including poly(vinyl pyrrolidone) (PVP), poly(dialyl dimethylammonium chloride) (PDDA), poly(styrene sulfonic acid) sodium salt (PSS), and poly(ethylene glycol) (PEG), have been reported to generate pH-responsive hydrogels when cross-linked by the TA−Fe(III) complex at pH ~ 6.16 However, studies on the potential biomedical applications of the hydrogels are still limited. In this study, supramolecular cross-links formed of TA, Fe(III), and PEG are described and utilized to create injectable hydrogels for controlled drug release. The physical properties of the hydrogels are assessed to determine the hydrogel’s suitability as an implant, which would require direct contact with biological tissues. The hydrogel was also stabilized using bovine serum albumin (BSA) to increase the hydrogel integrity.
and prolong the degradation duration. A cancer drug doxorubicin (DOX) was used as a drug model to study drug release. The effects of BSA, DOX, and basic concentrations on the swelling ratio, Young’s modulus, mesh size, and biodegradability are reported and supported by Fourier transform infrared (FTIR) results to elucidate the interactions of the hydrogel components. Insights into the releasing mechanism are described using the Weibull model. Cytotoxicity tests were also conducted to assess the hydrogel biocompatibility for biomedical applications.

**RESULTS AND DISCUSSION**

**Injectable Hydrogel Formation.** Injectable hydrogels rapidly formed as the basic precursor mixture of PEG, TA, and Fe(III) were simultaneously injected and mixed with 3 M NaOH using a dual syringe. The supramolecular interactions behind the hydrogel formation are described by Fan et al.16 Briefly, the interactions between PEG and TA are mainly influenced by the hydrogen bonds that link the other groups of PEG and the catechol groups of TA. Metal–ligand coordination occurs immediately when Fe(III) is added to the mixture. This interaction can be observed from the change of mixture color from light yellow to dark green.16 However, we found that the hydrogels formed from those basic precursors were insufficiently stable for sustained drug release. The hydrogels showed fast degradation, with >70% weight degradation in 1 day, and they had a comparatively low Young’s modulus (9.22 ± 2.27 kPa), as shown in Figure 1A.

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c01393)

Figure 1. (A) Young’s modulus and (B) mesh size of the hydrogels prepared using different concentrations of BSA. Letters indicate significant differences, analyzed using the Kruskal–Wallis test with Conover post hoc pairwise analysis (n = 3).

**Influence of Bovine Serum Albumin.** In contrast to hydrogel H-BSA0 formed of only PEG, TA, and Fe(III), all of the injectable hydrogels stabilized with BSA illustrated significantly different physical properties that indicated higher stability. According to Figure 1A, the Young’s modulus of the hydrogel significantly increased when BSA was added, regardless of BSA concentration. The fitted curve between the indentation depth and force is shown in Figure S1. The results suggest that the hydrogel possesses a remarkably stronger cross-link network as the BSA concentration increased. It is interesting to note that the Young’s moduli of the hydrogels are in a range that can match the moduli of different human internal tissues, such as the intestines (20–40 kPa), heart (10–15 kPa), and kidneys (5–10 kPa), suggesting its potential use as an implant material in biomedical applications. For the swelling ratio, only slight differences were found among the hydrogels H-BSA0 (1.15 ± 0.12), H-BSA75 (1.23 ± 0.03), H-BSA150 (1.24 ± 0.02), H-BSA300 (1.35 ± 0.01), and H-BSA750 (1.34 ± 0.01) (Figure S2). Figure 1B shows that the decreased mesh size implies that BSA caused denser polymeric network as the BSA concentration increased. The mesh size ranged from 80.44 ± 3.38 nm for H-BSA0 to 36.15 ± 0.26 nm for H-BSA750. Increased BSA concentrations therefore resulted in a higher Young’s modulus and denser cross-linking network. Even though H-BSA750 showed the highest modulus that matches some internal tissues, the preparation of a 750 μM BSA solution was found to be difficult and laborious due to its limited solubility. For this reason, H-BSA750 was not investigated further.

According to the physical properties, the results concur with previous studies that utilized BSA as a stabilizer to increase the robustness of PEG-based hydrogels.20,21 The higher hydrogel stability can be explained by hydrophobic interactions between PEG and BSA that occur in pockets of the BSA molecule.22 The FTIR results confirm the interaction between PEG and BSA that occurred from amides I and II, which shifted from 1644 cm⁻¹ (C＝O stretch) and 1531 cm⁻¹ (C＝N stretching couple with N–H bending mode) to 1640 and 1571 cm⁻¹, respectively, upon the addition of BSA (Figure 2). The different FTIR spectrum intensities have been previously explained to be the consequence of different levels of interactions between PEG and BSA.22 For low PEG concentrations, the intensity of amide bands I and II increases due to interactions between PEG and BSA. In contrast, high PEG concentrations result in decreased intensity due to the reduction of the protein α-helix content.22 Such PEG–BSA hydrophobic interactions cause higher numbers of cross-links and consequently result in a stronger polymeric network. Previous studies showed that the interactions between PEG and BSA could be significantly enhanced by the PEG chain length because of the higher hydrophobicity of the polymeric chain.22,23 Since the injectable hydrogel herein was formed of relatively high MW PEG (Mₙ = 20 000), it is reasonable that the effect of BSA on hydrogel stability was sufficiently important for BSA to become a critical factor for stabilizing the hydrogel and adjusted its physical properties.

**Influence of Alkaline Concentration.** The formation of supramolecular hydrogels cross-linked via metal–tannic acid interactions rapidly occurred when injecting the precursor with
3 M NaOH, as previously described. The supramolecular interaction is very responsive to environmental pH. However, to use hydrogel as an implant, a minimum alkaline concentration is desirable to avoid the potential for the concentrated solution to cause some degree of acute irritation to internal tissues. According to the Screening Information Dataset Initial Assessment Report (SIAR), NaOH becomes nonirritant to the skin and eyes at concentrations of 0.2–1.0% (w/v), equivalent to around 0.05–0.3 M. Therefore, 10-fold and 100-fold dilutions of the alkaline used for hydrogel formation were investigated to assess the effect of reduced alkaline concentrations on the hydrogel physical properties.

The results demonstrate that reduced alkaline concentrations at 0.3 and 0.03 M affected hydrogel formation. Different NaOH concentrations injected with the same precursor volume and injection rate yielded different overall appearances of the hydrogels, as shown in Figure 3A–C, indicating different hydrogel formation rates with no alteration to the supramolecular interactions, as confirmed by FTIR spectra (Figure S3). Lower alkaline concentrations resulted in slower hydrogel formation, leading to larger, but looser hydrogels. NaOH concentrations of 0.03, 0.3, and 3 M resulted in gelation times of 40 ± 7, 19 ± 5, and 5 ± 2 s, respectively. The physical properties were measured as swelling ratios (Figure S4) and Young’s moduli (Figure 3D). Although the swelling ratios were similar, the significantly lower Young’s moduli suggest that the hydrogels provide different mechanical environments when used as implants. Since reduced alkaline concentrations rendered slower hydrogel formation, this would be beneficial for deep tissue penetration, while the low modulus would be suitable for soft tissues—such as the brain (1–4 kPa)—to avoid microtears, which are usually caused by tissue-implant modulus mismatch. For physical structure, the mesh size became significantly larger from 38.18 ± 0.23 nm (3 M) to 74.12 ± 1.40 and 154.96 ± 2.94 nm for 0.3 and 0.03 M NaOH, respectively (Figure S5). Therefore, the results suggest that hydrogel stabilized with BSA can be prepared with the lowest alkaline concentration, which is more diluted than the reported nonirritant NaOH concentration.

**Influence of Doxorubicin.** In this study, doxorubicin (DOX) was used as a drug model due to the advantages of injectable hydrogels that allow it to be directly injected into a targeted cancerous site to provide localized treatment with reduced side effects compared to conventional chemotherapy. To compare biodegradability as an effect of drug concentration, H-BSA300—which possessed a high Young’s modulus—was selected as a representative hydrogel.

Similar to the previously described drugless hydrogel, the drug-loaded hydrogel could be rapidly formed by dual injection. There was an insignificant change in the hydrogel’s physical properties—especially the swelling ratio (Figure S6) and Young’s modulus (Figure S7)—for all drug concentrations from 0.086 to 0.344 mM. However, increased DOX concentrations significantly reduced the hydrogel mesh size (Figure S8). Minimal changes can be described by the interactions between BSA and DOX. Briefly, BSA and DOX can interact with each other to form a BSA–DOX complex via hydrophilic and hydrophobic forces using several amino acids in BSA and then stabilized by hydrogen bonds. The FTIR results show that amide bands I (1644 cm⁻¹) and II (1531 cm⁻¹) shifted to 1651 and 1537 cm⁻¹, respectively (Figure S9), confirming the interaction between BSA and DOX.

In other words, the drug was encapsulated in the hydrogel by partially binding to BSA, which was not a component of the cross-linking core. Therefore, the hydrogel’s physical properties were barely affected by drug loading, with the exception of mesh size. Therefore, the results suggest that besides stabilizing the hydrogel, BSA also acted as a drug carrier that retained DOX in the porous structure of the hydrogel.

In addition to physical properties, the biodegradability of the drug-loaded hydrogel was also investigated. Figure 4 shows that all of the drug-loaded hydrogels could be biodegraded in phosphate-buffered saline (PBS) at 37 °C over time. They all had two stages of degradation considering different degradation rates. The early stage of hydrogel degradation occurred at a faster rate and lasted approximately 4 days. The drug concentration clearly influenced the early stage and, consequently, indicated the percentage of hydrogel left prior to the degradation rate sharply reducing. The hydrogel with the highest drug amount—H-DOX344—showed the fastest early degradation rate and had 34.03 ± 0.6% by weight remaining by day 4. After day 4, all of the hydrogels began to slowly degrade up to at least 25 days (maximum experimental period). The slow degradation allowed the material to last more than 3 weeks, which covers the treatment cycle of typical chemotherapy. Because BSA is known to be stable in neutral environments and H-BSA rapidly degraded by >70% by weight in 1 day, the early stage was substantially inhibited by metal–phenolic unbinding. The BSA molecules were therefore...
crucial to delaying biodegradation by interacting with the polymer chains and had to be present in sufficient quantities in the hydrogel to exceed the number required to form BSA–DOX complexes. The results can be used as a guideline to tune for a desired biodegradation by balancing the amount of BSA and DOX, which also influence the physical properties and cancer treatment efficiency, respectively.

**Controlled Drug Release.** All formulas of the hydrogels could create a drug reservoir to provide controlled drug release with different release profiles (partly shown in Figure 5). The hydrogel drug release profiles were compared according to BSA and NaOH concentrations because the drug could directly bind to BSA, and the alkaline significantly influenced the hydrogel physical properties, as previously described.

According to the release profiles shown in Figure 5, the hydrogel prepared with the highest BSA concentration—H-BSA300—provided slower drug release than H-BSA75. These results correspond to the biodegradability, which occurred at a slower rate for hydrogels with more BSA molecules. According to Aguado et al., one BSA molecule can limitedly bind with only 1.5 DOX molecules.25 Therefore, the different release profiles might be explained by the different number of BSA molecules. The hydrogel prepared with least BSA concentration—H-BSA75—provided faster drug release because the matrix of the hydrogel was composed of fewer BSA molecules that could bind the drug. This meant that the encapsulated DOX was only retained in the matrix by the physical polymeric structure. The drug release mechanism was further investigated using four different mathematical models for curve fitting. According to the results in Table 1, the Weibull model provided the highest $R^2$ adjusted when fitted to the experimental data using eq 1

$$F(t) = F_{max}(1 - e^{-t/\alpha})$$

where $F$ is the fraction of the drug released at time $t$, $F_{max}$ is the maximum fraction of the drug released at infinite time, $\alpha$ is the scale parameter, which defines the time scale of the process, and $\beta$ is the shape parameter.26 Papadopoulou et al. described $\beta$ as an indicator of the release mechanism of small molecules from a polymer matrix. $\beta \leq 0.75$ indicates Fickian diffusion, while $0.75 < \beta < 1$ indicates the combined mechanism between Fickian diffusion and Case II transport, which is caused by polymer relaxation. $\beta > 1$ with the sigmoid shape of the fitted curve indicates the complex release mechanism.26 According to the fitted $\beta$ shown in Table 2, the BSA concentrations did not significantly affect the release mechanism. $\beta = 0.983 \pm 0.04$ and $0.999 \pm 0.03$ for H-BSA75-3M and H-BSA300-3M, respectively, indicate that both hydrogels released the drug using the similar combined release mechanism of Fickian diffusion and Case II transport. In other words, the hydrogels gradually released the drug by both diffusion and erosion.

For the influence of alkaline concentration, the non-tissue irritant 0.03 M NaOH yielded the hydrogel with a clearly faster drug release compared to the 3 M NaOH, for both BSA concentrations, as shown in Figure 5. This phenomenon could be described by the hydrogel formation rate. As described above, the low alkaline concentration leads to slow hydrogel formation and, consequently, resulted in a looser polymeric network with a larger mesh size compared to the hydrogel prepared with a high alkaline concentration. It is therefore reasonable that the drug molecules would move faster out of the matrix prepared with 0.03 M NaOH. According to the results in Table 2, in contrast to H-BSA75-3M and H-BSA300-3M, H-BSA75-0.03M and H-BSA300-0.03M both illustrated $\beta > 1$, indicating complex release mechanisms. This suggests that the drug released from the hydrogel prepared with 0.03 M NaOH was predominantly released in various multiple ways, potentially including diffusion, erosion, and the release of the BSA–DOX complex, which moved more freely in the polymeric network with a larger mesh size.

In addition to the influence of BSA and NaOH concentrations, the environmental pH was also found to govern the drug release profiles. At pH 7.4—representing the environmental pH of normal human cells—the hydrogels H-BSA300-0.03M and H-BSA300-3M experienced a drug release plateau around 4.10 ± 0.02 and 2.28 ± 0.03% of the total loaded drug, respectively, as shown in Figure 6. Meanwhile, at pH 6.4—representing the acidic cancerous environment—H-BSA300-0.03M and H-BSA300-3M showed drug release plateaus at higher percentages of 12.36 ± 0.17 and 4.83 ± 0.03%, respectively. The significantly higher drug release was due to the higher degree of the hydrogel degradation caused by the pH-responsive metal–tannic acid and PEG–tannic acid

![Figure 5](https://dx.doi.org/10.1021/acsomega.0c01393)

**Table 1. $R^2$ adjusted from Curve Fitting Using Higuchi, Probit, Weibull, and Logistic Models**

| pH  | samples     | $R^2_{adj}$ | Higuchi | Probit | Weibull | Logistic |
|-----|-------------|-------------|---------|--------|---------|----------|
| 6.4 | H-BSA300-0.03M | 0.5430 | 0.8810 | 0.997 | 0.8703 |
|     | H-BSA300-3M   | 0.7429 | 0.9083 | 0.992 | 0.8973 |
| 7.4 | H-BSA75-0.03M | 0.6878 | 0.8835 | 0.971 | 0.8716 |
|     | H-BSA75-3M   | 0.3503 | 0.8880 | 0.997 | 0.8814 |
|     | H-BSA300-0.03M | 0.6483 | 0.8495 | 0.988 | 0.8373 |
|     | H-BSA300-3M   | 0.5770 | 0.8951 | 0.994 | 0.8870 |
interactions. While the acidic environment predominantly determined the maximum cumulative release, the drug release mechanism remained rather dependent on the NaOH concentration during hydrogel formation, as shown in Table 2. However, interestingly, the β values of H-BSA300-0.03M and H-BSA300-3M at pH 6.4 were reduced and closer to each other compared to the ones at pH 7.4. This could be explained by BSA stability. As previously mentioned, one of the releasing ways in the complex release mechanism could be a drug release in the form of BSA–DOX complex. As BSA became less stable at pH 6.4, the BSA–DOX complexes formed to a lesser degree. Therefore, the release mechanism in the acidic environment was more determined by Fickian and Case II transport. The results demonstrate that the hydrogel released significantly higher quantities of DOX in the acidic environment, which represents pH of cancerous milieu, compared to the physiological environment. Such pH-dependent release suggests the material capability to cause less side effects in actual treatments and to serve as a smart pH-sensitive drug carrier.31,32

Furthermore, the drug release profiles demonstrate that the maximum cumulative drug release and drug release rate from all of the hydrogels were unexpectedly low when considering the constantly fast degradation of the hydrogel in the early stage. The maximum cumulative drug release from the hydrogels had plateaus ranging from only 2.28 ± 0.03% for H-BSA300-3M at pH 7.4 to a maximum of 12.36 ± 0.17% for H-BSA300-0.03M at pH 6.4, as shown in Figure 6. The results may be explained by drug encapsulation in the form of BSA–DOX complex, which is obviously larger than the free drug molecule. The complexes entrapped in the cross-link network require a longer duration to degrade and are gradually released as free drug molecules. However, the highest cumulative drug release from H-BSA300-0.03M at pH 6.4 was equal to 123.6 ± 1.7 µg, which still slightly exceeds the DOX concentration of 100 µg, which has been previously reported to effectively kill HeLa cells.33 Such results therefore suggest that the injectable hydrogel can be tuned to provide appropriate drug release profiles for controlled drug release, as seen by H-BSA300-0.03M. The overall results suggest that the physical properties and the drug release profiles were essentially governed by the supramolecular interactions underpinning the hydrogel formation, as summarized and illustrated in Scheme 1.

**Biocompatibility of Hydrogels.** Figure 7 demonstrates that all hydrogels resulted in >80% L929 fibroblast cell viability, indicating biocompatibility of the materials, according to ISO Standard 10993-5 (cytotoxic when cell viability <70%). H-BSA0 (93.66 ± 1.36%) and H-BSA300 (93.60 ± 1.99%) showed negligible cytotoxicity, while H-BSA300-DOX (88.17 ± 0.52%) had around 12% less cell number, compared to the negative control (Dulbecco’s modified Eagle’s medium (DMEM)). The cytotoxicity of H-BSA300-DOX was much less than the drug alone at the same drug concentration. This phenomenon can be explained by controlled drug release that retains drug molecules in a matrix and gradually releases them in the therapeutic window, avoiding too high drug levels that are toxic to cells and tissues. The hydrogel biocompatibility supports the great potential of the hydrogel to serve as a biomaterial for biomedical applications.

**CONCLUSIONS**

In this research, the researchers fabricated injectable pH-responsive hydrogels for controlled drug release using a combination of metal–phenolic–PEG and PEG–albumin interactions to create a cross-link network. It was found that the properties—including Young’s modulus, swelling ratio, and mesh size—could be tuned by varying the hydrogel component concentrations. The BSA molecules could interact with the PEG chains in their hydrophobic pockets and consequently stabilize the hydrogels. The diluted alkaline delayed the hydrogel formation and resulted in the hydrogels having a looser polymeric network. Since the drug DOX bound to BSA, more drug molecules caused faster early degradation. The differences between the hydrogel properties also influenced the controlled drug release profiles. The drug release rate became faster with decreased BSA and alkaline concentrations. In addition, the drug-loaded hydrogels were found to provide a cumulative DOX release under acidic conditions up to an effective drug concentration, indicating the optimum hydrogel formula to kill cancer cells. The supramolecular interactions underlying the hydrogel formation can be reasonably explained by experimental results, drug release...
models, and FTIR spectra. The hydrogels both with and without drug loading exhibited excellent in vitro biocompatibility. The overall results suggest that the injectable supramolecular hydrogels possess great potential as a biomaterial for controlled cancer drug release.

■ EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) (PEG, $M_n = 20\,000$ g/mol) was purchased from Sigma-Aldrich Chemical, Inc. Tannic acid (TA, reagent), anhydrous ferric chloride ($\text{FeCl}_3$, 98%), and phosphate-buffered saline (PBS, 0.01 M, pH 7.4) were purchased from Alfa Aesar (United Kingdom). Sodium hydroxide ($\text{NaOH}$) was purchased from Merck, Inc. (Germany). Bovine serum albumin (BSA, >98.0%) was purchased from Bio Basic, Inc. (Canada). Doxorubicin hydrochloride ($\text{C}_{27}\text{H}_{29}\text{NO}_{11}\cdot\text{HCl}$, 95%) was purchased from Tokyo Chemical Industry (Japan). Dulbecco’s modified Eagle’s medium (DMEM; Gibco), fetal bovine serum (FBS; Gibco), and penicillin–streptomycin were purchased from Thermo Fisher Scientific, Inc. Sterile flat-bottom 24-well plates and transwell inserts (pore size 0.8 μm) were purchased from Corning, Inc. All aqueous solutions were prepared using distilled water.

Hydrogel Formation. Hydrogel formation was performed using a modified method based on the process previously described. In this experiment, 0.75 mL of PEG (0.035 M) was mixed with 0.2 mL of TA (0.12 M) and briefly stirred. The solution was then adjusted to pH $\sim 5.5$, and 0.05 mL of $\text{FeCl}_3$ (0.79 M) was added. The total volume was then adjusted with 1.0 mL of distilled water and stirred until the solution became homogeneous. To study the influence of BSA, BSA was added to the solution to give different final concentrations of 0.75, 1.50, 3.00, and 7.50 mM. To study the drug release, a cancer drug, DOX, was used as a representative small molecule to investigate the hydrogel’s drug delivery capability. DOX was added to the solution to give different final concentrations of 0.086, 0.172, and 0.344 mM. The hydrogel was rapidly formed by injecting the mixture simultaneously with a solution of $\text{NaOH}$ at concentrations of 0.03, 0.3, and 3 M. After hydrogel formation, the hydrogel pH was measured and determined to be 7.8 (Mettler Toledo LE427-S7 pH puncture electrode). The hydrogels were stored in PBS at room temperature before characterization. Table 3 summarizes all of the hydrogel component variations.

![Figure 7.](image)

Figure 7. (A) Micrographs of L929 cells incubated with the transwell inserts of H-BSA0, H-BSA300, and H-BSA300-DOX, and a solution of DOX in DMEM; live and dead cells are shown in green and red, respectively. (B) Schematic of in vitro cytotoxicity test using a transwell insert. (C) Normalized cell viability by negative control (DMEM).

**Table 3. Hydrogel Components**

| Hydrogels | BSA (mM) | DOX (mM) |
|-----------|----------|----------|
| H-BSA0    |          |          |
| H-BSA75   | 0.75     |          |
| H-BSA150  | 1.50     |          |
| H-BSA300  | 3.00     |          |
| H-BSA750  | 7.50     |          |
| H-DOX86   | 3.00     | 0.086    |
| H-DOX172  | 3.00     | 0.172    |
| H-DOX344  | 3.00     | 0.344    |

Hydrogel Characterization. Swelling Ratio. The hydrogel swelling ratio is the mass or volumetric fractional increase of the hydrogel after water absorption. Here, we quantified both the mass and volume swelling ratios to indicate the polymeric network and mesh size. Briefly, the hydrogel was immersed in PBS and incubated at 37 °C for 24 h. The swollen hydrogel was weighed to obtain its swollen weight ($W_s$) and then dried in vacuum to obtain its dried weight ($W_d$). The mass swelling ratio ($Q_m$) was calculated using eq 2

$$Q_m = \frac{W_s}{W_d}$$

Meanwhile, the volume swelling ratio ($Q_v$) was calculated using eq 3

$$Q_v = 1 + \left(\frac{\rho_{\text{polymer}}}{\rho_{\text{solvent}}}\right)(Q_m - 1)$$

where $\rho_{\text{polymer}}$ is the density of the dry hydrogel (estimated to be 1.12 g/cm$^3$ for PEG) and $\rho_{\text{solvent}}$ is the solvent density (1 g/cm$^3$ for PBS). Young’s Modulus. The Young’s moduli of the hydrogel were evaluated by an Instron Universal Testing Machine. The test was conducted using a customized flat-end cylindrical rigid indenter (10 mm in diameter) with a 10 N load cell. The indentation rate was constant at 3 mm/min. As shown in eq 4, the relationship between force and indentation depth was used...
in the curve fitting to extract Young’s modulus using Microsoft Excel Solver.

\[ F = \frac{2ER\delta}{2 - \nu^2} \]  
(4)

where \( F \) is the force, \( E \) is the Young’s modulus, \( R \) is the radius, \( \delta \) is the indentation depth, and \( \nu \) is Poisson’s ratio (estimated to be 0.5 as an incompressible soft material).36–38

Mesh Size. Mesh size of the hydrogel was obtained from calculation using eq 5

\[ \xi = \frac{1}{2} \left[ (r_0^2) \right]^{1/2} \]  
(5)

where \( r_2 \) is the polymer volume fraction in the swollen equilibrium hydrogel, which relates to the reciprocal of \( Q_0 \), and \( (r_0^2) \) is the root-mean-square end-to-end distance of the polymer chain in the unperturbed state.39,40

Biodegradability. The biodegradability of the hydrogel was obtained by recording the dry weight over time after incubation. Briefly, the hydrogel was dried in vacuum and weighed until a constant initial weight (\( W_i \)) was obtained. The hydrogel was then incubated in PBS, pH 7.4, at 37 °C, for 24 h and dried for designated periods of 1, 2, 3, 4, 15, and 25 days to obtain the dried weight (\( W_f \)). The biodegradability percentage was calculated using eq 641

\[ \text{biodegradability (\%)} = \left( \frac{W_f - W_i}{W_i} \right) \times 100 \]  
(6)

FTIR Analysis. The infrared spectrum of the hydrogel was measured using a Fourier transform infrared spectrometer (Thermo Scientific Nicolet iS5 FTIR spectrometer) in the range of 4000–5500 cm\(^{-1}\).

Drug Release Study. Hydrogel precursors with 17.2 mM DOX were injected to form a hydrogel in a dialysis bag (Cellu-Sep T1, MWCO 3500) and then immersed in 2 mL of PBS at 37 °C. At designated time points, the solution was collected and replaced with fresh PBS. The drug concentration of the collected solution was analyzed by fluorescence intensity measurement using an Infinite M200 microplate reader (Tecan, Switzerland) with an excitation wavelength of 480 nm and an emission wavelength of 570 nm.42 The drug release mechanisms of the hydrogels were analyzed according to curve fitting between the experimental data and drug release models, including Higuchi,43 Probit,44,45 Weibull,46 and the Logistic model using DDSolver 1.0.44 The adjusted coefficient of determination (\( R_{adj}^2 \)) was used to indicate suitable model fitting.

Cytotoxicity Test. L929 mouse fibroblasts (ATCC, Manassas, VA) were used to evaluate the material cytotoxicity according to ISO Standard 10993-5.37 The cells were cultured in DMEM, 10% FBS, and 1% penicillin/streptomycin. An indirect contact test was performed using a transwell insert.48

The test was conducted for hydrogels H-BSA0, H-BSA300, and H-BSA300-DOX (loaded with 17.2 mM DOX). All hydrogels were formed by co-injecting 0.3 mL of hydrogel precursors with 0.03 M NaOH into a transwell insert placed on the top of a 24-well plate, which was pre-seeded to have ~80% confluent L929 cells at the bottom of each well. The hydrogel in the transwell insert and the cells were then incubated together at 37 °C with 5% CO\(_2\) for 24 h. Viable and dead cells were determined using Live/Dead Assay Kit (L-3224, Invitrogen, Life Technologies, Foster City, CA). Briefly, the cells were stained in a staining solution containing 2 M:4 \( \mu \)M calcein acetoxymethyl ester (calcein-AM)/ethidium homodimer-1 (EthD-1). Live cells convert calcein-AM into green fluorescent calcein, while EthD-1 penetrates damaged membranes of dead cells and, upon binding to nucleic acids, produces bright red fluorescence (excitation/emission, approximately 495/635). Micrographs were recorded using a fluorescence microscope (Cytell Cell Imaging System by GE Healthcare Life Sciences) and analyzed using NIH ImageJ (National Institute of Health, available at http://rsb.info.nih.gov/ij).

Statistical Analysis. The swelling ratio, mesh size, and Young’s moduli were analyzed using the Kruskal–Wallis test due to the limited sample size. Biodegradability and drug release profiles were analyzed using repeated measures ANOVA. Data were considered to be statistically significant at a p value of 0.05. All statistical analyses were performed using MedCalc statistical software. All experiments were done in triplicate, and the results reported as mean ± standard deviation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01393.

Fitted curve between the indentation depth and force of the hydrogels with different concentrations of BSA (Figure S1); swelling ratio of the hydrogels with different concentrations of BSA (Figure S2); FTIR spectra of the hydrogels formed using different NaOH concentrations (Figure S3); swelling ratio of the hydrogels formed using different NaOH concentrations (Figure S4); mesh size of the hydrogels formed using different NaOH concentrations (Figure S5); swelling ratio of the hydrogels loaded with different DOX concentrations (Figure S6); Young’s moduli of the hydrogels loaded with different DOX concentrations (Figure S7); mesh size of the hydrogels loaded with different DOX concentrations (Figure S8); and FTIR spectra of DOX, H-BSA, and H-BSA–DOX (Figure S9) (PDF)

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Notes

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