Autoclaving pHEMA-Based Hydrogels Immersed in Deionized Water has No Effect on Physicochemical Properties and Cell Behaviors

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ABSTRACT: Hydrogels based on poly-(2-hydroxyethyl methacrylate) (pHEMA) have been widely used as biomaterials in tissue engineering due to their biocompatibility, hydrophilicity, and low friction coefficient. The terminal sterilization of hydrogels is a critical step in clinical applications. However, regulations and standardization for the sterilization of hydrogels based on pHEMA are still lacking. In this study, we explored six sterilization methods on pHEMA-based materials (A1: pHEMA, A2: pHEMA copolymerizes with acrylic acid, and A3: pHEMA copolymerizes with acrylic acid and further coordinated with iron ions), such as gamma irradiation, 75% ethanol, ultraviolet (UV), ethylene oxide (EtO), and autoclaving with or without deionized water (autoclaving-H\textsubscript{2}O or autoclaving-dry). Combining results from the multifaceted approaches with assessment, pHEMA-based hydrogels can be completely sterilized via the autoclaving-H\textsubscript{2}O method analyzed by sterilized testing. The physicochemical properties and cell behavior of sterilized hydrogels were not influenced by this sterilization approach, validated by Fourier transform infrared (FT-IR) spectroscopy and tensile tests. The pHEMA-based hydrogel sterilized by the autoclaving-H\textsubscript{2}O method also had no effect on the cell behavior evaluated by in vitro cytotoxicity experiments and caused no evident inflammatory reaction in tissue in vivo implantation experiments. However, it was also found that there were still some defects in the A2 and A3 groups as biomaterials possibly because of an inappropriate proportion of formulations or raw material used in exploring sterilization methods. These findings have implications for the improvement and clinical application of pHEMA-based hydrogels.

1. INTRODUCTION
Since the invention of the first known hydrogel as a possible biomaterial in 1960, advances in science and technology, as well as extensive research, have resulted in increased exploration of innovation and application of biomaterials, allowing the development of adaptable biomaterials that allow for advancements in healthcare.\textsuperscript{1} Hydrogels have gained a lot of attention since then and have been used in a variety of applications, for example, in controlled release, tissue engineering, wound repair, ophthalmic lenses, sensors, coatings, and implants.\textsuperscript{2,−10} Several research studies have described the synthesis of hydrogels and their biological evaluation by cytotoxic or cell viability tests in the field of biomaterials.\textsuperscript{11}\textsuperscript{−10} However, there is still a lack of regulations and consistency in sterilization for materials used in novel medical approaches.\textsuperscript{11}

It is noteworthy that before hydrogels are put into clinical application, it is most important to focus on the terminal sterilization of hydrogel-based biomaterials. However, fewer than 100 studies have been published in the last decade focusing on hydrogel sterilization. Thus, the implications of sterilizing treatments on hydrogels’ intrinsic properties remain unexplored.\textsuperscript{12} Therefore, we summarized six sterilization methods to find the best one, which not only causes little damage to the material but is also efficient and convenient.

2-hydroxyethyl methacrylate (HEMA) is photopolymerizable, allowing for spatial and temporal control of mechanical characteristics\textsuperscript{13} with a range of formulations and methodologies reported in the literature with Young’s moduli ranging from \(<100\text{ kPa}\)\textsuperscript{14} to 1.5 GPa.\textsuperscript{15} poly-(2-hydroxyethyl methacrylate) (pHEMA)-based hydrogels have been extensively explored for biomedical applications because of their swellability, oxygen permeability, and biocompatibility.\textsuperscript{16,−18} Although pHEMA is widely used in a variety of fields, including artificial corneas and cardiac tissue engineering, it has weak mechanical qualities as well as limited protein adsorption and cell adhesion.\textsuperscript{19} Acrylic

Received: May 18, 2022
Accepted: July 21, 2022
Published: August 30, 2022
2. RESULTS

2.1. Synthesis and Characterization of Hydrogels. All these hydrogels (A1: pHEMA, A2: pHEMA copolymerized with AA, A3: pHEMA copolymerized with AA and further coordinated with iron ions) were obtained under the same polymerization conditions, especially in the A1 and A2 groups. For the A2 group, the introduction of AA enhanced the hydrogen bonding, resulting in the transition from ductility to brittleness. For the A3 group, to improve the mechanical properties of the pHEMA/AA hydrogels, further chemical reactions were carried out before immersion in deionized water. Hydrogen bonding and iron ion coordination further enhance the toughness of the hydrogel, and the robust coordination of ferric ions leads to a tighter polymer network. The finished product is shown in Figure 1A (group 0). Meanwhile, the impact of sterilization on the morphology of the material was initially explored. As shown in Figures 1A and 2, we observed that the A1/A2 group developed 75% ethanol-soaked morphological changes and lost its structure because AA tends to self-polymerize and cause swelling. As a result, all samples treated with 75% ethanol were eliminated without additional biological or chemical analysis. When the hydrogels were immersed in phosphate-buffered saline (PBS) for 48 h, the extracted liquid pH of each group was separately compared with that of the untreated group, and there were no significant differences between the autoclaving-H2O group and untreated group (Figure 1B), which demonstrated that the autoclaving-H2O sterilization method has no effect on the sterilized hydrogel morphology. The hydrogels in this work refer to the equilibrated hydrogels.

2.2. Tensile Test. To further analyze the effect of different sterilization treatments on the structure and mechanical properties, the hydrogels were systematically subjected to tensile strength measurements. The tensile strength at the break of non-sterilized hydrogels was 0.15, 0.31, and 3.6 MPa in A1/A2/A3 groups, respectively. After sterilization by autoclaving-H2O, the tensile strength at the break of sterilized hydrogels was 0.06, 0.1, and 1.91 MPa in A1/A2/A3 groups, respectively. Compared to all other sterilization treatments, autoclaving-H2O sterilization can minimize the mechanical deformation and maintain the structure of the hydrogel network, suggesting that H2O may have a protective effect on hydrogels during the sterilization process. The typical tensile stress–strain curves of the hydrogels under tensile tests are shown in Figure 2. The excellent tensile elasticity and stretchability of the A3 group are revealed from the tensile stress–strain curves. The tensile strength, fracture strain, and toughness showed great changes in the irradiation group, in which A3 became too brittle to be tested (Figure 2C), and in the ethylene oxide (EtO) group and UV group, the indicators of A1 were significantly lower than those of the others (Figure 2E,F). In contrast, there was little difference in other sterilized groups compared with the untreated group, and the tensile stress–strain curves for autoclaving in a dry environment were jagged (Figure 2D), indicating that the sterilized hydrogel showed a loss of water. Thus, the above three sterilization methods were excluded. It is worth noting that autoclaving-H2O sterilization showed little influence on the structure and mechanical properties.
2.3. Sterilization Testing. Considering UV as a common sterilization method in multiple hydrogel materials and the results above, the efficiency of sterilization was further evaluated in UV and autoclaving-$H_2O$ approaches. FTM and SGA were selected to test the growth of bacteria or fungi, and the results are shown in Figure 3. Colonies were observed on Sabouraud 2% glucose agar after 3 days in both the positive control and UV groups of the three hydrogels, while there was no appearance in the negative control and autoclaving treatment (Figure 3A). After 7 days of culture, UV irradiation clouded the thioglycollate broth media with the A1 hydrogel, indicating that bacteria proliferated in the tube (Figure 3B–A1). It was unexpected, however, that even 15 days later, the media were clear and light yellow with the A2 and A3 hydrogels in neither the experimental groups nor the control groups (Figure 3B–A2/A3). In conclusion, hydrogels were completely sterilized by autoclave-$H_2O$ sterilization, and UV radiation was ineffective in removing microorganisms from the hydrogel scaffold. The influence of autoclaving sterilization on hydrogel structures will be shown later.
2.4. Infrared Spectroscopy. As shown in the Fourier transform infrared (FT-IR) spectrum (Figure 4), hydrogels sterilized by soaking in deionized water for autoclaving and those with no treatment were analyzed and compared. The FT-IR spectrum of these three pHEMA-based hydrogels exhibited a major characteristic band at about 3300 cm$^{-1}$, which was assigned to O-H stretching vibration, and a characteristic peak at approximately 1600 cm$^{-1}$ was associated with the stretching vibration of the C=O groups, indicating that the sterilized hydrogel successfully contained hydroxyl groups and carbonyl groups and retained the properties of the hydrogel. The O-H characteristic peaks of sterilized hydrogels (A1–A3) were observed at 3332.9, 3319.9, and 3301.1 cm$^{-1}$, respectively, and O-H peaks of sterilized hydrogels (A1–A3) were observed at 3330.1, 3315.6, and 3309.7 cm$^{-1}$, respectively. Compared with pHEMA hydrogels (A1 group), the peaks of O-H shifted to low wavenumbers, demonstrating the stronger hydrogen bonding in A2 and A3 groups. Also, both the peaks of O-H and C=O of sterilized hydrogels showed a little shift in wavenumbers and appeared in the same range compared with unsterilized hydrogels. This demonstrated that this sterilization method has no effect on the chemical functional groups of pHEMA-based hydrogels.

2.5. Cytocompatibility Measurements. To further investigate the potential of the three hydrogels (pHEMA, pHEMA/AA, and pHEMA/AA/Fe$^{3+}$) for clinical applications, the cytocompatibility of hydrogels was assessed by in vitro culture of HUVECs (human umbilical vein endothelial cells) and in vivo subcutaneous implantation experiments.

2.5.1. In vitro Cytotoxicity. The viability and proliferation of HUVECs were shown using cell counting kit-8 (CCK-8) assay, and cells seeded without hydrogels served as a control. Cell viability was as high as 53% in the A1 group, and undesirable outcomes were observed in the A2 and A3 groups (Figure 5B). To further study the compatibility and visualize the cell proliferation and live/dead viability assays, cells were cocultured with hydrogels for 1, 3, and 5 days. After 3 and 5 days of culture, in the A1 group, the cell survival rate increased from 68 to 84% (Figure 5C), showing a good growth trend. It should be noted that some cells were lost with the removal of the hydrogel instead of the toxicity of the hydrogel. Representative live/dead fluorescence images are shown in Figure 5A. After 1 day of culture, in the A1 group, the live cell (green) density and very few dead cells (red) were similar to those in the control group, indicating that the A1 hydrogel is not cytotoxic. However, compared with normal cells, the morphology of cells in A2 and A3 groups were markedly altered; the cells became smaller, shrunk, and more rounded. AA is usually more acidic, which could be one of the reasons causing this results. After 5 days of culture, cells in A1 and the control group proliferated and spread homogeneously until nearly full coverage was achieved, with only a few dead cells being observed during this extended culture, further demonstrating that hydrogels were sterilized completely by autoclave-H$_2$O sterilization. In contrast, in the A2 and A3 groups, cell proliferation was inhibited, the number of dead cells increased, and the viable cell rate was only about one-fifth of that of cells from the control. This result was consistent with the abovementioned CCK-8 results and pH test results.

Figure 3. Efficiency of two sterilization treatments on hydrogels. The autoclave group is referred to autoclaving (hydrogels soaked in deionized water). (A) SGA, white colonies were observed in the positive control and UV sterilization and (B) FTM, white and flocculent precipitates were observed in the positive control and UV sterilization in the A1 group, and the medium in the A2 and A3 groups remained clear and light yellow.

Figure 4. FT-IR spectrum of three hydrogels before and after being treated by autoclaving-H$_2$O.
The reason for this was probably the impact of material synthesis rather than the sterilization method on the cellular behavior, and these results also suggested the direction of the follow-up improvement of hydrogel synthesis in their future application.

2.5.2. In vivo Implantation Experiment. After in vivo implantation for 1 month, the tissue demonstrated good wound healing. Additionally, a histological examination was carried out to determine the inflammatory response of skin tissue implanted with sterilized hydrogels (hydrogel samples sterilized by the autoclaving-H₂O method). The magnified images of HE staining showed that no distinct inflammatory properties were observed in the hydrogel-treated groups (Figure 6), indicating that the implanted hydrogels did not cause an inflammatory response. The results show that the pHEMA-based hydrogels have good compatibility in tissue in vivo; furthermore, the hydrogel sterilized by this approach did not cause an evident inflammatory reaction in tissue, allowing it possible to be used as a tissue filler.

3. DISCUSSION

Scientists must have a better knowledge of the clinical problems that require solutions and refocus their attention on
fundamental research rather than developing a novel smart hydrogel and then exploring its applications. For current clinically available hydrogels, what calls for special attention is their safety and efficacy; the literature is scarce regarding hydrogel sterilization, and there is no universal set of methods for sterilizing hydrogels. Hence, in this study, we have tried six sterilization methods for pHEMA-based hydrogels, on one hand, to select an optimal method for clinical applications in the future; on the other hand, we want to know if any aspect needs to be improved after sterilization.

Attention has been drawn to the fact that the formula of hydrogel synthesis determines its properties. A2 (pHEMA/AA) is synthesized by copolymerization of HEMA and AA, but AA is easy to self-polymerize, which usually leads to inhomogeneities, and this may be one of the reasons for the swelling in 75% ethanol. In the synthesis of A3 (pHEMA/AA/Fe \(^{3+}\)), iron ions are coordinated, which leads to an increase in the modulus. It is difficult to say that these factors have no effect on biocompatibility. However, obtaining more specific information on the causes of observed changes is critical for a better understanding of sterilization’s effects and for assisting in the selection of the optimal sterilizing procedure for each system.

For these three hydrogels, it was found that the unsterilized hydrogel can also exhibit mold growth even when stored at 4 °C. As reported in previous studies, disinfection of polymeric hydrogel can also exhibit mold growth even when stored at 4 °C and in Sabouraud 2% glucose agar (SGA) (Sigma-Aldrich, St. Louis, MO, USA) (Figure 2D) compared with the control group. In the sterility test, it was shown that immersion in deionized water for autoclaving was efficient to avoid contamination (i.e., bacteria). From what has been found in the results above, UV irradiation can only remove the bacteria from the surface of the hydrogel but cannot completely sterilize it.

The cytotoxicity evaluation based on the three hydrogels demonstrated that there was some cytotoxicity in the A2 (pHEMA/AA) and A3 (pHEMA/AA/Fe \(^{3+}\)) groups because the acidic environment and high modulus environment were not conducive to cell growth. It was also a possible association with the absence of clouding in thioglycollate broth media in both the A2 (pHEMA/AA) and A3 (pHEMA/AA/Fe \(^{3+}\)) groups. However, according to the procedure outlined in the ISO 10933-5:2009 standard, it has been established that a cytotoxic effect is considered when cell viability drops by more than 30%. The viability was higher than 53% in the A1 group, and the results showed that autoclaving did not damage their properties.

According to the exploration of sterilization methods of hydrogels, a higher concentration of acid balance and appropriate reduction of the modulus should be employed in future studies. Overall, this study provides further insight into the pHEMA-based material design with consideration of the effect of terminal sterilization.

4. CONCLUSIONS
Sterilization of hydrogels is extremely important for meeting practical clinical needs. In this work, we have demonstrated that pHEMA hydrogel, pHEMA/AA, and pHEMA/AA/Fe\(^{3+}\) hydrogels can be sterilized by immersing in deionized water for autoclaving without affecting their physicochemical properties and cell behaviors. However, pHEMA/AA and pHEMA/AA/Fe\(^{3+}\) hydrogels showed cytotoxicity when co-cultured with cells, which may be associated with their synthetic materials and conditions rather than the effect of sterilization, and they may have promising potential for their use in tissue engineering or other biomedical applications after improvement. Here, we provide an optimal sterilization method of autoclaving pHEMA-based hydrogels immersed in deionized water with the advantages of good sterilization effects, low cost, and high efficiency.

5. MATERIALS AND METHODS

5.1. Materials. AA and photoinitiator 2-hydroxy-2-methyl-phenylacetone were purchased from TCI (Shanghai) Chemical Industrial Development Co., Ltd. HEMA was provided by Adamas, China. Iron(III) chloride hexahydrate (FeCl\(_3\)·6H\(_2\)O) was supplied by Tianjin KERMEL Chemical Reagent Co., Ltd. The aqueous solution used in the experiment was prepared with homemade deionized water.

5.2. Hydrogel Synthesis. 5.2.1. Synthesis of pHEMA. A certain amount of monomer of HEMA and the photoinitiator were dissolved in deionized water and then poured into the mold and reacted under UV irradiation at 300 W for 2 h after deoxygenation, obtaining the copolymer of HEMA, which was then immersed into deionized water for 24 h to obtain the equilibrated hydrogels—A1.

5.2.2. Synthesis of HEMA and AA Copolymer (pHEMA/AA). Similarly, pHEMA/AA was synthesized according to the method described for the A1 group. The difference is that a certain amount of monomer of AA, HEMA, and photoinitiator was prepared first, and A2 was obtained after the same synthesis steps.

5.2.3. Synthesis of pHEMA/AA Coordination with Iron Ions (pHEMA/AA/Fe\(^{3+}\)). The hydrogels were obtained by a two-step method. After obtaining the copolymer of HEMA and AA, the copolymer was soaked in a solution of 0.1 M ferric chloride (FeCl\(_3\)) at 60 °C for 5 h, obtaining the as-prepared coordinate hydrogels, and then immersed in deionized water for 24 h to prepare the equilibrated hydrogels—A3.

5.3. Sterilization. The three pHEMA-based hydrogels were sterilized by the following methods: (1) gamma sterilization (irradiated hydrogels with 25 kGy), (2) disinfection with 75% ethanol for 2 h, (3) UV radiation for 90 min (UV lamp = 30 W, wavelength = 254 nm, distance = 30 cm), (4) sterilization with ethylene oxide for 5 h, and (5) autoclaving at 121 °C for 15 min, including two conditions (the hydrogels were placed separately in a dry environment and soaked in deionized water). These methods were applied to six equal batches of the three polymeric materials, each receiving a single method.

5.3.1. Sterility Testing. The sterilized hydrogel samples were tested for sterility in fluid thioglycollate medium (FTM) (Sigma-Aldrich, France) for bacterial growth for 15 days at 37 °C and in Sabouraud 2% glucose agar (SGA) (Sigma-Aldrich, France) for fungal cultivation for 7 days at 37 °C. Unsterilized samples served as a positive control, and culture media alone served as a negative control. Every 3 days, the clouding of the
FTM and the appearance of the colonies on Sabouraud agar were examined, indicating contamination and ineffective sterilization.

5.4. Mechanical Property Measurements (Tensile Test). The mechanical properties of the samples treated with different sterilization methods were tested using a universal testing machine (Instron 3367, Instron Instrument Co., Ltd). A1−A3 were cut into rectangular samples to be tested (10 mm wide, 50 mm long, 1 mm thick) for tensile testing at room temperature, and the tensile rate was fixed at 100 mm/min. Each sample under test was repeated five times, and its average value was taken.

5.5. FT-IR Spectroscopy. The molecular structure of the hydrogels was characterized by a FT-IR (VERTEX70, USA KINO Industry Co., Ltd). The samples of A1−A3 treated with different sterilization methods were tested by the KBr tabletting method. The test range was from 400 to 4000 cm$^{-1}$, and the test temperature was room temperature.

5.6. Cytocompatibility Measurements. 5.6.1. In vitro Cytotoxicity. To quantify and evaluate the effect of the hydrogels on cell survival, the viability of HUVECs was evaluated using CCK-8 assay, which allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays (Sigma-Aldrich, USA).

HUVECs were seeded at a density of roughly 5000 cells per well in 96-well plates and allowed to adhere for 24 h before the tests. The cells were cocultivated with a series of the three kinds of hydrogels in sample disks with a diameter of 4 mm and a thickness of 1 mm at 37 °C for 24 h. Next, the hydrogels were removed, and the abandoned medium was aspirated. The CCK reagent was added to each well at a ratio of 1:9 (10 μl) with the medium. The cells were incubated at 37 °C for 2 h, and the absorbance at 450 nm was measured to evaluate cell viability using a microplate reader (USA). Similarly, CCK-8 assays for 1, 3, and 5 days were further applied to the three hydrogels to evaluate cell proliferation.

The proliferation percentage of the CCK-8 reagent was calculated using the following equation

$$%\text{proliferation} = \frac{OD_{\text{test}} - OD_{\text{blank}}}{OD_{\text{control}} - OD_{\text{blank}}} \times 100$$

Optical Density: OD (test) corresponds to the optical density of the test group (with cells, CCK solutions and hydrogels), OD (control) corresponds to the control group (with cells, CCK solutions and no hydrogels), and OD (blank) corresponds to the blank group (with medium, CCK solutions and no cells).

Cell Live/Dead Assay: HUVECs were seeded on 24-well plates at a density of 2.4 × 10$^4$ cells/well and cocultured with sterilized hydrogels after 24 h of incubation for 1, 3, and 5 days. Then, the hydrogels were removed, and the reagent was added with FluoroQuench AO/EB (USA). Fluorescence images were taken using an inverted fluorescence microscope (OBSERVER D1/A1X10 cam HRC, Zeiss, Germany). The green channel depicts live cells, while the red channels depict dead cells.

5.6.2. In vivo Implantation Experiment. To evaluate the biocompatibility of the sterilized hydrogels with tissue in vivo, the hydrogels (a diameter of 8 mm) were implanted into the subcutaneous dorsum of 3-month-old male Bama miniature pigs. Hydrogels were prepared and sterilized by autoclaving-H$_2$O sterilization. One month later, the samples with the surrounding tissues were taken for H&E-stained frozen sections and observed under an optical microscope.
ACKNOWLEDGMENTS

The research leading to these results had received funding from the National Natural Scientific Foundations of China (82070640) and the 1.3.5 project for disciplines of excellence, West China Hospital Sichuan University (no. ZYPY20005 and no. ZYJC21014).

REFERENCES

(1) Koetting, M. C.; Peters, J. T.; Steichen, S. D.; Peppas, N. A. Stimulus-responsive hydrogels: Theory, modern advances, and applications. *Mater. Sci. Eng., R* 2015, 93, 1–49.
(2) Calò, E.; Khutoryanskiy, V. V. Biomedical applications of hydrogels: A review of patents and commercial products. *Eur. Polym. J.* 2015, 65, 252–267.
(3) Hoffman, A. S. Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* 2002, 54, 3–12.
(4) Peppas, N.; Hilt, J.; Khademhosseini, A.; Langer, R. Hydrogels in biology and medicine: From molecular principles to bionanotechnology. *Adv. Mater.* 2006, 18, 1345–1360.
(5) Peppas, N. A.; Bures, P.; Leobundung, W.; Ichikawa, H. E. Hydrogels in pharmaceutical formulations. *Eur. J. Pharm. Biopharm.* 2000, 50, 27–46.
(6) Peppas, N. A.; Sahlin, J. J. Hydrogels as mucoadhesive and bioadhesive materials: a review. *Biomaterials* 1996, 17, 1553–1561.
(7) Aziz, M. A.; Cabral, J. D.; Brooks, H.; McConnell, M. A.; Fitzpatrick, S. C.; Hanton, L. R.; Moratti, S. C. In vitro biocompatibility and cellular interactions of a chitosan/dextran-based hydrogel for post-surgical adhesion prevention. *J. Biomed. Mater. Res., Part B* 2015, 103, 332–341.
(8) Duffy, C.; Venturato, A.; Callanan, A.; Lilienkampf, A.; Bradley, M. Arrays of 3D double-network hydrogels for the high-throughput discovery of materials with enhanced physical and biological properties. *Acta Biomater.* 2016, 34, 104–112.
(9) Franco, R. A.; Min, Y. K.; Yang, H. M.; Lee, B. T. Fabrication and biocompatibility of novel bilayer scaffold for skin tissue engineering applications. *J. Biomater. Appl.* 2013, 27, 605–615.
(10) Zhang, M.; Li, X. H.; Gong, Y. D.; Zhao, N. M.; Zhang, X. F. Properties and biocompatibility of chitosan films modified by blending with PEG. *Biomaterials* 2002, 23, 2641–2648.
(11) Escudero-Castellanos, A.; Ocampo-García, B. E.; Domínguez-García, M. V.; Flores-Estrada, J.; Flores-Merino, M. V. Hydrogels based on poly(ethylene glycol) as scaffolds for tissue engineering application: biocompatibility assessment and effect of the sterilization process. *J. Mater. Sci.: Mater. Med.* 2016, 27, 176.
(12) Galante, R.; Pinto, T. J. A.; Colaço, R.; Serro, A. P. Sterilization of hydrogels for biomedical applications: A review. *J. Biomed. Mater. Res., Part B* 2018, 106, 2472–2492.
(13) Boazk, E. M.; Greene, V. K.; Auguste, D. T. The effect of heterobifunctional crosslinkers on HEMA hydrogel modulus and toughness. *PLoS One* 2019, 14, No. e0215895.
(14) Zhu, J.; Wang, J.; Liu, Q.; Liu, Y.; Wang, L.; He, C.; Wang, H. Anisotropic tough poly(2-hydroxyethyl methacrylate) hydrogels fabricated by directional freezing redox polymerization. *J. Mater. Chem. B* 2013, 1, 978–986.
(15) Arima, T.; Hamada, T.; McCabe, J. F. The Effects of Cross-linking Agents on Some Properties of HEMA-based Resins. *J. Dent. Res.* 1995, 74, 1597.
(16) Andrade, J. Hydrogels in Medicine and Pharmacy: N. A. Peppas (Editor), CRC Press, Boca Raton, FL, Vol. III, Properties and Applications, 208 pages, $110.00. *J. Contr. Release* 1989, 10, 225.
(17) Sun, Y. M.; Huang, J. J.; Lin, F. C.; Lai, J. Y. Composite poly(2-hydroxyethyl methacrylate) membranes as rate-controlling barriers for transdermal applications. *Biomaterials* 1997, 18, 527–533.
(18) Compañ, V.; Guzmán, J.; Riande, E. A potentiotstatic study of oxygen transmissibility and permeability through hydrogel membranes. *Biomaterials* 1998, 19, 2139–2145.
(19) Yan, T.; Sun, R.; Li, C.; Tan, B.; Mao, X.; Ao, N. Immobilization of type-I collagen and basic fibroblast growth factor (bFGF) onto poly (HEMA-co-MMA) hydrogel surface and its cytotoxicity study. *J. Mater. Sci.: Mater. Med.* 2010, 21, 2425–2433.
(20) Ma, Y.; Hua, M.; Wu, S.; Du, Y.; Pei, X.; Zhu, X.; Zhou, F.; He, X. Bioinspired high-power-density strong contractile hydrogel by programmable elastic recoil. *Sci. Adv.* 2020, 6, No. eabd2520.
(21) Ceylan, E.; Odabaş, M. Novel adsorbent for DNA adsorption: Fe (3+)-attached sporopollenin particles embedded composite cryogels. *Artif. Cells, Nanomed., Biotechnol.* 2013, 41, 376–383.
(22) Ahmed, M.; Punshon, G.; Darbyshire, A.; Seifalian, A. M. Effects of sterilization treatments on bulk and surface properties of nano-composite biomaterials. *J. Biomed. Mater. Res., Part B* 2013, 101, 1182–1190.
(23) Lee, S. C.; Kwon, I. K.; Park, K. Hydrogels for delivery of bioactive agents: a historical perspective. *Adv. Drug Delivery Rev.* 2013, 65, 17–20.
(24) Faucautano, A.; Buttafava, A.; Montanari, L.; Cilurzo, F.; Conti, L.; Genta, I.; Valvo, L. Radiation-induced free radical reactions in polymer/drug systems for controlled release: an EPR investigation. *Radiat. Phys. Chem.* 2003, 67, 61–72.
(25) Naves, A. F.; Motay, M.; Méirindol, R.; Davi, C. P.; Felix, O.; Catalani, L. H.; Decher, G. Layer-by-Layer assembled growth factor reservoirs for steering the response of 3T3-cells. *Colloids Surf., B* 2016, 139, 79–86.
(26) Pires, F.; Ferreira, Q.; Rodrígues, C.; Morgado, J.; Ferreira, F. C. Neural stem cell differentiation by electrical stimulation using a cross-linked PEDOT substrate: Expanding the use of biocompatible conjugated conductive polymers for neural tissue engineering. *Biochim. Biophys. Acta* 2015, 1850, 1158–1168.
(27) Rizwan, M.; Chan, S. W.; Comeau, P. A.; Willett, T. L.; Yim, E. K. F. Effect of sterilization treatment on mechanical properties, biodegradation, bioactivity and printability of GelMA hydrogels. *Biomed. Mater.* 2020, 15, 065017.