

Research Article

Preparation and Characterization of Polyamidoamine G2.0-Hematin as a Biocatalyst for Fabricating Catecholic Gelatin Hydrogel

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In this study, we report that an enzyme-mimicking biocatalyst polyamidoamine (PAMAM) dendrimer G2.0-hematin (G2.0-He) was fabricated successfully. The chemical structure of G2.0-He was verified by ¹H NMR and FT-IR spectroscopy. G2.0-He exhibited a size distribution from 11.6 ± 1.7 nm to 12.5 ± 2.9 nm and a zeta potential from 32.5 mV to 25.6 mV along with the enhancement of the hematin conjugation degree. The relative activity of G2.0-He was evaluated based on pyrogallol oxidation reactions at pH = 7. The results showed that G2.0-He was more stable than horseradish peroxidase (HRP) enzyme in high H₂O₂ concentrations. The HRP-mimic ability of G2.0-He was also confirmed by the catalyzation when preparing catecholic gelatin hydrogels under mild conditions. Moreover, our results also revealed that these hydrogels performed with excellent cytocompatibility in an *in vitro* study and could be used as a potential scaffold for adhesion and proliferation of fibroblast cells. The obtained results indicated that G2.0-He is a suitable platform for altering the HRP enzyme in several biomedical applications.

1. Introduction

Hydrogels are recognized as excellent 3D matrices that can mimic cell and tissue culture environments; thus, hydrogels have become a tremendous motivator in biomedical research fields [1–4]. Because of the following requirements for tissue regeneration, namely, biocompatibility, biodegradability, and versatile applications, various injectable hydrogel systems have been introduced. Recently, phenolic polymer-based hydrogels have been extensively studied [3–5]. An interesting aspect of a hydrogel is its injectability; a hydrogel system could be *in situ* formed after injecting the polymer solution into a defective site [5]. Currently, the application of a perox-

idase enzyme in the preparation of injectable hydrogels is suggested as an attractive technique [5]. The peroxidase enzyme could accelerate the oxidation of phenolic hydroxyl groups to form a chemical crosslinking, subsequently resulting in the formation of a hydrogel network at the desired time that is suitable for implantation surgeries [5, 6]. Among them, horseradish peroxidase (HRP) is one of the most commonly used enzymes for fabricating a phenolic gelatin hydrogel [6, 7]. The change in the oxidation stage of hem groups (the active site of HRP) in the presence of an oxidant agent (H₂O₂) induces the formation of free radical phenolic substrates leading to the crosslinking of the aromatic ring by C-C and C-O coupling, and ultimately leading to hydrogel

formation [8–12]. This is the exquisite action of the HRP enzyme in the preparation of the phenolic gelatin hydrogel; however, it is a fact that the HRP enzyme becomes quickly inactivated during phenol oxidation and polymerization due to reaction conditions (e.g., an active site intermediate compound reacts with excess H_2O_2) [13]. In addition, the HRP enzyme suffers from some intrinsic drawbacks, such as high cost; difficulty in mass production, purification, and storage; low stability; and sensitivity towards atmospheric conditions, which in turn result in limited availability for a wide usage in clinical applications [13, 14]. Consequently, the pursuit of alternative platforms that can accomplish those challenges has brought the introduction of various enzyme mimics, also called mimicking enzymes.

It has been found that hematin, a hydroxyferritoporphyrin obtained through the decomposition of hemoglobin with one of the axial coordination sites of the Fe (III) center, has the analog structure of the prosthetic iron protoporphyrin IX found in the active site of HRP [15]. Various studies have explored and proved the potential of hematin as a prospective candidate for playing a catalytic role to the HRP enzyme [15–20]. However, the usage of hematin as a catalyst for phenolic hydrogelation has been restricted due to its low solubility and aggregation at neutral or acidic pH. Previously, a study had been conducted to dissolve hematin in alkaline buffer at pH = 10.0, subsequently neutralized to 7.4 for further uses; however, its applications were partially limited [17]. Therefore, different functionalization strategies, such as chitosan-g-hem [17], poly(ethylene glycol)-hematin-catalyzed (PEG-hematin) polyaniline (Pani) [18], or even its inclusion into poly(ethylene glycol)-block-poly(4-vinylpyridine) (PEG-b-P4VP) micelles [19], have been attempted.

Motivated by these studies, we aim to synthesize a soluble hematin-modified generation 2.0 polyamidoamine dendrimer (G2.0-He). In the past two decades, dendrimers have emerged as a novel class of polymeric materials, thanks to their nano-sized platforms, high solubility, and monodispersed properties [20]. These characteristics give them a branched architecture that enables them to flexibly modify themselves in numerous ways [21]. It is well known that cationic PAMAM dendrimers exhibit several advantages in controlling drug and protein delivery properties, such as their high drug-loading capacity, highly electrostatic interaction with anionic bioactive molecules, enhanced hydrophobic and hydrophilic drug solubility and bioavailability, and easy functionalization of their external groups [22]. In addition, the high density of peripheral NH_2 groups in cationic PAMAM dendrimers could enhance the possibility of chemical covalent formation between dendrimers and hematin [23]. It can be expected that the combination of hematin and PAMAM G2.0 could overcome the intrinsic drawbacks of both because of their partner's outstanding properties. Therefore, we evaluated the activity of G2.0-He as an alternative catalyst to substitute the HRP enzyme in the *in situ* formation of a catecholic gelatin hydrogel.

2. Experimental Procedure

2.1. Materials. Hematin (MW 633.49 g/mol), 3,4-dihydroxyphenethylamine hydrochloride, horseradish peroxidase

(HRP, 216 U·mg⁻¹), guaiacol ($\text{C}_7\text{H}_8\text{O}_2$, MW 124.14 g/mol), and 1,2,3-trihydroxybenzene (pyrogallol) were purchased from Sigma-Aldrich (USA). Dimethylsulfoxide (DMSO) was purchased from Merck Chemicals GmbH (Darmstadt, Germany). Hydrogen peroxide (H_2O_2), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, MW 191.71 g/mol), and N-hydroxysuccinimide (NHS) were purchased from Acros Organics (USA). PAMAM dendrimer G2.0 and catecholic gelatin (Gel-Dop) were prepared at the Institute for Applied Materials Science. All other chemicals are of the analytical grade.

For the cell study, human fibroblast cells were purchased from ATCC (USA). The culture media Dulbecco's Modified Eagle's Medium (DMEM) high glucose, fetal bovine serum (FBS), trypsin-EDTA (0.25%), and penicillin-streptomycin (10,000 U/mL) were purchased from Sigma-Aldrich (Singapore). Phosphate-buffered saline (PBS, 1x) was purchased from Gibco. Other culture wells were ordered from Corning. Cell culture flasks and plates were ordered from Corning. The cytotoxicity assay was performed with sulforhodamine B (SRB) assay (Ab235935) and dual-staining Live/Dead assay via acridine orange (AO, Alfa Aesar, USA) and propidium iodide (PI, Sigma-Aldrich).

2.2. Synthesis of PAMAM Dendrimer G2.0-Hematin.

PAMAM dendrimer G2.0-hematin was prepared using the carbodiimide coupling reagent as illustrated in Figure 1. Firstly, hematin (100 mg, 0.16 mmol) was dissolved in 50 mL DMF, and G2.0 was dissolved in 50 mL methanol according to the molar ratio: G2.0 – He = 1 – 1; 1 – 2. The excess EDC/NHS (molar ratio: hematin/EDC/NHS = 1/2/2) was employed to activate the carboxylic group of hematin, which subsequently reacted with the amine group of PAMAM G2.0. Then, hematin/EDC/NHS was added dropwise into the G2.0 solution under stirring. The reaction was kept at room temperature under nitrogen for 24 h in the dark. After that, the product was dialyzed against methanol using a membrane with a molecular weight cut-off (MWCO) of 1,000 Daltons until the unreacted hematin was completely removed. Vacuum distillation was used to eliminate methanol at 45°C. The products were redissolved in DI water and finally lyophilized to get G2.0-He without impurities [17]. G2.0-He was characterized by ¹H NMR and Fourier transform infrared (FT-IR) spectroscopy. Ultraviolet-visible (UV-Vis) spectroscopy was used to quantify hematin conjugated on the PAMAM G2.0 surface. Via the absorption spectrum of hematin, the λ_{max} at 404 nm was defined and used to calculate the conjugation degree [24]:

$$\text{Conjugation} = \frac{\text{weight of He in G2.0-He}}{\text{weight of the feeding He}} \times 100\%. \quad (1)$$

Furthermore, dynamic light scattering (DLS, SZ-100 nanoparticle series instruments, Horiba) was measured to determine the hydrodynamic size and the zeta potential of G2.0-He.

2.3. Preparation of Catecholic Gelatin (Gel-Dop). The preparation of Gel-Dop was conducted following Fu et al.'s process

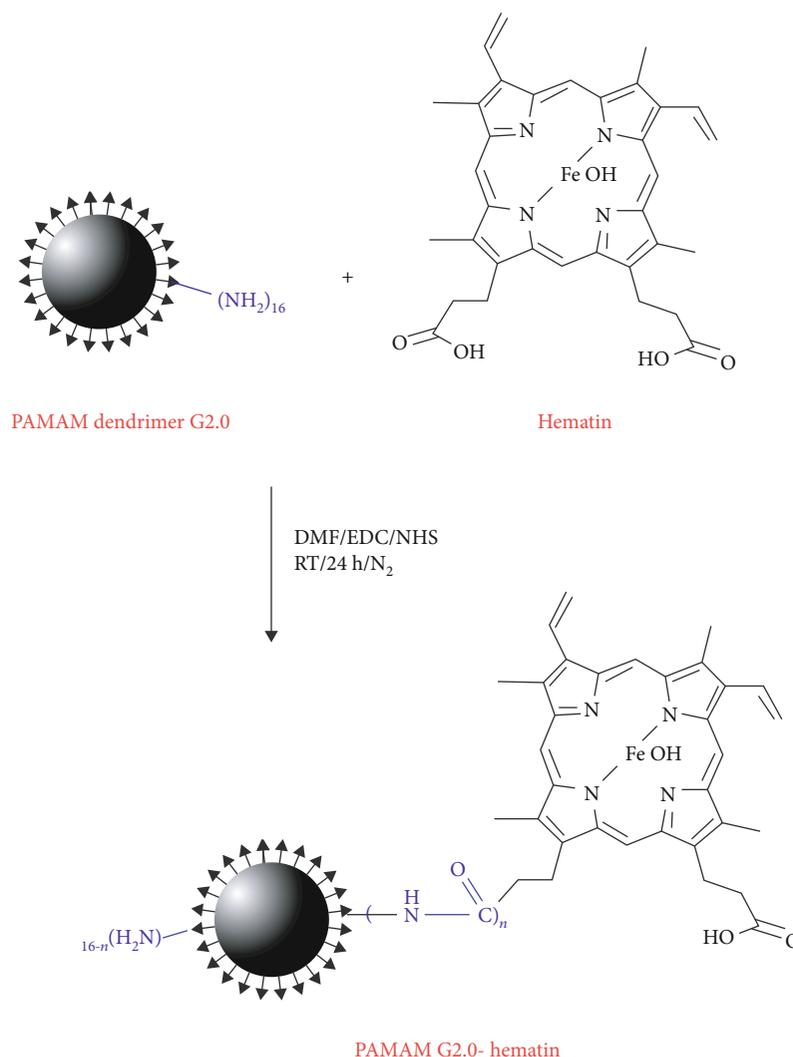


FIGURE 1: Schematic illustration of the of PAMAM dendrimer G2.0-hematin preparation.

[25]. Briefly, gelatin (2 g) was dissolved in 100 mL of DI water under stirring at 50°C, followed by the precise addition of dopamine solution. After 30 minutes, EDC (0.5 g, 0.00261 mol) and NHS (0.5 g, 0.00434 mol) were added to the mixture. The pH value was maintained at 5.5 during the reaction for 24 h at room temperature. The reaction solution was later dialyzed in DI water for 3 days before lyophilization. The product was verified using proton nuclear magnetic resonance spectroscopy (¹H NMR).

2.4. Catalytic Activity on Pyrogallol. The catalytic activity of G2.0-He at pH = 7.0 was evaluated via pyrogallol oxidation reaction using UV-Vis spectroscopy. A mixture containing 700 mg/L pyrogallol and 50 mg/L G2.0-He, H₂O, and H₂O₂ for a total volume of 3.5 mL was added into a quartz cell [17]. The concentration of H₂O₂ that was investigated varied from 0.1 to 100 mM [15]. HRP (0.01 U/mL) was used as a control. The increasing absorbance was recorded at 420 nm after 180 s since the addition of H₂O₂. The tracked absorbance was used to calculate the relative activity of G2.0-He and HRP at each H₂O₂ concentration, respectively [17].

The catalytic activities were exhibited as the median values of three measurements.

2.5. Gelation Time Test. In this study, different concentrations of Gel-Dop and H₂O₂ were used to obtain the optimal condition for the gelation time test of the hydrogels. Basically, the hydrogels were prepared by dissolving Gel-Dop and G2.0-He in pH 7.0 solutions, and the gelation time was counted from the time hydrogen peroxide (H₂O₂) was added to the solutions until nonflowing gel behavior was observed. After a series of experiments to study the effect of Gel-Dop and H₂O₂ concentrations on the gelation time, the Gel-Dop solutions and H₂O₂ concentrations were fixed at 10 wt% and 12 mM, respectively, while the concentration of G2.0-He varied from 0.2 to 1.2 wt%. Then, the optimal condition would be chosen to examine the effect of H₂O₂ concentration. For the HRP enzyme, its concentration was changed from 0.0002 to 0.001 wt% in the solutions containing 10 wt% Gel-Dop and 12 mM H₂O₂ [26]. Experiments were performed three times at each condition, and the data was expressed by mean ± SD.

2.6. Cytotoxicity Test. The toxic profiles of the G2.0-He-mediated Gel-Dop hydrogels were determined using an indirect method, which was previously described by Zhang et al. [27]. Human fibroblast cells were seeded into 96-well plates with a density of 2×10^4 cell/well. The cells were incubated at 37°C, 90% humidity, and 5% CO₂ condition. After an incubation time of 24 h allowing cells to be adherent, a newly completed DMEM (10% FBS, 1% penicillin-streptomycin) containing a series concentration of leachates of hydrogels (50%, 20%, and 10% *v/v* corresponding to 0.5 mg/mL, 0.2 mg/mL, and 0.1 mg/mL, respectively) was added, and cells were incubated for another 4 h and 24 h. Along with the extracted Gel-Dop hydrogel, the cytotoxicity of G2.0-He (with the same concentration used in hydrogelation) as well as the buffer (PBS 1x) used to extract hydrogel were also involved in this experiment. The untreated cells incubating with the completed DMEM only was used as the negative control, while the cell culture with doxorubicin (0.1 mM) was considered as the positive control. At the designed time (4 h and 24 h), the SRB kit (Ab235935) was applied in each well following the guidance of the manufacturers. The percentage of viable cells was calculated regarding untreated cells. All the experiments were repeated with three independent replications.

We also performed another cytotoxicity assay for validation using AO/PI dual staining. In this study, the fibroblast cells (2×10^5 cells) were cultured in a 35 mm culture dish (Thermo Fisher Scientific™ Nunc). After 24 h of incubation time similar with the previous condition, the culture medium was removed and the new culture medium containing the leachates of hydrogels (50% *v/v*), G2.0-He (100 ppm), and PBS 1x was then added into each culture dish. These culture dishes were incubated at 37°C, 90% humidity, and 5% CO₂ for a further 24 h. Afterward, 2 μ L working solution (AO/PI) was added to each dish and incubated for 15 minutes in a CO₂ incubator. PBS 1x was used to remove the free dye in the media, and the newly completed media was supplied prior to microscope observation. Confocal (Dragonfly, Oxford Instrument, England) was used to observe the morphology and evaluate the live and dead cells through dual channels (525 nm and 617 nm).

3. Results and Discussion

3.1. Characterization of G2.0-He and Gel-Dop

3.1.1. ¹H NMR and FT-IR Spectrum of G2.0-He. Synthesis of G2.0-He was carried out by conjugating the carboxylate groups of hematin and the amine groups of PAMAM G2.0 using EDC/NHS. Successful synthesis of G2.0-He was verified by ¹H NMR and FT-IR spectroscopy. The ¹H NMR spectrum (Figure 2(a)) of G2.0-He exhibits the typical proton peaks of PAMAM G2.0 at $\delta \sim 3.717$ ppm (a) ($-\text{CH}_2-\text{CH}_2-\text{NH}-$) and $\delta \sim 2.837$ ppm (b) ($-\text{CH}_2-\text{CH}_2-\text{NH}_2-$) [28]. The spectrum shows the signal at $\delta \sim 1.877$ ppm belonging to the methyl protons in the hematin structure (c) [29]. Figure 2(b) reveals the correlative characterizations of G2.0-He in a comparison with G2.0 and hematin, confirming the successful conjugation of G2.0-He. The FT-IR spectrum of

G2.0-He (1-1) exposes the vibration of the porphyrin ring of heme at a range from 1000 to 1650 cm⁻¹. In addition, ring stretching vibrations mixed strongly with C-H in-plane bending at 1031 cm⁻¹ and 1439 cm⁻¹. The C-N stretching mode of amide III is at 1255 cm⁻¹, and for CH₃, the deformation of C-H and N-H is at 1384 cm⁻¹. Amide II bands arise from C-N stretching and CHN bending vibrations at 1544 cm⁻¹ [30, 31]. For amide band I, the “footprint” for the generation of G2.0-He, the C=O stretching vibration of the amide C=O is predominantly shown at 1651, 1661, and 1727 cm⁻¹. C-H stretching vibrations of methyl (CH₃) and methylene (CH₂) groups are exhibited at the range of wavenumbers from 2800 to 3100 cm⁻¹. Also, the stretching O-H asymmetric structure in the remaining carboxylate groups of hematin appears at 3454 cm⁻¹ [31]. Besides, the FT-IR spectrum of G2.0-He (1-2) displays the correlation to G2.0-He (1-1) as well. Generally, the ¹H NMR and FT-IR data are consistent in consolidating the successful synthesis of PAMAM dendrimer G2.0-hematin.

3.1.2. ¹H NMR Spectrum of Gel-Dop. The ¹H NMR spectrum of Gel-Dop (Figure 2(c)) appears as signals of the aromatic ring of dopamine at $\delta \sim 6.822$ ppm (a), $\delta \sim 6.757$ ppm (b), and $\delta \sim 6.594$ ppm (c). The peaks at $\delta \sim 3.274$ ppm (d) and $\delta \sim 2.745$ ppm (e) are attributed to the protons of ($-\text{C}-\text{CH}_2-$) and ($-\text{CH}_2-\text{NH}-$), respectively [32]. Besides, the spectrum exposes the resonance peaks of aromatic protons of phenylalanine and other typical protons of amino acids in gelatin at 7.340-7.406 ppm, and the signals at 0.9-4.6 ppm are assigned to the alkyl protons of gelatin [33]. Regarding the results, the successful conjugation of dopamine onto the gelatin backbone was verified for use in preparing catecholic gelatin hydrogels.

Figure 3(a) displays the UV-Vis spectra of 3 ppm hematin, G2.0-He (1-1), and G2.0-He (1-2) in DMSO with the absorbance peak of 404 nm; thus, the conjugation degree of hematin was measured and calculated at this absorbance peak. The conjugation of G2.0-He (1-1) reaches 86%, which is about 22% higher than that of G2.0-He (1-2) (Figure 3(b)); hence, G2.0-He (1-1) was preferred to investigate the effect of catalytic ability when comparing to the HRP enzyme in forming catecholic gelatin hydrogels. The difference in the conjugation of G2.0-He (1-1) and G2.0-He (1-2) can be explained by the impactation of the steric effect of loaded hematin and the effect of hematin on the entrapment efficiency.

The combination of PAMAM G2.0-hematin brings benefits in two directions: improvement in hematin solubility and the toxicity minimization of PAMAM G2.0. Given the presence of amine groups on the PAMAM G2.0 surface, hematin-covered PAMAM G2.0 yields higher solubility compared to pure hematin. In addition to this, hematin-covered PAMAM G2.0 also solves the membrane disruption problem caused by the positive charge of these amine groups. Here, we investigated G2.0-He with four molar ratios (1-1, 1-2, 1-4, and 1-6). According to our results, G2.0-He (1-1) and (1-2) were well soluble, while the others were precipitated. Thus, G2.0-He (1-1) and (1-2) were selected for further observation and investigation. As shown in Figure 4, generally, the more

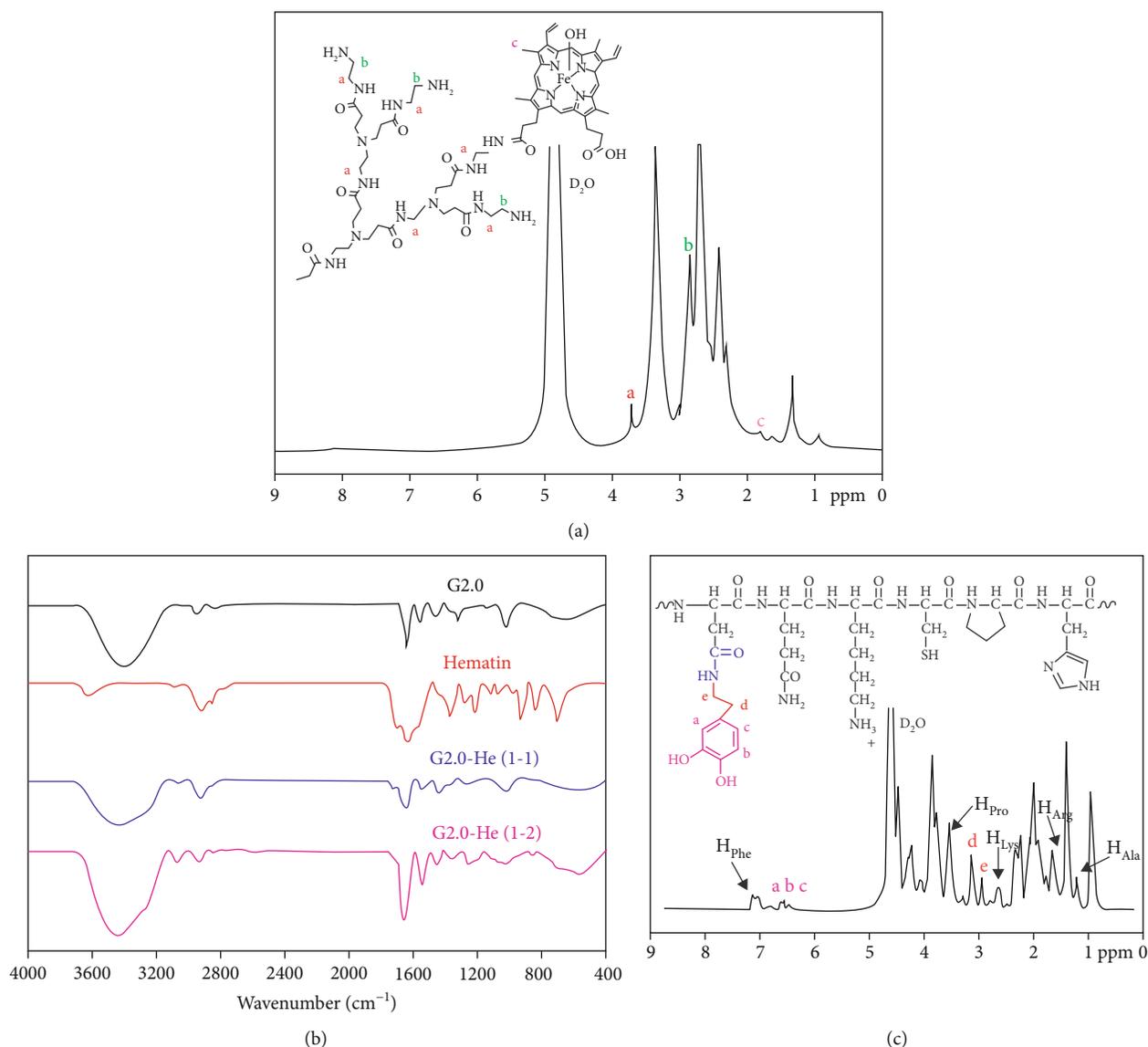


FIGURE 2: ¹H NMR spectra of G2.0-He (a); FT-IR spectra of PAMAM G2.0, hematin, and G2.0-He (b); ¹H NMR spectra of Gel-Dop (c).

hematin attached to PAMAM G2.0, the higher size distribution and the lower the zeta potential obtained. Specifically, the median size distribution values of PAMAM G2.0, G2.0-He (1-1), and G2.0-He (1-2) are 7.5 ± 1.3 nm, 11.6 ± 1.7 nm, and 12.5 ± 2.9 nm, respectively; and their median zeta potential values are 35.4 mV, 32.5 mV, and 25.6 mV, respectively. According to our results, it is suggested that the increment of hematin conjugated onto the PAMAM G2.0 surface decreases the number of amine groups leading to steric hindrance, thereby, resulting in charge balance and size enlargement.

3.2. HRP-Mimic Activity of G2.0-He. The HRP-mimic catalytic activity of G2.0-He was investigated via oxidation of pyrogallol in comparison to native HRP under H₂O₂ conditions. The courses of the wavelength of the oxidation of pyrogallol/H₂O₂ catalyzing by G2.0-He in physiological buffer pH 7.4 were indistinguishable from those of native HRP.

Impressively, the spectrum of the oxidative reaction of pyrogallol/H₂O₂ with the help of G2.0-He shows the appearance of absorbance at 420 nm corresponding to the characteristic peak of the oxidized product of pyrogallol [13] (Figure 5), verifying that G2.0-He has a peroxidase-like function.

Furthermore, the H₂O₂-concentration-dependent absorbance change for both G2.0-He and native HRP enzyme was investigated. As shown in Figures 5(a) and 5(b), the absorbance of pyrogallol/H₂O₂/G2.0-He solutions at $\lambda = 420$ nm increased in accordance with the increase of H₂O₂ concentrations. With the native HRP enzyme, the catalytic courses of the oxidation of pyrogallol were divided into two parts following the change of H₂O₂ concentration. The characterized peak of purpurogallin at $\lambda = 420$ nm increased the absorbance values when H₂O₂ concentration increased from 2 mM to 10 mM (Figure 5(c)). However, the addition of 30 mM H₂O₂ to the pyrogallol oxidation catalyzed by the native HRP enzyme decreased the signal of the pyrogallol

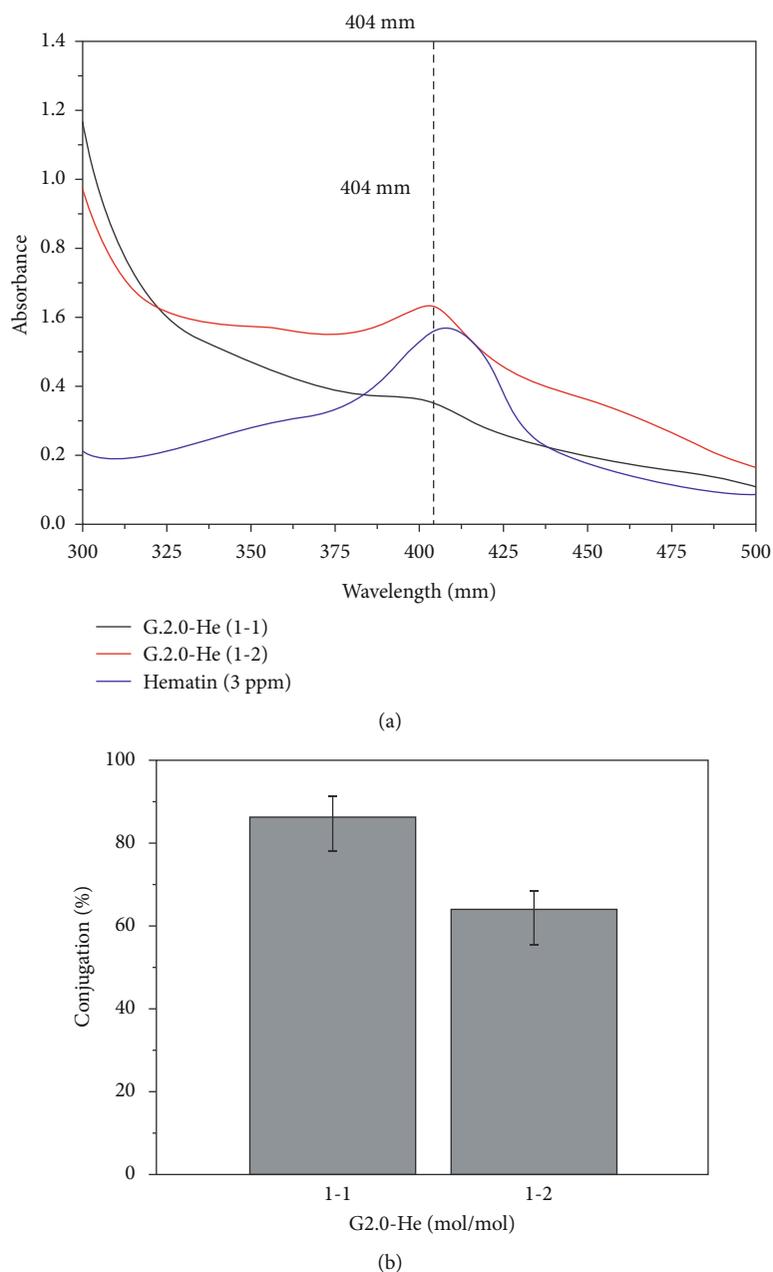


FIGURE 3: UV-Vis spectra of hematin and G2.0-He (a) and conjugation of G2.0-He (b).

oxidation production. The level of the oxidative reaction also dwindled when H_2O_2 concentration was further increased to 100 mM. The comparison of the relative activity of G2.0-He and the native HRP in the function of H_2O_2 is displayed in Figure 5(d). The relative activity of the HRP enzyme increased from 82% to 100% along with the increasing H_2O_2 concentration in the range from 2 to 10 mM, while it significantly decreased when the H_2O_2 concentration was higher than 10 mM. These results express that the HRP enzyme suffers inactivation when the concentration of H_2O_2 is further increased up to 30 mM, which is consistent with previous studies [15, 17]. G2.0-He was found to possess outstanding H_2O_2 stability over a wide range of peroxide concentrations from 2 to 100 mM. The relative catalytic

increase from 19% in 2 mM H_2O_2 to 85% in 50 mM H_2O_2 then reaches approximately 100% in 100 mM H_2O_2 . The reason for this could be derived from the intrinsic peroxidase property of hematin molecules. Consequently, G2.0-He is available in a wide range of applications given its high stability.

3.3. G2.0-He-Mediated Gelation of Catecholic Gelatin Hydrogel. Gel-Dop, a polymer with phenol functionalities, can be crosslinked by HRP/ H_2O_2 or G2.0-He/ H_2O_2 systems. As a result of assuming these systems, phenol radicals will be produced, then they will be coupled with each other, either at the C-C or C-O positions, and crosslinks will be formed between the polymer chains (Figure 6(a)). Figure 6(a) shows

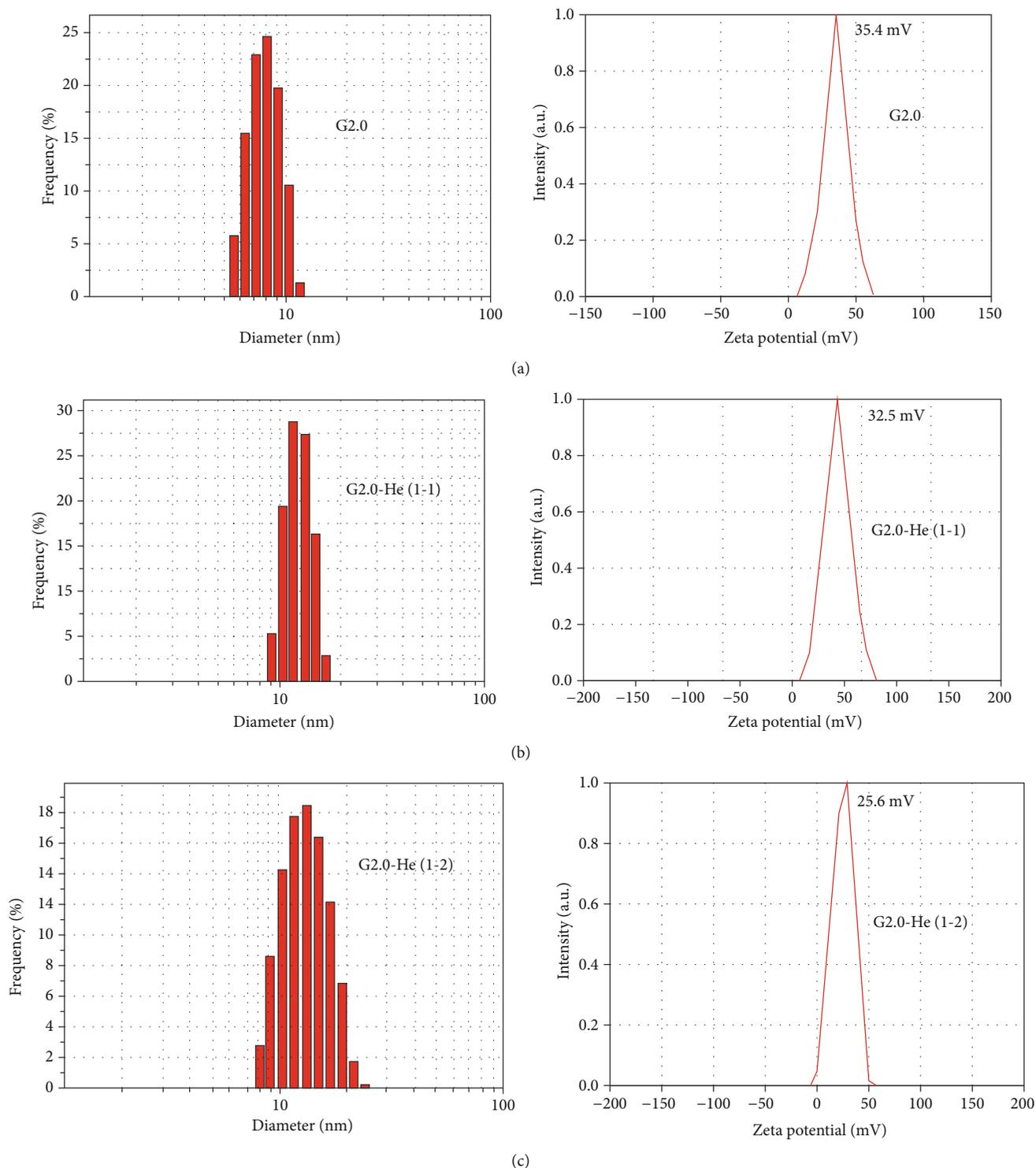
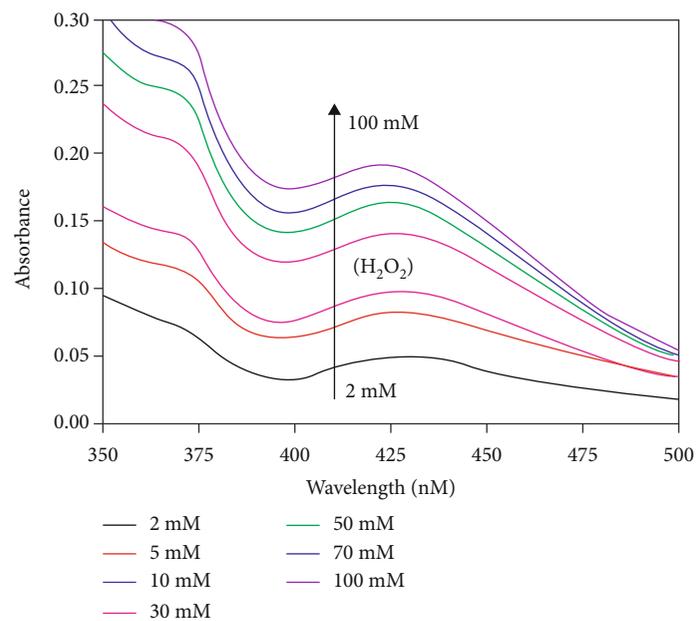


FIGURE 4: Size distribution and zeta potential: PAMAM G2.0 (a), G2.0-He (1-1) (b), and G2.0-He (1-2) (c).

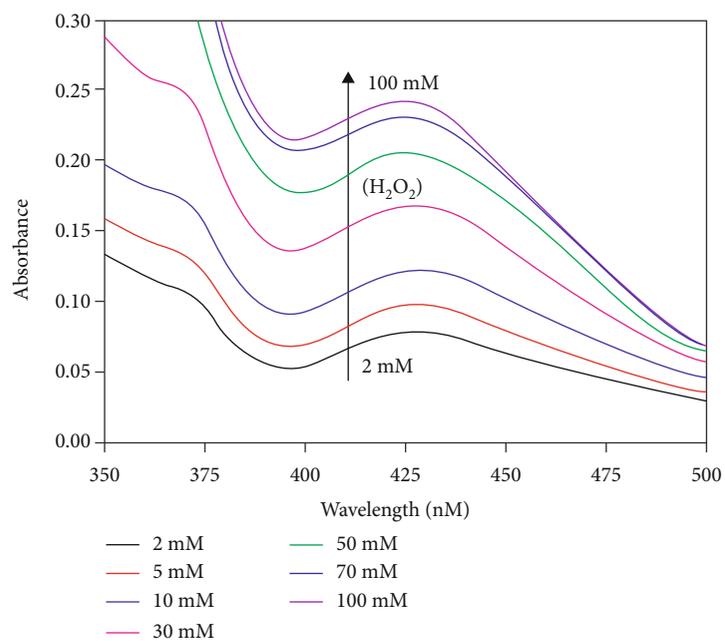
a photograph of a Gel-Dop/G2.0-He solution before and after the *in situ* gelation. Before the addition of a catalyst (HRP or G2.0-He), the solution remains in a liquid state and flows when tilted. After brief incubation with catalyst, the solution turns into a nonflowing state.

To study the effect of catalyst concentration, the gelation of Gel-Dop in the presence of various concentrations of HRP

and G2.0-He (1-1) was examined. The results show that the gelling rate increases with increments of catalyst concentrations. For the HRP enzyme, the time to complete the gelation of 10 wt% Gel-Dop was 271 s at 12 mM H_2O_2 and 0.0002% HRP. The gelation time decreased from 217 ± 8 s to 15 ± 2 s following increasing HRP concentrations of up to 0.001% (Figure 6(b)). Similar to HRP, the enhancement of G2.0-He

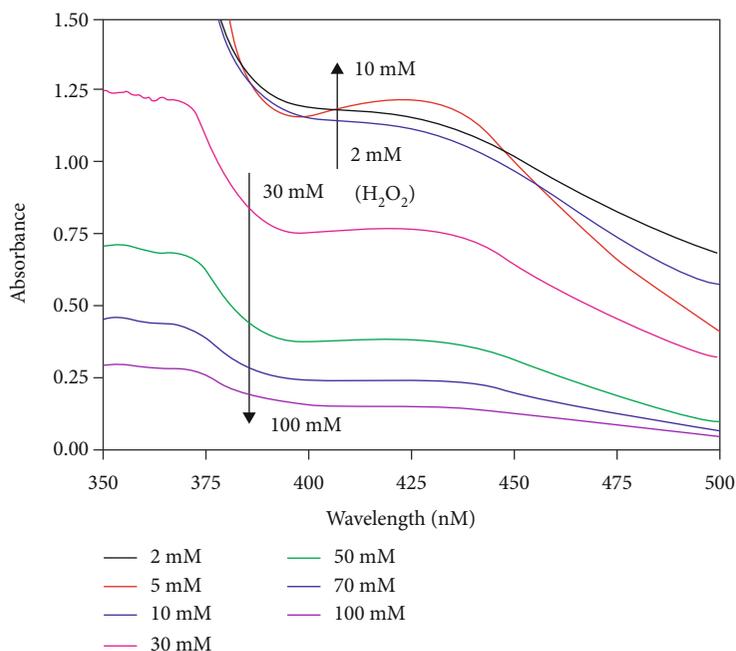


(a)

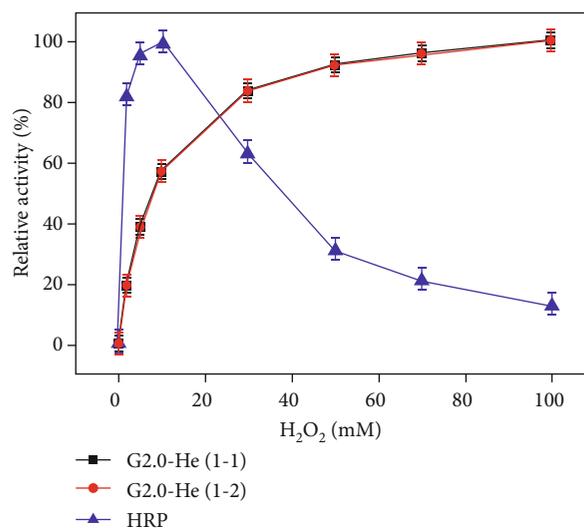


(b)

FIGURE 5: Continued.



(c)



(d)

FIGURE 5: The absorbance of G2.0-He (1-1) (a), G2.0-He (1-2) (b), and HRP (c) and the relative activity of G2.0-He (1-1) and (1-2) and HRP (d) for the pyrogallol oxidation reactions with the H_2O_2 concentration varying from 2 to 100 mM.

concentration from 0.03 to 0.12 wt% results in a decrease of gelation time from 695 ± 28 s to 45 ± 8 s (Figure 6(c)), in which the gelation time significantly declined in the early state (when the concentration of G2.0-He was from 0.03 to 0.09 wt%) and then slightly changed when G2.0-He (1-1) concentration increased to 1.2 wt%, as shown in Figure 6(c). However, at the same oxidative reagent concentration of 12 mM H_2O_2 , it required Gel-Dop catalyzed by G2.0-He a longer time to complete the crosslinking process than HRP. A higher amount of G2.0-He (above 0.07% v/v) was needed to reach similar activity levels when induced by the native HRP enzyme. Because the catalytic activity of G2.0-He is based on the concentration of H_2O_2 , the effect

of H_2O_2 on the gelation of Gel-Dop catalyzed by G2.0-He was investigated. As expected, the gelation time decreased from 150 ± 12 s to 55 ± 4 s when H_2O_2 concentration increased from 3 to 12 mM; the fastest gelation time in this examination was approximately 28 ± 3 s in 30 mM H_2O_2 (Figure 6(d)). According to previous studies [15, 16], a higher amount of H_2O_2 (above 30 mM) was used to boost the catalytic activity of hematin. It has been noted that using a higher amount of H_2O_2 to control the gelation time of phenol motif polymers such as Gel-Dop brings with it a remarkable risk to the health of the cells, thereby, limiting the widespread use of hematin in biological hydrogels [4]. In this study, aside from controlling gelation time by adjusting H_2O_2 concentration,

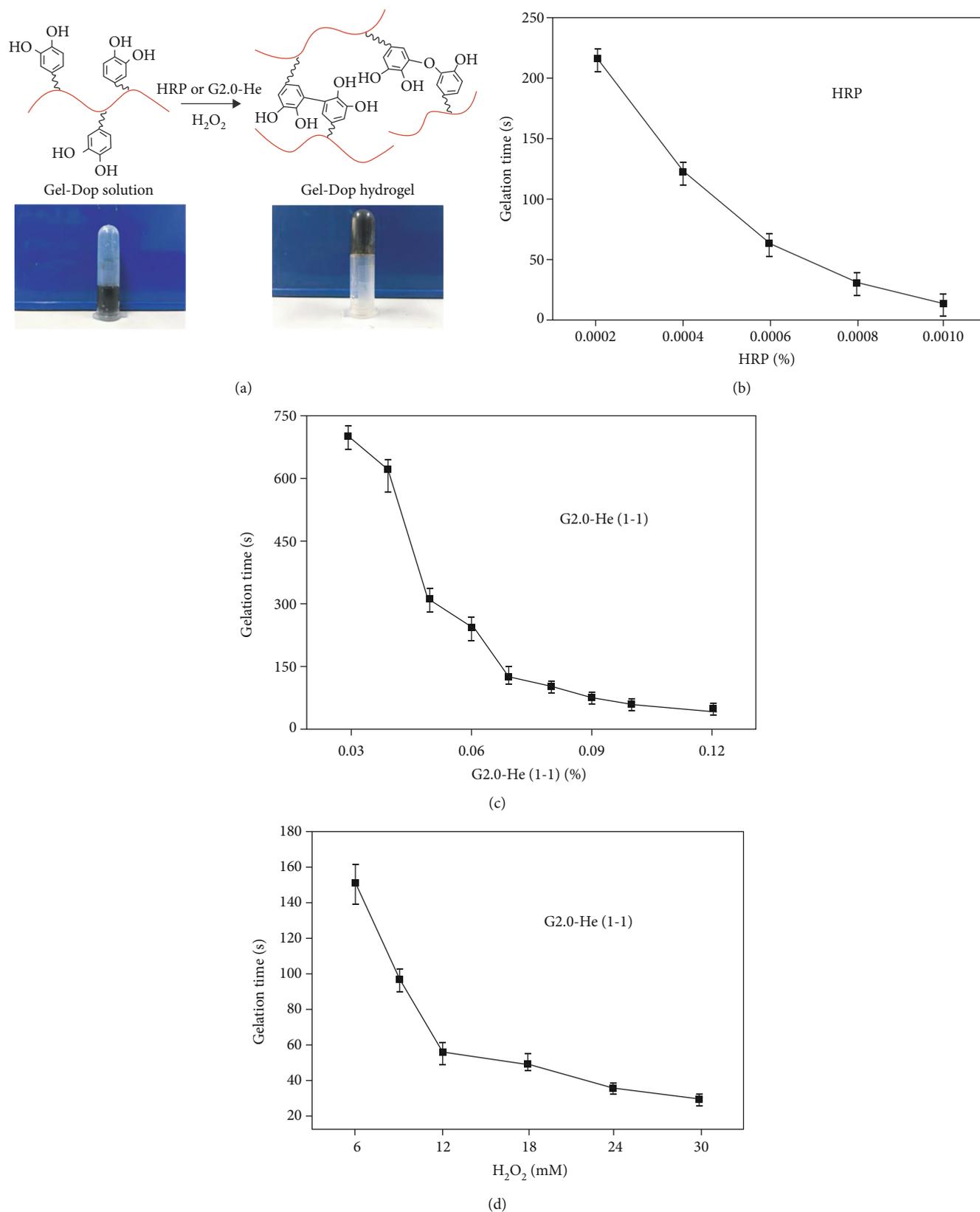


FIGURE 6: Schematic illustration of Gel-Dop hydrogel formation and photographs of the Gel-Dop/G2.0-He solution before and after gelation (a). The gelation time for forming Gel-Dop hydrogels catalyzed by HRP in the function of enzyme concentration (b) and by G2.0-He (1-1) in the change of either G2.0-He (c) and H_2O_2 concentration (d).

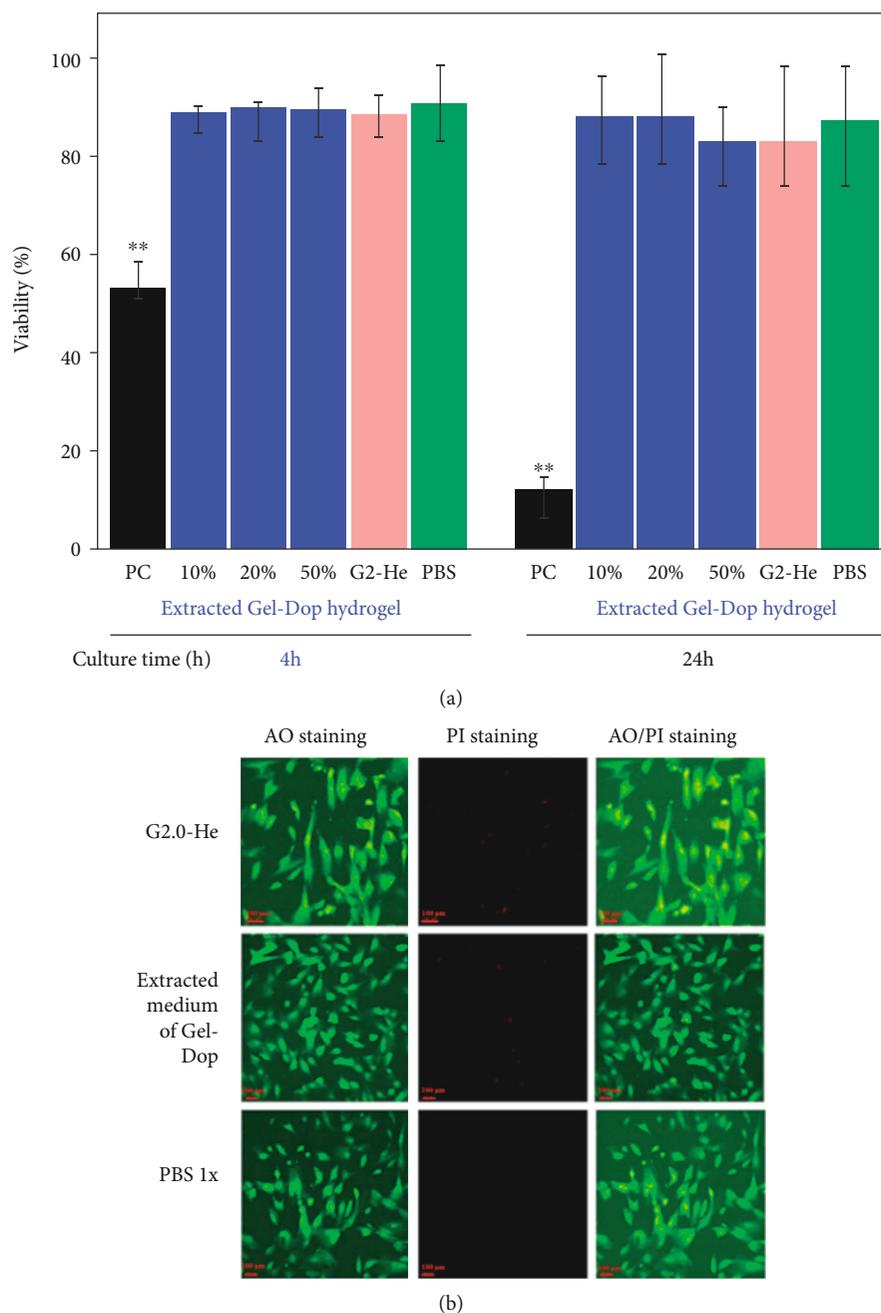


FIGURE 7: Cytotoxicity evaluation of instituting or forming Gel-Dop hydrogels through G2.0-He under H_2O_2 conditions. (a) Viability (% with the cell culture in DMEM) of human fibroblast cells incubated with various concentrations of Gel-Dop hydrogel extract, G2.0-He, positive control (doxorubicin 0.1 mM), and PBS 1x using prepared samples for 4h and 24h. (b) Fluorescence image of human fibroblast cells cultured with G2.0-He, 50% extracted medium of Gel-Dop, and PBS 1x for 24h. Data are presented as mean \pm SEM of three independent replicated results. ** $p < 0.01$.

the gelation time could also be controlled by varying G2.0-He concentrations. The maximum concentration of hematin in the same study was around 0.08% *w/w*, whereas, in this study, the concentration of G2.0-He could be up to 1.2 wt%. Hence, our findings support the application of hematin as HRP mimics in biotechnology, specializing in biohydrogelation.

To prove the potential application of Gel-Dop hydrogel formation with the help of G2.0-He as a scaffold for cell stud-

ies, the cytotoxicity of the obtained Gel-Dop was investigated using human fibroblast cells by SRB assay and Live/Dead staining assay. Human fibroblast cells were incubated with different concentrations of Gel-Dop hydrogel extract (10%, 20%, and 50% *v/v*), and their cytotoxicity was then compared to G2.0-He, positive control (doxorubicin, 0.1 mM), and PBS 1x (a solution used to extract hydrogels for the first time). After the first 4h of coculture with the positive control, the viability of cells were reduced to half as compared to

untreated cells, about $53.39 \pm 6.01\%$ (Figure 7(a)). As the culture time was prolonged, the viability of fibroblast cell culturing with doxorubicin plunged to $12.14 \pm 2.66\%$. On the contrary, there was no significant difference observed in % viability of cells with the Gel-Dop hydrogel extract (10%, 20%, and 50% v/v), G2.0-He, and PBX 1x (Figure 7(a)); levels of the viability of fibroblast cells were approximately 90% for untreated cells after 4 h or 24 h culture ($p > 0.01$). Likewise, the data from the Live/Dead staining assay with AO/PI was consistent with the SRB assay (Figure 7(b)). Fibroblast cells were alive (green cells, >90%), and an unremarkable red fluorescent signal (the death marker) was observed in 50% extracted hydrogel, G2.0-He, and PBS 1x. Altogether, the Gel-Dop hydrogelation using G2.0-He has almost no adverse impact on cell viability suggesting that it can be potentially used as a scaffold in tissue regenerated application.

4. Conclusion

In conclusion, this study was successful in preparing PAMAM dendrimer G2.0-hematin, which can overcome the poor aqueous insolubility of hematin. Besides, our synthesized G2.0-He exhibits the same catalytic activity as the HRP enzyme but is more stable in a high H_2O_2 concentration condition. Therefore, G2.0-He could be employed in fabricating hydrogels based on its catalytic ability, similar to the HRP enzyme, and these hydrogels are highly cytocompatible and able to serve as an excellent scaffold. Overall, according to our findings, G2.0-He could be considered an efficient horseradish peroxidase mimetic catalyst for biochemical analysis and fabrication of biomedical materials.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that no conflict of interest exists regarding the publication of this paper.

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