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ARTICLE *in* BIOMACROMOLECULES · AUGUST 2010

Impact Factor: 5.75 · DOI: 10.1021/bm100623k · Source: PubMed

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Hematin is an Alternative Catalyst to Horseradish Peroxidase for In Situ Hydrogelation of Polymers with Phenolic Hydroxyl Groups In Vivo

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Received June 5, 2010; Revised Manuscript Received July 2, 2010

Hematin, an iron-containing porphyrin used in the management of porphyria attacks, was evaluated as an alternative catalyst to horseradish peroxidase (HRP) for in situ gelation of polymers with phenolic hydroxyl (Ph) moieties in vivo. An aqueous solution of gelatin derivative with Ph moieties was gellable in the presence of both hematin and H_2O_2. A total of 98.6% adhesion of L929 fibroblast cells 4 h after seeding and their similar morphology to those on substrate coated with unmodified gelatin indicated no obvious substrate cytotoxicity. High cytocompatibility of the gelation process under conditions inducing gelation within 20 s was demonstrated by 95.0% viability of enclosed cells in vitro. Furthermore, no adverse effects of hematin were found compared with HRP by histological observation of cutaneous tissue surrounding in situ formed gels. The versatility of hematin for gelation of a variety of polymers possessing Ph groups was demonstrated by the gelation of a carboxymethyl cellulose derivative.

Introduction

In situ fabrication of hydrogels in vivo provides advantages for a variety of biomedical applications such as tissue engineering and drug delivery.[1] For example, therapeutic molecules and cells containing hydrogels can be placed in any shape of defect with less surgical trauma by injecting as pregelled solution compared with implantation of preformed hydrogels. An absolute requirement of in vivo applications is that the gelation conditions are mild and compatible with surrounding tissues, that is, harmless for living mammalian cells. Recently, enzymatic gelation has attracted attention as an effective route for fabricating hydrogels under mild conditions for mammalian cells.[2−6] In particular, the horseradish peroxidase (HRP)-catalyzed gelation system has been under intense study after the report by Kurisawa et al. of the subcutaneous gelation of aqueous solution containing a hyaluronic acid derivative possessing phenolic hydroxyl (Ph) moieties.[5] HRP catalyzes the oxidation of donors using H_2O_2, resulting in polyphenols linked at the aromatic ring by C−C and C−O coupling between Ph moieties. The effectiveness of the gelation system has been demonstrated for a variety of polymers possessing Ph moieties derived from biocompatible polysaccharides[7−10] and proteins.[11,12] Despite reported results indicating the feasibility of the gelation system, a concern for future clinical application is the potential risks of using HRP of plant origin in vivo. As far as we know, HRP is not available for clinical use as a safe material for administration in vivo.[13]

In the present study, we aimed to evaluate the feasibility of hematin as an alternative catalyst to replace HRP for in situ gelation of polymers with Ph moieties. Hematin obtained through decomposition of hemoglobin is a Fe(III) compound (Figure 1) with a structure similar to the prosthetic iron protoporphyrin IX found in HRP. One of the axial coordination sites of Fe(III) is occupied by a hydroxide ion instead of the histidine residue found in HRP.[14] Hematin has been used as a catalyst for the oxidative polymerization of phenol compounds.[15,16] One attractive point of hematin for in situ gelation in vivo is that the material has already been used clinically as a safe material to treat porphyric attacks.[17−19] Following issues at least should be satisfied for using hematin as a catalyst for in situ hydrogelation in vivo: (1) Gelation resulting from hematin-catalyzed reaction is accomplished in the short enough period to form hydrogels at the injected sites. (2) The gelation process and the resultant substrates are not harmful for surrounding tissues.

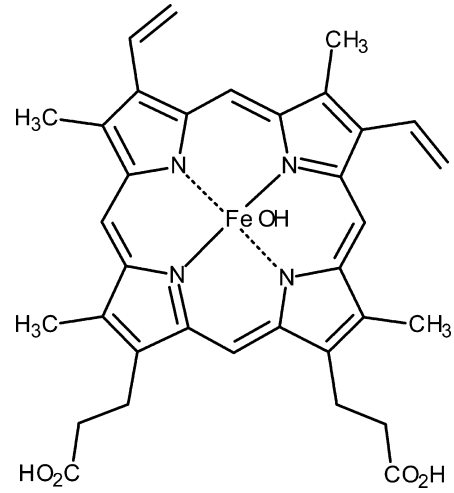

Figure 1. Structure of hematin.

In this paper, we first demonstrate the possibility of using hematin for gelation of the solutions containing polymers

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10.1021/bm100623k
Published on Web 07/15/2010

possessing Ph moieties using a carboxymethylcellulose derivative (CMC-Ph)[9] and a gelatin derivative (Gelatin-Ph).[11] We then demonstrate the feasibility for in vivo applications by showing the controllability of gelation profiles, cellular compatibility in vitro by encapsulating cells in gels, and biocompatibility in vivo by subcutaneous injection into mice using gelatin-Ph as a model polymer.

Materials and Methods

Materials. Porcine hematin, sodium carboxymethyl cellulose (CMC, 400−800 cP at 2% aqueous solution at 25 °C), and gelatin (type A from porcine skin, 300 Bloom) were obtained from Sigma (St. Louis, MO). Horseradish peroxidase (HRP; 170 units/mg) and 30% (w/w) H_2O_2 aqueous solution were obtained from Wako Chemicals (Osaka, Japan) and Kanto Chemicals (Tokyo, Japan), respectively. Gelatin-Ph and CMC-Ph were synthesized using previously reported methods through conjugation with tyramine using aqueous-phase carbodiimide activation chemistry.[9,11] CMC-Ph and gelatin-Ph contained 10×10^{-7} Ph moieties/mg of CMC and 2.0×10^{-7} Ph moieties/mg of gelatin, respectively. Mouse fibroblast L929 (RCB1451), provided by the Riken Cell Bank (Tsukuba, Japan), was used to evaluate cellular adhesion on gels and the effect of the gelation process on cells. Cells were grown in minimum essential medium (MEM, Wako Chemicals, Osaka, Japan) supplemented with 5% (v/v) fetal bovine serum, 1×10^5 units/L penicillin, and 100 mg/L streptomycin. The cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2.

Gelation. It is difficult to dissolve hematin in neutral and lower pH solutions; thus, hematin was first dissolved in 100 mM disodium hydrogen phosphate solution (pH 10.5) and the pH was then adjusted to 7.4 by gradually adding 100 mM sodium dihydrogen phosphate solution (pH 4.4). CMC-Ph or gelatin-Ph was dissolved in calcium-free Krebs Ringer Hepes buffer solution (CF-KRH, pH 7.4) at 3.0% (w/v). The solution was mixed with 1/9 volume of the hematin solution in a glass vessel (15 mm in diameter). After stirring, 1/10 vol of 100 mM H_2O_2 solution or CF-KRH not containing H_2O_2 was added under stirring. The total volume of solution in the glass vessel was 1.5 mL. The final concentrations of hematin were 0.02 and 0.1% (w/v) in the CMC-Ph and gelatin-Ph systems, respectively. Gelation was evaluated by turning the glass vessels upside down after 20 min of standing at room temperature.

Catalytic Profile at pH 7.4. The catalytic profile of hematin at pH 7.4 was measured using pyrogallol as a substrate.[20] The assay mixture containing 5.0 mM pyrogallol, 0.01 mg/mL hematin, and H_2O_2 in a total volume of 3 mL was poured into a quartz cell. The reaction was initiated by the addition of H_2O_2, and the increase in absorbance at 420 nm was recorded. The concentration of H_2O_2 was changed from 0.1 to 100 mM. HRP (0.01 unit/mL) was used as a control. Relative catalytic activities under different H_2O_2 concentrations were calculated by normalizing the increase in absorbance at 420 nm at each H_2O_2 concentration by the maximum values detected at 100 and 1 mM H_2O_2 for hematin and HRP, respectively. The data for catalytic profiles are expressed as the average of three measurements.

Gelation Time. Hematin was dissolved in 100 mM phosphate buffer solution (PBS, pH 7.4) by the above method. To this solution, gelatin-Ph was suspended and dissolved by heating at 60 °C. The mixture solution was poured into a 48-well plate at 400 μL/well. Subsequently, 100 μL of H_2O_2 in CF-KRH was poured into the well and stirred at 80 rpm using magnetic stirrer bars (10 mm length, 4 mm diameter). Formation of the gel state was signaled when the magnetic stirring was hindered and the surface of the solution swelled. The final contents of hematin and gelatin-Ph were fixed at 0.05 (w/v) and 3.0% (w/v), respectively. The H_2O_2 concentration was varied in the range 1−100 mM. A system containing HRP at 2.4 units/mL was used as a control.

Cell Studies In Vitro. Compatibility of the resultant gel and gelation process with mammalian cells was studied using two different systems: adhesion and morphology of adherent cells on a preformed gelatin-Ph gel and viability of cells enclosed in a gelatin-Ph gel through the hematin-catalyzed reaction involving H_2O_2. A preformed gelatin-Ph gel was prepared for adhesion and morphology studies from a mixture of 0.05% (w/v) hematin and 10% (w/v) gelatin-Ph. The mixture was poured into a 6-well plate at 1 mL/well. After 1 h of standing in a refrigerator, 2 mL of 10 mM H_2O_2 solution was poured onto the gel to form hematin-catalyzed cross-links. After 24 h of standing, the resultant gelatin-Ph gel sheet was rinsed several times using CF-KRH and medium. L929 cells were then seeded at 5.0×10^5 cells/well. After 4 h of incubation at 37 °C in a CO_2 incubator, percentages of adhered cells were determined from the number of cells floating in the medium. A 6-well plate coated using unmodified gelatin was used as a control substrate. The percentages of cell adhesion were expressed as the average of measurements for six separate wells.

Encapsulation of cells was performed using a mixture solution containing 0.05% (w/v) hematin, 10% (w/v) gelatin-Ph, 4 mM H_2O_2, and L929 cells at 2×10^5 cells/mL, giving about 15 s of gelation after mixing at 4 °C: First, a mixture of hematin, gelatin-Ph, and L929 cells was poured into a 6-well plate at 1 mL/well. Immediately after adding H_2O_2 solution to the well, the mixture was stirred gently and spread to cover the surface at the bottom of wells. After 30 min of standing at room temperature for formation of cross-links between Ph moieties, medium was poured into the wells at 5 mL/well. After 1 h of standing at 37 °C, the medium was replaced with fresh medium. Fluorescence staining using a fluorescence double-staining kit (Cellstain, Dojindo, Kumamoto, Japan) was performed after a further 12 h of incubation at 37 °C. A total of 6−8 fluorescence microscopy fields (×40), containing more than 100 cells per field, were randomly selected in four separate experiments. The number of living (stained green) and dead (stained red) cells were counted to determine the viability of encapsulated cells.

In Vivo Animal Tests. To demonstrate the in situ formation of gels through hematin-catalyzed reaction in vivo and to test for the presence of histological reactions toward the resultant gels, gels were prepared based on a method reported for in situ gelation of gelatin-Ph through HRP-catalyzed reaction.[11] Briefly, a mixture containing 0.1% (w/v) hematin, 5.0% (w/v) gelatin-Ph, and 5 mM H_2O_2, after about 25 s of gelation after mixing at room temperature, was subcutaneously injected into five male DDY mice (6 weeks old) using a 26-gauge needle, under anesthesia with pentobarbital. The mice were euthanized 7 days after the injection by an overdose of anesthetic. The cutaneous tissues containing the gels were excised and fixed using glutaraldehyde/paraformaldehyde solution. Subsequently, the samples were dehydrated and embedded in paraffin wax. Sections were stained with hematoxylin and eosin.

All protocols using the animals were carried out according to the recommendations of the Kyushu University manual entitled "Guide for the Care and Use of Laboratory Animals".

Results

Catalytic Properties of Hematin at pH 7.4. First, we checked the catalytic profiles of hematin under mild conditions for mammalian cells. As shown in Figure 2, we confirmed gelation of CMC-Ph and gelatin-Ph solutions through a hematin-catalyzed reaction consuming H_2O_2 at pH 7.4 and room temperature. Based on the result, we compared the catalytic profiles of hematin with HRP under different H_2O_2 contents by oxidation of pyrogallol. HRP showed the highest catalytic activity at 1 mM H_2O_2, and this decreased with increasing H_2O_2 concentration (Figure 3). Hematin showed a completely different catalytic behavior with HRP. The catalytic activity of hematin increased with increasing H_2O_2 concentration from 0.1 to 50 mM and showed constant value after further increase until 100 mM. The catalytic activity of 1.0 mg hematin for oxidizing pyrogallol was equivalent to 7.25 units HRP at 5 mM H_2O_2.

Gelation Profiles. One unique phenomenon of gelation through the HRP-catalyzed reaction compared with general catalytic reactions is that the time required for gelation increases

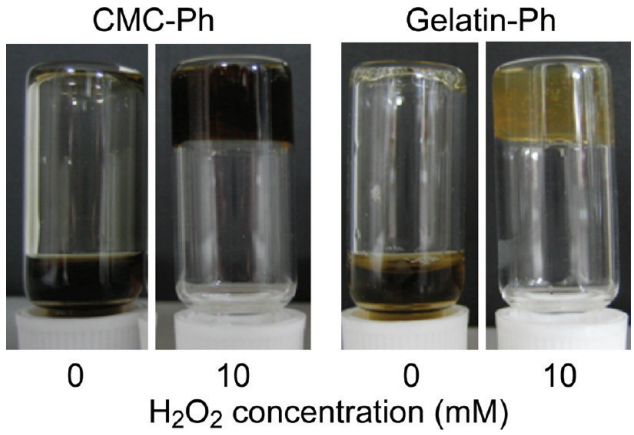

Figure 2. Photographs of glass vessels turned upside down 20 min after mixing 3.0% (w/v) CMC-Ph or 3.0% (w/v) gelatin-Ph containing hematin in the absence and presense of H_2O_2. The content of hematin in CMC-Ph and gelatin-Ph solutions were 0.02 and 0.1% (w/v), respectively.

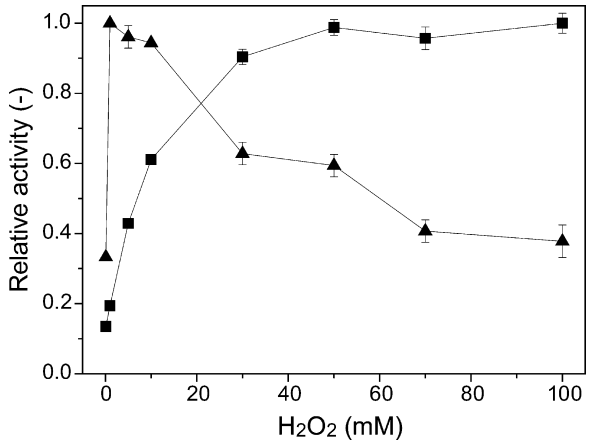

Figure 3. Dependence of catalytic activities of (■) hematin and (▲) HRP on H_2O_2 concentration for oxidation of pyrogallol. Relative activity: Values normalized by the maximum activities detected for hematin and HRP, respectively. Bars: SD ($n = 3$).

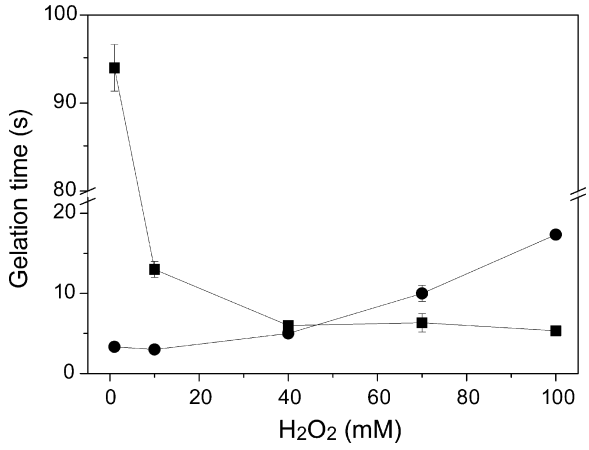

Figure 4. Dependence of gelation time on H_2O_2 concentration of 3.0% (w/v) gelatin-Ph solution by (■) 0.05% (w/v) hematin and (●) 2.4 units/mL HRP catalyzed reactions. Bars: SD ($n = 3$).

with increasing H_2O_2 concentration, despite consuming H_2O_2 for oxidization, as shown in Figure 4.[8,10,11] Thus, we compared the effect of H_2O_2 on gelation time using the hematin-catalyzed reaction with the system using HRP. The gelation time by hematin-catalyzed reaction at 1 mM H_2O_2 was 95 s. In contrast to the gelation catalyzed by HRP, the gelation time by hematin-catalyzed reaction decreased to 6 s when the H_2O_2 concentration increased to 40 mM. The gelation time of the hematin system showed almost constant values in the range 40 to 100 mM H_2O_2.

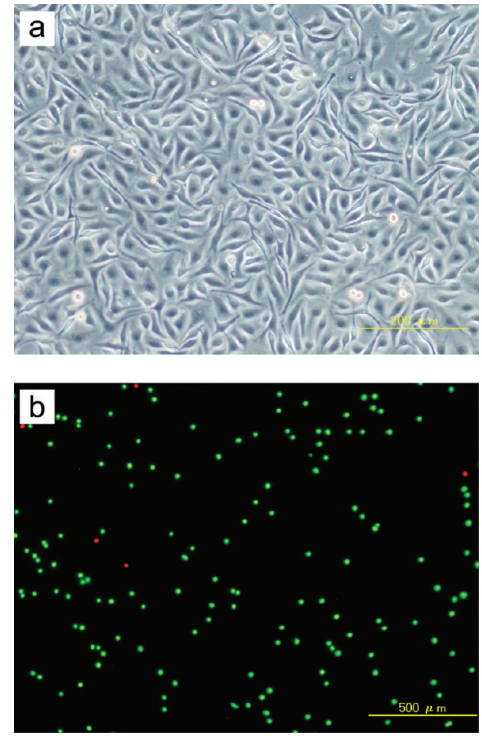

Figure 5. Photographs of L929 cells (a) adhering to gelatin-Ph gel sheets after 4 h of seeding and (b) enclosed in a gelatin-Ph gel after overnight incubation in a CO_2 incubator with medium at 37 °C. Live and dead cells in (b) show green and red fluorescence, respectively. Percentages cells adhering to gelatin-Ph gels sheets and living cells in gelatin-Ph gels were 98.6 ± 1.2% ($n = 6$) and 95.0 ± 4.6% ($n = 4$), respectively. Bars: (a) 200 μm and (b) 500 μm.

Compatibility with Cells and Surrounding Tissues. We evaluated the biocompatibility of gels prepared through hematin catalysis using two methods. First, we seeded L929 cells onto the gels. After 4 h of seeding, 98.6 ± 1.2% ($n = 6$) of the cells were adhered to the gel. The value was almost the same as those adhered onto a dish coated with unmodified gelatin, 98.6 ± 1.5% ($n = 4$). In addition, the morphology of cells spreading on the gel (Figure 5a) was the same as those on the dish coated with unmodified gelatin (data not shown).

Next, we studied the viability of cells enclosed in gel through hematin-catalyzed reaction consuming H_2O_2, giving gelation for about 15 s. The resultant gelatin-Ph gels did not melt even after 12 h of incubation at 37 °C and the viability of the cells enclosed in the gels was 95.0 ± 4.6% ($n = 4$; Figure 5b).

Finally, based on these results, we studied the compatibility with surrounding tissues using five mice. In all animals, the retention of gels formed through subcutaneous injection of a mixture of hematin, gelatin-Ph, and H_2O_2 was confirmed at 7 days after injection, as shown in Figure 6a. In contrast, gels were not observed in any of the animals injected with a mixture of hematin and gelatin-Ph in the absence of H_2O_2 (Figure 6b). Figure 6c shows the stained section of cutaneous tissue containing the gels 1 week after in situ gelation in vivo using hematoxylin and eosin. The surrounding tissues did not show necrosis, while leukocytes, including lymphocytes, fibroblast cells and a layer of thin fibrous capsules, were found on the surface of the gel. Macrophages with phagocytized hematin were also observed around the gels.

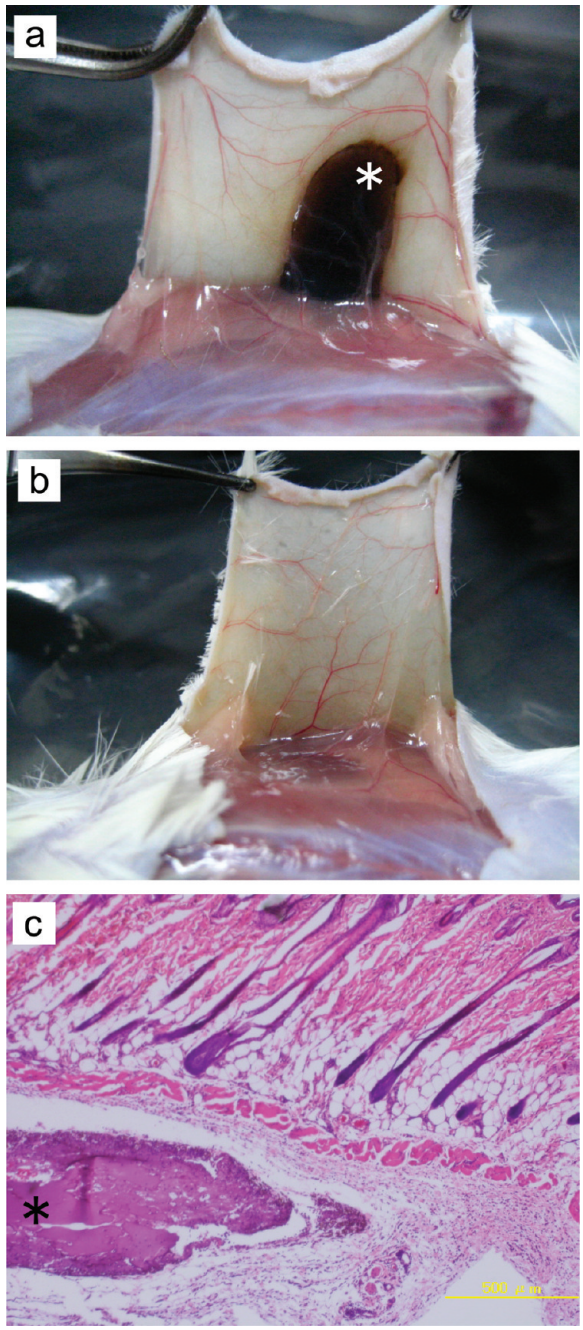

Figure 6. (a) Subcutaneous in situ gelled gelatin-Ph gel 1 week after the injection of a solution containing 0.1% (w/v) hematin, 5.0% (w/v) gelatin-Ph, and 5.0 mM H_2O_2. (b) Subcutaneous site injected with a solution containing 0.1% (w/v) hematin and 5.0% (w/v) gelatin but not containing H_2O_2. (c) Hematoxylin and eosin-stained slice of cutaneous tissues containing the gels (×40) shown in (a); * in (a) and (c) indicates remaining gel.

Discussion

The focus of the present research was to investigate the feasibility of hematin as an alternative catalyst to HRP for in situ gelation of polymers with Ph moieties in vivo. An attractive point of hematin is that it has historically been dosed through intravenous injection as a rational, safe, and effective therapy in porphyric attacks.[17−19] Hematin has been used as a catalyst for the oxidative polymerization of phenol compounds.[15,16] However, as far as we know, there have been no reports of hydrogelation using hematin for applications in the biomedical field, including in situ gelation in vivo.

The first stage of our investigation evaluated the catalytic activity of hematin under mild conditions for mammalian cells, pH 7.4 and room temperature. We compared the dependence of hematin catalytic activity on H_2O_2 concentration with that of HRP. The main trend we observed was that there was increase in the catalytic activity of hematin with increasing H_2O_2 concentration (Figure 3). In contrast, catalytic activity of HRP decreased with increasing H_2O_2 concentration, resulting in a longer gelation time (Figure 4), consistent with our previous results.[8,10,11] The constant values of catalytic activity of hematin between 40 and 100 mM H_2O_2 clearly indicate that hematin is stable even under excess H_2O_2. The higher tolerance of hematin to H_2O_2 compared with HRP, resulting in a shorter time of gelation (Figure 4), can be explained by the more stable oxidized form of the free heme in hematin.[14] One of the axial coordination sites of Fe(III) is occupied by a hydroxide ion, rather than the histidine residue found in HRP. We have not shown results of the effects of hematin and gelatin-Ph concentrations because their tendencies were the same with those obtained for HRP, as is easily expected from the usual kinetics of chemical reactions. Gelation time could be shortened by increasing the contents of hematin and gelatin-Ph. Including the results for the effects of hematin and gelatin-Ph concentrations, important information obtained from the study was that gelation time of the system using hematin is controllable, the same as with HRP systems, even within 20 s.

We then investigated the cellular compatibility of the gels obtained through the hematin-catalyzed reaction. We did not find any adverse effects on cells in contact with the gel, as indicated by their having the same adhesion rate (>95%) and morphology as cells adhering onto a substrate covered with unmodified gelatin (Figure 5a). In addition, 95.0% cell viability 12 h after enclosing them in gels prepared under conditions that induced gelatin about 15 s clearly demonstrates the possibility of obtaining gels at injected sites in vivo with less toxicity of hematin-catalyzed reaction.

In the third and final stage of our investigation, we evaluated the histological reaction to the gelatin-Ph gels formed at the injected site through hematin-catalyzed reaction. Successful gelation in vivo was demonstrated by the absence of gels at the site injected with a mixture of hematin and gelatin-Ph in the absence of H_2O_2 (Figure 6b). In addition, clearly visible border between the remaining gel and the neighbor tissues (Figure 6a) indicates a rapid gelation at the injected site as expected from the results in vitro. To our knowledge, this is the first paper showing in situ gelation using hematin as a catalyst for cross-linking between Ph moieties in vivo. High biocompatibility of this in situ gelation system and the resultant gels was revealed by the outer appearance and the histological sections of cutaneous tissues surrounding the gels (Figure 6a): There was not any visible redness and swelling characteristic of inflammation. Histological sections of cutaneous tissues containing the gels showed that gels did not induce necrosis in the surrounding tissues (Figure 6c). Although we found leukocytes, including lymphocytes, fibroblast cells, and a layer of thin fibrous capsules on the surface of the gels, such reactions were also observed for gelatin-Ph gels obtained through HRP-catalyzed reaction,[11] and no significant adverse reaction was found compared with previous gels. An obvious difference compared with previous results reported using HRP was the existence of macrophages with phagocytized hematin around the gels. This may result in iron deposition around the injected site. It is beyond the scope of this study to speculate on whether this cellular and fibrillar response will be regarded as positive

or negative for specific applications. The formation of similar thin fibrous capsules has been reported for a variety of feasible biomaterials.[21,22] In addition, both positive and negative aspects of these responses have been reported.[22−24] Cellular and fibrillar responses can confer stability and elicit a persistent augmentative effect from biomaterials,[23] whereas the formation of fibrous capsules can also hinder molecular exchange between encapsulated materials and surrounding tissues.[24] The important finding obtained from the study of histological reactions was that the use of hematin as an alternative to HRP did not induce specific adverse effects in surrounding tissues.

Conclusions

This study demonstrates the feasibility of hematin as an alternative to HRP for in situ gelation of polymers with Ph moieties in vivo for drug delivery and tissue engineering applications. The in vitro results demonstrate that the dependence of gelation on H_2O_2 concentration differed from that of gelation by HRP but it was similarly controllable. In addition, the possibility of gelation by hematin under nonharmful conditions for mammalian cells was also demonstrated by a cell-encapsulation study. In vivo results from histological analysis of the tissues surrounding gels obtained by subcutaneous in situ gelation by hematin demonstrate there was no specific harmful effect of using hematin.

Acknowledgment. This research was supported by a Grant-in-Aid for Young Scientists (B), 22760610, from the Ministry of Education Culture Sports Science and Technology of Japan.

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BM100623K