Formation of Fibrinogen-Based Hydrogels Using Phototriggerable Diplasmalogen Liposomes

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We report the triggered release of Ca2+ from liposomal compartments to induce rapid gelation of protein-based hydrogels. Phototriggerable liposomes were designed by entrapment of CaCl2 within liposomes composed of 38:57:5 diplasmenylcholine (DPPlsC):disteroylphosphatidylcholine (DSPC):bacteriochlorophyll (Bchl). These liposomes release >80% of their entrapped Ca2+ within 15 min when irradiated at 800 nm (800 mW/cm²). A precursor solution, containing liposomes suspended in aqueous human fibrinogen and transglutaminase (TGase), remained fluid for several hours in the dark, but gelled rapidly when exposed to 800 nm excitation, as a result of photosensitized Ca2+ release and TG-induced fibrinogen cross-linking. TGase and hrFXIII activities, determined using a fluorimetric dansylcadaverine assay, were found to depend strongly on irradiation time and reaction temperature. SDS-PAGE of the photolyzed reaction mixture revealed that gelation arises from enzyme-catalyzed cross-linking of predominately the α and γ chains of fibrinogen. This approach to the phototriggered formation of protein hydrogels creates new opportunities for biomaterials applications in drug delivery, tissue engineering, and wound healing.

INTRODUCTION

Development of new strategies and materials to facilitate rapid and efficient wound healing and localized drug delivery are major thrusts in biomaterials research. Fibrinogen hydrogels are potentially important biomaterials for drug delivery applications. Protein-based hydrogels such as these can be created by exploiting various Ca2+-dependent enzymes to catalyze the enzyme-mediated cross-linking of polypeptide precursors (1, 2). One such family of Ca2+-dependent enzymes are the transglutaminases, which promote the formation of cross-links between glutamine and lysine residues on neighboring proteins (3–7) (Figure 1a). An area of considerable interest is the development of injectable biomaterials that undergo a rapid solid-gel transformation in-situ to form semisolid hydrogels. These materials may be potentially useful for site-directed drug delivery, wound healing, and in the emerging field of tissue engineering (8). Light activation is a particularly promising way to achieve in-situ formation of biomaterials since it provides a very broad range of adjustable parameters (e.g., pulse duration, intensity, pulse cycle, and wavelength) that can be optimized for biological compliance and clinical efficacy. Moreover, fiber optic light sources are common clinical devices that can offer a convenient means for triggering useful chemical reactions within confined regions. For example, UV and visible light activated photopolymerization systems have been developed for rapid in vivo gelation of monomeric liquids for drug delivery and tissue repair (9–11).

Efforts in our laboratories have been aimed at developing stimuli-responsive liposome strategies for site-specific drug delivery (12–21) and in-situ formation of resorbable biomaterials (22–27). The ability of stimuli-responsive liposomes to release sequestered reagents in response to an applied stimulus (temperature, light, pH, etc.) (15, 16) can be exploited to trigger in-situ formation of mineral-, polymer-, and peptide-based biomaterials.

In this work, we use phototriggered release of liposomal Ca2+ as an initiator of enzyme-mediated formation of fibrinogen hydrogels. The basic strategy of this approach is to utilize calcium-loaded phospholipid vesicles to physically isolate calcium ions from Ca2+-dependent enzymes contained within the surrounding aqueous medium (Figure 1b); photoactivated release of Ca2+ converts the enzyme from an inactive to an active state. Previously, Wymer et al. used this concept to photochemically control the activity of a calcium-dependent phospholipase A2 in a cascade triggering approach (20). This work employs Ca2+-dependent transglutaminase (TGase)3 enzymes (protein–glutaminemine γ-glutamyltransferase, EC 2.3.2.13), which catalyze an acyltransfer reaction between the γ-carboxamide groups of peptide-bound glutamine residues and the α-amino groups of lysine residues in proteins (3–7). In this case, photoactivated release of Ca2+ from liposomes results in the cross-linking of a soluble protein (fibrinogen) through the formation of ε-(γ-glutamyl)lysine isopeptide side-chain bridges as shown in Figure 1. This paper describes a method for producing transglutaminase-cross-linked hydrogels based on photooxidative triggering of Ca2+ release from diplasmalogen (DPPlsC) liposomes, with subsequent

1 Abbreviations used: Bchl, bacteriochlorophyll; Ca–FTE, calcium-loaded freeze–thaw-extruded vesicles; Ca–IFV, calcium-loaded interdigitation fusion vesicles; DPPlsC, 1,2-Di-(1-Z-O-hexadecenyl)-sn-glycero-3-phosphocholine; DSPS, 1,2-Disteryl-sn-glycero-3-phosphocholine; hrFXIII, human recombinant Factor XIII; HBS, HEPES-buffered saline; 10 mM HEPES, 0.3 M NaCl, 1 mM EDTA, pH 7.4; TGase, tissue transglutaminase.

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activation of transglutaminase-catalyzed cross-linking of fibrinogen. The gelation rate and physical properties of these hydrogels have been characterized by dansylamine fluorescence assay, SDS–PAGE, and cryogenic scanning electron microscopy (C–SEM) to determine the rate and extent of protein cross-linking.

**MATERIALS AND METHODS**

**Materials.** Diplasmenylcholine (DPPlsC) was synthesized as described (21). Human recombinant Factor XIII (hrFXIII) was generously supplied by ZymoGenetics Corporation (Seattle, WA). 1,2-Disteroyl-sn-glycerol-3-phosphatidylcholine (DSPC), bacteriochlorophyll a (Bchl), Arsenazo III (AIII), NaCl, CaCl₂, human fibrinogen (93% clottable), tissue transglutaminase (TGase), dithiothreitol (DTT), HEPES, and EDTA were all used as received.

**Preparation of Liposomes.** Calcium chloride-loaded phospholipid vesicles were prepared by the interdigitation–fusion approach as previously described (23, 28). A lyophilized powder of 38:57:5 (mol %) DPPlsC:DSPC:Bchl was hydrated in the presence of 0.2 M CaCl₂ for 15 min at 65 °C with occasional vortexing. The resulting vesicle suspension was sonicated under an inert atmosphere at 65 °C for 30 min, cooled to room temperature, and then centrifuged at 3000g for 3 min to remove metal particles released from the sonicator probe tip. Absolute ethanol was added to the supernatant to achieve a final ethanol concentration of 4 M. The solution was mixed vigorously and then incubated at 22 °C for 15 min. Ca²⁺-encapsulated interdigitation–fusion vesicles (Ca⁻IFVs) were obtained by flushing the suspension with dry Ar at 65 °C for 60 min and at 23 °C for an additional 30 min to remove the remaining EtOH. Extrasvesicular Ca²⁺ was removed from the liposome suspension by repeated centrifugation (20000g) for 3 min with isotonic 0.3 M NaCl solution containing 1 mM EDTA in 10 mM HEPES at pH 7.4, HBS and decanting of the supernatant. This process was repeated until Ca²⁺ was no longer detected in the supernatant using the Arsenazo III spectrophotometric assay (see below) (29). The Ca⁻IFV’s were resuspended in isotonic HBS solution to a final lipid concentration of 20 mg/mL and then stored at 4 °C until use. The average Ca⁻IFV diameter, determined via quasielastic light scattering using a Coulter N4–Plus instrument and the manufacturers’ analysis software, was approximately 1.9 μm.

The amount of Ca²⁺ encapsulated within the Ca⁻IFV was determined using the calcium sensitive dye, Arsenazo III (AIII, λmax = 656 nm), which undergoes a color change upon complexation with Ca²⁺ (Ca²⁺-AIII, λmax = 656 nm) (29). A 5 μL aliquot of Ca⁻IFV suspension was added to 3 mL of AIII solution (0.2 mM AIII in 20 mM HEPES, 0.3 M NaCl, pH 7.4) in a 1 × 1 cm cuvette. The mixture was vigorously stirred for 30 s with a micromagnetic stir bar and then the UV absorption at 656 nm measured. Subsequent addition of 10% Triton X-100 (30 μL) to this mixture leads to vesicle disruption and complete release of Ca²⁺. Control experiments showed that this assay was linear over 5–100 μM calcium concentration ranges.

Figure 1. A: Glutamine-lysine cross-linking reaction catalyzed by calcium-dependent transglutaminase (TGase). B: Conceptual illustration of the photoinduced fibrinogen gelation process.
Light-Triggered Ca\(^{2+}\) Release from Ca–IFV. Liposome solutions (1.5 mL in 13 × 100 mm culture tubes) were illuminated using a SDL 820 Al–GaAs diode laser (Spectro Diode Labs, San Jose, CA, \(\lambda_{em} = 800\) nm) coupled to a 1 mm diameter optical fiber, the emitting end of which was mounted perpendicular to the side plane of an irradiation cuvette. Incident light intensities were measured using a MAX 5100 power meter (Moleclectron Detector, Portland, OR). A 10 \(\mu\)L aliquot of liposome sample was added to 3 mL of ALII solution. Release of Ca\(^{2+}\) from the liposomes was followed as a function of time by measuring the absorption intensity at 656 nm before and after the addition of 10% Triton X-100 solution and the amount of Ca\(^{2+}\) released calculated as previously described (27).

Light-Activation of hrFXIII and Liver TGase via Ca–IFVs. The activity of hrFXIII and liver TGase in the presence of Ca–IFVs was determined using the fluorometric dansylcadaverine assay (30), modified to account for the presence of Ca–IFVs (see below). Sample temperature control was provided by a water-jacketed cell holder, which was connected to a thermostated water bath. A nitrogen atmosphere (50 \(\mu\)M in 0.29 mM NaCl, pH 9.0: 1.2 mL), Tris buffer (50 mM in 0.175 mM NaCl, pH 7.5: 1.0 mL), and monodansylcadaverine (2 mM in 50 mM Tris buffer, pH 7.5: 40 \(\mu\)L) were combined in a 1 cm × 1 cm quartz fluorescence cuvette. After temperature equilibration, 0.2 mL of dimethylcasein (2% in distilled H\(_2\)O, cooled to 5 °C) was added. The solution temperature initially dropped by approximately 1 °C, but returned to the set temperature within 1–2 min. Thrombin (500 units/mL; 20 \(\mu\)L) was then diluted with 0.1 mL of distilled water and immediately combined with 10 \(\mu\)L of hrFXIII (10 mg/mL), and the entire volume transferred to the monodansylcadaverine-containing cuvette. DTT (50 \(\mu\)L in 50% glycerol) was then added, followed by a volume of the Ca–IFV suspension (35 mM Ca\(^{2+}\); 60 \(\mu\)L). Time-dependent fluorescence data collection was then started (\(\lambda_{em} = 360\) nm; \(\lambda_{ex} = 500\) nm; slit widths = 20 nm). After 5 min incubation in the dark, the samples were illuminated at 800 nm as described above. The distance between the end of optical fiber and the solution surface was approximately 3 cm. Incident light power was measured at the surface of the suspension.

Light-Triggered Gelation of Precursor Suspensions. Fluid hydrogel precursor solutions were prepared by suspending Ca–IFV in a solution containing TGase and fibrinogen. The hydrogel precursor solution was produced by first combining 200 \(\mu\)L of human fibrinogen solution (50 mg/mL in HBS), 10 \(\mu\)L of TGase (0.02 units/\(\mu\)L), and 10 \(\mu\)L of 0.5 M DTT in a 10 × 13 cm culture tube, followed by addition of 200 \(\mu\)L of Ca–IFV solution with mixing. The culture tube was immersed in a water bath held at 37 °C and the tip of the laser fiber positioned 3 cm away from the surface of the precursor solution. The laser power was then adjusted to give the desired power density without introducing heating effects.

Transglutaminase-catalyzed cross-linking of fibrinogen was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (31). TGase solution (10 \(\mu\)L) was combined with Ca–IFV’s (200 \(\mu\)L) and fibrinogen solution (2 mg/mL in HBS; 200 \(\mu\)L), and the samples were irradiated for 40 min at 37 °C. Aliquots of the reaction mixture were withdrawn at 10 min intervals during the irradiation and immediately analyzed by SDS–PAGE.

Cryogenic Scanning Electron Microscopy (C–SEM). Samples were frozen by plunging into N\(_2\) slush. The samples were then fractured and sublimated at −70 °C for 30 min prior to sputter-coating for 4 min with Au at approximately −160 °C. Samples were imaged at −140 °C in a JEOL JSM-840 SEM using a 4 kV accelerating voltage. The structures observed were consistent in numerous areas viewed.

RESULTS

Thompson et al. have previously shown that Ca-loaded DPPlsC:Bchl liposomes can rapidly release Ca\(^{2+}\) upon irradiation at 800 nm (20, 32). This study employed the same photooxidatively based triggering chemistry, except that DSPC was used as the major membrane lipid component to keep the dark leakage rates low and minimize the amount of DPPlsC required (Figure 2). Phototriggered calcine release experiments showed that formulations containing 38% DPPlsC were capable of releasing more than 80% of their cargo when irradiated for 30 min at 800 nm, 1 W/cm\(^2\) (Figure 3). To further reduce the lipid requirements of this system, we used the interdigitation fusion technique to make large vesicles that were capable of very high trapping efficiencies. This approach enabled 53 mM Ca\(^{2+}\) encapsulation efficiencies using only 20 mg/mL of total lipid. By comparison, liposomes prepared by conventional freeze–thaw-extrusion (FTE) methods were capable of encapsulating only 13 mM Ca\(^{2+}\) at the same lipid concentration. As shown in Figure 4, more than 90% of the entrapped Ca\(^{2+}\) was released upon irradiation of 38:57:5 DPPlsC:DSPC:Bchl Ca–IFV at 800 nm for 45 min.

Our approach to phototriggered triggering of hydrogel formation is based on the well-known dependence of TGase activity on free Ca\(^{2+}\) concentrations (7). We first sought to verify this dependence using Ca–IFV to initiate hrFXIII and TGase activity by photooxidative triggering. For this purpose, the fluorescence based assay that monitors monodansylcadaverine incorporation into casein (30) was employed. This assay is based on fluorescence intensity increases that result when the dansyl group becomes enzymatically coupled to casein via amide bond formation. Figures 5 and 6 show the fluorescence intensity increases as a function of time for reaction mixtures containing N’N’-dimethylcasein, Ca–IFV, and either liver TGase (Figure 5) or hrFXIII (Figure 6) at both 24 °C and 37 °C. At 24 °C with 100 s irradiation time, the fluorescence intensity of both enzymes rises only slowly during the first 60 min (Figure 5 and 6). However, the fluorescence intensity rises rapidly at 37 °C, indicating that hrFXIII and TGase more efficiently catalyze the incorporation of monodansylcadaverine into N’N’-dimethylcasein via a photoinitiated release of Ca\(^{2+}\) at higher temperature.

To test the concept of phototriggered TGase-catalyzed fibrinogen gelation, Ca–IFV were mixed with fibrinogen and liver TGase to form a precursor solution which did not gel in the dark at 37 °C for 120 min. Mild irradiation (0.4 W/cm\(^2\)) of these samples at 37 °C, however, led to the formation of a dense gel within 40 min (Figure 7a). Gelation time could be reduced to 15 min by increasing the laser power to 0.8 W/cm\(^2\) or by increasing the TGase concentration to 0.6 units (data not shown). Cryo-SEM analysis (Figure 7b and 7c) of the irradiated sample from

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3 The Ca\(^{2+}\) concentrations reported here, 53 mM and 13 mM, for Ca–IFV and Ca–FTE, respectively, refer to the bulk Ca\(^{2+}\) concentrations (i.e., after complete escape of Ca\(^{2+}\) from the inner vesicle compartment due to Triton X-100 addition).
Figure 7a reveals the formation of a microporous network structure whose pores are approximately the diameter of the Ca\(^{2+}\)IFV. The pores of this hydrogel material may be imprinted by the vesicle precursors, i.e., the vesicles themselves may serve as both the Ca\(^{2+}\) source and as a template for the TGase-catalyzed cross-linking reaction. SDS–PAGE analysis of photoinitiated Ca\(^{2+}\)IFV/TGase/fibrinogen samples was performed as a function of irradiation time to observe the TGase-induced changes in protein MW upon cross-linking (Figure 8). For samples incubated in the dark at 37 °C, SDS–PAGE revealed no observable protein cross-linking through 40 min, as indicated by the persistence of the fibrinogen \(\alpha\), \(\beta\), and \(\gamma\)-bands. By contrast, irradiation of the samples at 800 nm (400mW/cm\(^2\), 37 °C) resulted in extensive loss of both the \(\alpha\) and \(\gamma\) chains of fibrinogen within 10 min, as well as a gradual loss of the \(\beta\)-band over 40 min. At the same time, weakly staining higher molecular weight bands emerged, along with a densely stained, very high molecular weight band which did not penetrate the gel.

**DISCUSSION**

The goal of this project is to formulate aqueous protein solutions, which can expeditiously gelate upon transglutaminase catalysis in the presence of Ca\(^{2+}\) ions. Our method employs phototriggered liposomal release of Ca\(^{2+}\) to initiate the rapid enzymatic cross-linking of fibrinogen solutions to form hydrogels. Phototriggerable liposomes were designed by entrapping CaCl\(_2\) within liposomes composed of 38:57:5 DPPIslsC:DSPC:Bchl. These liposomes...
could release ~90% of entrapped Ca$^{2+}$ when irradiated at 800 nm for 30 min (Figure 4). Photochemical control of TGase enzyme and FXIII activity was clearly demonstrated using the dansylcadaverine enzyme assay as shown in Figures 5 and 6. When Ca–IFV's were used as the sole source of Ca$^{2+}$, virtually no enzyme activity was observed in the dark control sample (i.e., nonirradiated). FXIII and TGase became active, however, in the presence of the millimolar concentrations of Ca$^{2+}$ that were released by the Ca–IFV after irradiation at 800 nm for only 100 sec at 24 °C. As expected, higher enzyme activities were observed after irradiation of the suspensions at 800 nm for 100 sec at 37 °C. The phototriggered Ca$^{2+}$ release from Ca–IFV was used to activate enzyme-catalyzed cross-linking of pro-
teins to form hydrogels within 40 min, as confirmed by visual observations and SDS–PAGE experiments. Figure 8 showed that the intensity of the α- and γ-bands decreased when the suspensions were irradiated at 800 nm for 10 min. Densely stained wells at the origins of lanes 5–7 indicate the formation of macromolecules in the cross-linking reaction that cannot penetrate into the gel. When higher laser power or TGase concentrations were applied to the precursor solution at 800 nm and at 37 °C, gelation time was reduced to 15 min.

Commercially available, fibrin-based adhesives produce strong, biodegradable gels very quickly (<10 min) and are used as surgical glues, tissue sealants, and drug delivery devices (33). These materials consist of fibrinogen, FXIII, thrombin, and Ca^{2+} and are typically delivered via a dual syringe device that separates fibrinogen and FXIII from Ca^{2+} and thrombin during storage. Mixing of the components during discharge from the syringe results in thrombinization of fibrinogen to create fibrin, which self-assembles into a gel that is later cross-linked by Ca^{2+}-activated FXIII. This sequence of reactions closely mimics the manner in which fibrin and activated FXIII are formed in vivo (4). Through extensive cross-linking of the protein substrate by FXIII, resistance to both mechanical and enzymatic degradation is imparted to the material (34). Our approach relies on Ca^{2+}-activation of FXIII as well; however, there are several key differences between the fibrinogen hydrogels described in this paper and conventional fibrin tissue adhesives. For example, our formulations do not contain thrombin, which plays a key role in formation of both fibrin and activated FXIII in vivo. Because of this, the molecular (and supramolecular) structure of the fibrinogen gels are expected to be distinctly different from fibrin gels. Furthermore, the absence of thrombin could be an advantage in clinical use, since many current fibrin tissue formulations utilize bovine thrombin, which can induce anaphylactic and autoimmune responses in patients (35).

CONCLUSIONS

We recently reported the use of near-infrared (NIR) sensitive liposomes to trigger gelation of a self-assembling peptide (26); however, to the best of our knowledge, this is the first example of triggered protein hydrogel formation using NIR light. Since NIR has good tissue penetration relative to visible or UV light, one can conceivably (1) trigger the gelation of these suspensions in a noninvasive fashion using an external NIR source, (2) temporally modulate the release of drugs from NIR-sensitive liposomes entrapped within an implanted hydrogel, (3) produce hydrogels that possess unique gradient or microstructural properties. These experiments demonstrate that this approach to the phototriggered formation of protein hydrogels creates new opportunities for biomaterials applications in drug delivery, tissue engineering and wound healing. Experiments to explore these opportunities are currently in progress.

ACKNOWLEDGMENT

This work was supported by grants from NIH GM55266 & DE 13030. The authors thank ZymoGenetics, Inc. for their generous donation of hrFXIII.

LITERATURE CITED


