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Facile Formation of Dynamic Hydrogel Microspheres for Triggered Growth Factor Delivery

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Abstract

Dynamic hydrogels have emerged as an important class of biomaterials for temporal control over growth factor delivery. In this study we formed dynamic hydrogel microspheres from protein-polymer conjugates using an aqueous two phase suspension polymerization process. The aqueous two phase suspension polymerization process enabled rapid microsphere formation without the use of an organic phase, surfactants, mechanical strain, or toxic radical initiators. The microspheres' size distribution was modulated by varying the protein-polymer conformation in the pre-polymer solution. Notably, the protein's ligand-induced, nanometer scale conformational change translated to maximum hydrogel volume changes of $76 \pm 10\%$. The magnitude of the microspheres' volume change was tuned by varying the crosslinking time and ligand identity. After characterizing the microspheres' dynamic properties, we encapsulated two important therapeutic proteins, vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2), in the hydrogel microspheres and characterized how the microspheres' dynamic properties controlled their release. Significantly, the aqueous two phase suspension polymerization process enabled high encapsulation efficiencies ($65.8 \pm 4.8\%$ and $79.5 \pm 3.0\%$ for VEGF and BMP-2, respectively). Also, the microspheres' ligand-induced volume change triggered VEGF and BMP-2 release at specific, predetermined times. There are hundreds of proteins that undergo well characterized conformational changes that could be processed into hydrogel microspheres via aqueous two phase suspension polymerizations. Therefore, this approach could be used to form dynamic, growth factor-releasing hydrogel microspheres that respond to a broad range of specific biochemical ligands.

Keywords

Drug delivery; Emulsions; Vascular Endothelial Growth Factor; Bone Morphogenetic Protein

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1. Introduction

Temporal control over growth factor delivery has become a central research area in biomaterials science. The timing of growth factor signaling is a critical regulator of important *in vivo* and *in vitro* processes, and is tightly controlled during wound healing. In one pertinent study, Aiba-Kojima *et al.* measured no detectable vascular endothelial growth factor (VEGF) at wound sites 0–2 days post surgery. After 2–4 days post surgery the local VEGF concentration increased, but not dramatically. During the final stage of wound healing, on days 4–6 post surgery, the VEGF concentration dramatically increased [1]. The timing of VEGF expression is also important during other *in vivo* processes, including tissue regeneration[2] and angiogenesis [3]. In addition, temporal control over VEGF delivery is crucial during *in vitro* stem cell differentiation. For example, Keller and colleagues used the growth factor expression profile during primitive streak development as a guide to develop a protocol for human embryonic stem cell (hESC) differentiation into cardiovascular progenitor cells. First, hESCs were cultured in the absence of VEGF for 6 days, then VEGF was added to the culture media for 8 days to induce progenitor cell differentiation [4]. This concept of precisely timed growth factor presentation using tissue development as a guide has also been harnessed to direct stem cell differentiation down the ectodermal[5] and endodermal lineages *in vitro* [6]. Taken together, these studies suggest that temporal control of growth factor delivery, over the course of days, is a critical capability for regenerative medicine therapies, drug screening approaches, and fundamental biology studies.

Recently, researchers have developed dynamic biomaterials to control the timing of growth factor release. Hydrogels are particularly well suited for controlled growth factor delivery applications because they can be formed from base materials that do not denature proteins, processed into clinically relevant geometries, and their physiochemical properties can be tuned to control the rate of growth factor release [7]. Researchers have formed dynamic hydrogels that respond to non-specific physical and chemical stimuli, including electric fields [8], light [9], temperature [10], mechanical strain [11], and pH [12]. Several of these stimuli have tuned growth factor release from hydrogel networks by varying the hydrogel network mesh size (ξ), electrostatic interactions between the hydrogel and protein, and convective fluid flow out of hydrogel networks [7]. For example, VEGF release from alginate matrices has been modulated by mechanical loading-induced convective fluid flow[11] and electrostatic interactions between negatively charge alginate and positively charged VEGF [13]. Recent studies have extended this concept to enable hydrogel responsiveness to specific biochemical ligands. For example, we and others have formed dynamic hydrogels based on specific biochemical ligand-induced protein conformational changes, and we have used these dynamic hydrogels to modulate growth factor release [14–21]. These studies indicate that the physiochemical properties of hydrogel networks can be tuned to control protein release.

Hydrogel microspheres formed using mechanical[22–23] and solution phase processes[15,24] have been a particularly attractive biomaterial platform for controlled growth factor delivery applications. Solution phase crosslinking techniques have enabled rapid and scalable microsphere production. Water-in-oil suspension polymerization techniques have been used to form hydrogel microspheres, but the presence of organic solvents [25], shear forces [26], and surfactants[27–28] could denature growth factors included in the pre-polymer solution. For example, Crommelin and colleagues observed that a water-in-oil emulsion denatured 35% of an included model protein [29]. Precipitation/dispersion techniques have been used to form dynamic hydrogel microspheres with tunable network properties, but their precipitation polymerization required temperatures greater than 37°C [30], which could denature encapsulated proteins. Alternatively, Hennink and

colleagues have developed a microsphere fabrication approach to form hydrogel microspheres based on the phase separation of hydrophilic polymers, including polyethylene glycol (PEG) and dextran, in aqueous solutions [31]. This approach did not use chemicals or physical forces that could denature and encapsulated therapeutic protein. Therefore, developing this aqueous two phase suspension polymerization approach could be an attractive method to form hydrogels for controlled drug delivery applications

We have previously demonstrated that a common pharmaceutical excipient, raffinose, promoted PEG-protein-PEG conjugates' stability during lyophilization [14]. When the lyophilized powder was reconstituted in solution, a ternary system formed that contained PEG-protein-PEG conjugates, raffinose, and water. In the current study, we hypothesized that this ternary system would undergo phase separation, and the resulting aqueous two phase suspension could be photopolymerized to form microspheres in a similar manner to the previously reported ternary system of dextran, PEG, and water. Furthermore, we hypothesized that a dynamic protein component would allow these microspheres to undergo temporally triggered volume changes and, in turn, temporally controlled growth factor release.

As a demonstration of our proposed approach, we formed PEG-calmodulin-PEG (PEG-CaM-PEG) conjugates with terminal acrylate groups, then lyophilized the PEG-CaM-PEG conjugates in the presence of raffinose. PEG-CaM-PEG conjugates were chosen as the building blocks for our dynamic hydrogels because CaM's nanometer scale conformational change has translated to macroscopic hydrogel volume changes, and terminal acrylate groups can participate in chain growth polymerizations [14–18]. VEGF and BMP-2 were chosen as a model growth factors for controlled release studies because of their important roles in angiogenesis and osteogenesis, respectively [32]. Although the aim of this manuscript was to control the timing of VEGF and BMP-2 release, our results suggested that aqueous two phase suspension polymerizations of dynamic polymer-protein-polymer conjugates could be used to deliver other important therapeutic molecules.

2. Materials and Methods

2.1 PEG-CaM-PEG Synthesis and Characterization

Recombinant CaM(T34C, T110C) was expressed and purified using previously established protocols [14,16–18]. Purified CaM was reacted with poly(ethylene glycol) diacrylate ($M_n=3400$) (PEGDA₃₄₀₀) to form PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates. PEGDA₃₄₀₀ was synthesized from hydroxyl-terminated PEG₃₄₀₀ (Fisher Scientific, Fairlawn, NJ) using previously described techniques [18,33]. Unless stated otherwise, raffinose (Sigma-Aldrich, St. Louis, MO) was combined with purified PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates after phenyl Sepharose column purification (GE Healthcare, Chalfont St. Giles, United Kingdom) at a concentration of (0.2mg raffinose/mg CaM). This solution was subsequently frozen in liquid N₂ and lyophilized. The fractional mass of PEG₃₄₀₀-CaM-PEG₃₄₀₀ in the lyophilized solid was evaluated using the MicroBCA assay (Pierce, Rockford, IL). The molecular weight of PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates was evaluated using a REFLEX II MALDI-TOF mass spectrometer (Bruker, Billerica, MA, USA).

2.2 Dynamic Microsphere Formation

PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres were formed using an aqueous two phase suspension polymerization. Unless stated otherwise, lyophilized solids that contained PEG₃₄₀₀-CaM-PEG₃₄₀₀+ raffinose were reconstituted in aqueous buffers containing 0.05% Irgacure 2959 (Ciba, Basel, Switzerland) by gently mixing with a positive displacement pipette (Gilson, Madison, WI, USA) for 10 seconds. In these studies ϕ is defined as the initial (w/v)

concentration of PEG₃₄₀₀-CaM-PEG₃₄₀₀ in the pre-polymer solution. Buffers used to form these pre-polymer solutions included Ca²⁺ buffer (20mM MES, 150mM NaCl, and 10mM CaCl₂, pH 6.3), EGTA buffer (20mM MES, 150mM NaCl, and 10mM EGTA, pH 6.3), and Mg²⁺ buffer (20mM MES, 150mM NaCl, and 10mM MgCl₂, pH 6.3). Alternatively, pure PEG pre-polymer solutions were formed that contained 10–25% (w/v) PEGDA₅₇₅ (Sigma-Aldrich St. Louis, MO), PEGDA₃₄₀₀, or PEGDA₈₀₀₀, raffinose, and 0.05% Irgacure 2959. For one set of experiments, lyophilized PEG₃₄₀₀-CaM-PEG₃₄₀₀ and raffinose were reconstituted in Ca²⁺ buffer with 0.05% Irgacure 2959 in a microfuge tube. Photographs of the solution were acquired at predetermined times.

Unless stated otherwise, pre-polymer solutions were dispensed immediately after mixing between two glass microscope slides separated by 1mm spacers. The glass slides were pre-treated with Sigmacote (Sigma-Aldrich, St. Louis, MO) using the manufacturer's instructions. The pre-polymer solutions were photopolymerized with 4mW/cm² 365nm UV light and the resulting hydrogels were transferred into Ca²⁺ buffer. After reaching their equilibrium swollen volumes, microspheres were exposed to the CaM ligands trifluoperazine (TFP) or chlorpromazine (CPZ). PEG₃₄₀₀-CaM-PEG₃₄₀₀ microsphere volume changes were characterized with ImageJ software (Freeware, NIH, Bethesda, MD) using photomicrographs acquired with an inverted microscope (Olympus, Center Valley, PA, USA) controlled by SimplePCI software (Hamamatsu, Sewickley, PA, USA). Microsphere volume changes were calculated based on measured microsphere diameters assuming isotropic volume changes [17].

2.3 Influence of Raffinose Concentration on Phase Separation

20% PEG-CaM-PEG solutions were formed in Ca²⁺ buffer with varying raffinose concentrations and 0.05% Irgacure 2959. Pre-polymer solutions were mixed for 10 seconds and then centrifuged for 10 minutes. Aliquots from the top and bottom phases were acquired with a positive displacement pipette. The protein concentration from each phase was quantified using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA).

2.4 VEGF and BMP-2 Encapsulation and Release

VEGF was encapsulated into PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres during photopolymerization. First, pre-polymer solutions were prepared that contained 15ng of previously frozen human recombinant VEGF (Generous Gift of the National Cancer Institute) or BMP-2 (R&D Systems, Minneapolis, MN) with 0.1% bovine serum albumin (BSA) and a 1:200 fraction of I¹²⁵ labeled growth factor (Perkin Elmer, Waltham, MA). After crosslinking, the microspheres were loaded into a column with a protein filtration insert (Grace, Deerfield, IL) and washed with Ca²⁺ buffer to remove any unencapsulated therapeutic protein. Then, microspheres were treated with either Ca²⁺ buffer continuously, cycles of a 5 minute TFP exposure then three Ca²⁺ buffer washes, or they were maintained in Ca²⁺ buffer for 24 hours then subjected to cycles of a 5 minute TFP exposure and three Ca²⁺ buffer washes. The mass of VEGF or BMP-2 encapsulated and released from the microspheres was measured with a Packard Cobra II Gamma Counter (Perkin Elmer, Waltham, MA). The fraction of VEGF in its native conformation after encapsulation and release from the hydrogel microspheres was quantified using a modified immunoassay (R&D Systems, Minneapolis, MN) in which VEGF binding to VEGF-specific antibodies is dependent upon VEGF maintaining its native conformation [34]. The mass of VEGF that bound during the immunoassay was normalized to the mass of VEGF released measured for each experimental condition using the I¹²⁵-VEGF radiotracer.

3. Results

PEG was covalently reacted with recombinant CaM(T34C, T110C) to form PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates. PEGDA₃₄₀₀'s terminal acrylate groups underwent a Michael-type addition reaction with the mutant cysteine residues in CaM(T34C, T110C), and this reaction proceeded to completion as demonstrated by MALDI-ToF mass spectrometry (Supplementary Figure 1). PEG₃₄₀₀-CaM-PEG₃₄₀₀ microsphere formation was dependent on the ϕ of PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose in the pre-polymer solution. Pre-polymer solutions containing ϕ s of 15% PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose and 17.5% PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose formed macroscopic hydrogel disks when photopolymerized (Figure 1A–B). Alternatively, pre-polymer solutions containing ϕ s of 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose and 22.5% PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose formed hydrogel microspheres (Figure 1C–D). PEG-diacrylate pre-polymer solutions without CaM did not form microspheres under any condition tested (data not shown). Also, no phase separation was observed in solutions containing pure CaM and raffinose (Supplementary Figure 2). Pre-polymer solutions containing a ϕ of 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀ that was lyophilized without raffinose formed macroscopic hydrogel disks (Figure 1E).

The stability of the aqueous two phase suspension affected the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. After mixing, two distinct phases formed in the pre-polymer solution over the course of 2.5 hours (Figure 2A–F). Immediately after mixing, microspheres formed with an average diameter of $11 \pm 8 \mu\text{m}$. 30 minutes after mixing, microspheres formed with an average diameter of $11 \pm 6 \mu\text{m}$, which was not statistically different than the immediately formed microspheres. After 60 minutes, two distinct pre-polymer phases were visible and the microspheres' average diameter was statistically smaller than the immediately formed microspheres (Figure 2C–F, I–M). Although the average diameter of the microspheres decreased after 60 minutes, a limited population of large microspheres still formed (Figure 2I–L). After 90 minutes, microspheres formed from the bottom phase were statistically smaller than microspheres formed from the top phase (Supplementary Figure 3A). After 120 minutes, microspheres formed from the top and bottom phases did not have statistically different average diameters (Supplementary Figure 3B–C).

The raffinose concentration controlled the induction of phase separation in the pre-polymer solution and the partitioning of the PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates between the two phases. In the absence of raffinose no phase separation was observed. Increasing the raffinose concentration in the pre-polymer solution increased the concentration of PEG-CaM-PEG in the top phase and decreased the concentration of PEG-CaM-PEG in the bottom phase (Figure 3).

CaM's conformation in the pre-polymer solution affected the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres formed with a mean diameter of $15 \pm 9 \mu\text{m}$ in Ca^{2+} buffer, in which CaM is known to favor its "extended dumbbell" conformation[35]. In contrast, the microspheres' average diameter increased to $65 \pm 36 \mu\text{m}$ when formed in EGTA buffer or $32 \pm 18 \mu\text{m}$ when formed in Mg^{2+} buffer, in which CaM is known to favor its "apo-conformation" (Figure 4) [36].

Other easily variable initial processing conditions, including the photoinitiator concentration and mixing time, also affected the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. Pre-polymer solutions that did not contain Irgacure 2959 did not form hydrogels (data not shown). Increasing the photoinitiator concentration produced microspheres with smaller average diameters (Figure 5A). Increasing the mixing time decreased the microspheres' average diameter. For example, pre-polymer solutions mixed for 1 second formed

microspheres with an average diameter of $13 \pm 11 \mu\text{m}$. Alternatively, pre-polymer solutions mixed for 60 seconds formed microspheres with an average diameter of $3 \pm 1 \mu\text{m}$ (Figure 5B).

The photopolymerization time affected the swelling of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. Microspheres crosslinked for longer periods of time underwent smaller magnitude increases in swelling, which in this study was evaluated by the mean diameter of the swollen microspheres after 24 hours in Ca^{2+} buffer. For example, the mean diameter of microspheres crosslinked for 0.5 minutes was $15 \pm 9 \mu\text{m}$ after swelling, while the mean diameter of microspheres that were crosslinked for 3 minutes was $10 \pm 5 \mu\text{m}$ after swelling (Figure 6).

The magnitude of the microspheres' ligand-induced volume change was modulated by the photopolymerization time and ligand identity. Increasing the photopolymerization time produced microspheres with decreasing magnitudes of ligand-induced volume changes ($p < 0.05$, t test). 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose microspheres underwent ligand-induced volume decreases of $47 \pm 9\%$ for microspheres crosslinked 0.5 minutes, $33 \pm 8\%$ for microspheres crosslinked 1 minute, and $27 \pm 8\%$ for microspheres crosslinked 3 minutes (Figure 7A). The magnitude of the microspheres' ligand-induced volume changes could also be tuned using different CaM-specific ligands ($p < 0.05$, t test). For example, after 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀+raffinose microspheres were crosslinked for 0.5 minutes, TFP induced a $47 \pm 9\%$ volume decrease and CPZ induced a $76 \pm 10\%$ volume decrease. Also, CPZ induced faster ligand-induced volume changes than TFP (Figure 7B).

Importantly, the aqueous two phase suspension polymerization approach facilitated high VEGF encapsulation efficiencies, and CaM's ligand-induced conformational change controlled the timing of VEGF release. Up to $65.8 \pm 4.8\%$ of the VEGF included in the pre-polymer solution was encapsulated into the PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. The photopolymerization time did not have a significant effect on the encapsulation efficiency of VEGF into PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres ($p > 0.05$, t test) (Figure 8A). The timing of VEGF release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres was controlled by varying the timing of the microspheres' exposure to TFP. Microspheres formed from $5 \mu\text{l}$ of pre-polymer solution released $6.6 \pm 0.8 \text{ ng}$ of VEGF when they were exposed to TFP at $T=0$, and $6.6 \pm 0.6 \text{ ng}$ of VEGF when they were first maintained in Ca^{2+} buffer for 24 hours and then exposed to TFP (Figure 8B). Also, without TFP, there was limited release of VEGF (Figure 8B-C). Together, these data demonstrated that VEGF release could be triggered at a specific time in response to a specific biochemical ligand. TFP also triggered an increase in the VEGF release rate multiple times from the same population of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres (Figure 8C). However, the mass of VEGF released with each additional cycle of TFP exposure was decreased, likely due to VEGF depletion from the hydrogel network. Increasing the photopolymerization time decreased the VEGF release rate from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres ($p < 0.05$, t test). Microspheres that were photopolymerized for 0.5 minutes released $6.6 \pm 0.8 \text{ ng}$ of VEGF after a five minute TFP exposure, while microspheres that were photopolymerized for 3 minutes released $2.5 \pm 0.4 \text{ ng}$ of VEGF after a five minute TFP exposure (Figure 8D). Increasing the photopolymerization time decreased the fraction of encapsulated VEGF released from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres ($p < 0.05$, t test). Microspheres photopolymerized for 0.5 minutes released $91.9 \pm 8.1\%$ of their encapsulated VEGF after 4 cycles of TFP exposure, while microspheres crosslinked for 3 minutes released $53.1 \pm 3.5\%$ of their incorporated VEGF after 4 cycles of TFP exposure (Figure 8E). The fraction of VEGF in its native conformation was significantly decreased after encapsulation and TFP-triggered release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres ($p < 0.05$, t test) (Figure 8F).

The aqueous two phase suspension polymerization also facilitated the encapsulation and release of BMP-2 from dynamic hydrogel microspheres. $79.5 \pm 3.0\%$ of the BMP-2 in the pre-polymer solution was encapsulated into the hydrogel microspheres, which was statistically greater than VEGF's encapsulation efficiency ($p < 0.05$, t test) (Figure 9B). The timing of BMP-2 release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres was also controlled by varying the timing of the microspheres' exposure to TFP. Microspheres released $27.0 \pm 3.5\%$ of their encapsulated BMP-2 when they were exposed to TFP at $T=0$. TFP exposure triggered the release of an additional $23.7 \pm 4.2\%$ of the encapsulated BMP-2 when the microspheres were first maintained in Ca^{2+} buffer for 24 hours and then exposed to TFP (Figure 9C). TFP exposure triggered the release of BMP-2 multiple times from the same population of microspheres. However, TFP induced a smaller fraction of the encapsulated BMP-2 released compared to the triggered fraction of VEGF released (Figure 9D).

4. Discussion

The PEG-CaM-PEG synthesis and purification approach used in this study could be used to form a broad range of other protein-polymer conjugates. There are many proteins with surface exposed cysteines that could participate in similar Michael-Type addition reactions [37]. In this study, excess PEGDA was removed using column chromatography, which has been extensively used to purify proteins [38]. Therefore this general approach could be used to purify other protein-polymer conjugates. After purification, raffinose was added to the PEG₃₄₀₀-CaM-PEG₃₄₀₀ solution at a concentration which has previously been shown to preserve CaM's activity during lyophilization [14]. Sugars, such as sucrose, raffinose, and trehalose, have previously been used in the pharmaceutical industry to maintain the activity of a diverse set of proteins during lyophilization [39–40]. Therefore, in this approach the sugar achieved two distinct and important functions: 1) the induction of phase separation for microsphere synthesis; and 2) stabilization of the protein against denaturation. In order to take advantage of these two functions in future studies of protein-polymer conjugates, it will be important to include optimized concentrations of sugars during processing.

Emulsion formation was dependent upon the establishment of immiscible PEG₃₄₀₀-CaM-PEG₃₄₀₀ and raffinose solution phases. In the current study, macroscopic hydrogel disks were formed from solutions that contained lower ϕ s of PEG₃₄₀₀-CaM-PEG₃₄₀₀ + raffinose, while hydrogel microspheres were formed from solutions that contained higher ϕ s (Figure 1A–D). Stenekes *et al.* observed a similar trend for mixtures of dextran and PEG, in which microsphere formation was dependent upon the polymers' concentrations in solution [41]. In this current study, PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates did not form microspheres in the absence of raffinose (Figure 1E). Also, pre-polymer solutions that contained only PEG-diacrylate and raffinose did not form microspheres in any condition examined (data not shown). Furthermore, no phase separation was observed in pre-polymer solutions containing pure CaM and raffinose (Supplementary Figure 2). These results suggested that unfavorable interactions between PEG₃₄₀₀-CaM-PEG₃₄₀₀ and raffinose may induce the formation of an aqueous emulsion. Together, these observations suggested that two immiscible aqueous phases formed, a dispersed phase and a continuous phase, and that the PEG₃₄₀₀-CaM-PEG₃₄₀₀ concentration was too low in the continuous phase to form hydrogel networks (Figure 1F).

The phase separation's stability affected the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. Over the course of 150 minutes two distinct phases developed which contained a PEG₃₄₀₀-CaM-PEG₃₄₀₀ rich top phase (Figures 2 and 3). Microspheres derived from the bottom phase decreased in size and increased in polydispersity at later time points (Figure 3). As time progressed, the microspheres' mean size decreased which was indicative of coarsening dominated by coalescence instead of Ostwald Ripening [42]. Similar results

have been reported for other PEG containing hydrogel microspheres formed using an aqueous two phase suspension polymerization [43]. 90 minutes after mixing, microspheres from the bottom phase formed with an average diameter of $5\pm 9\mu\text{m}$ and microspheres from the top phase formed with an average diameter of $10\pm 6\mu\text{m}$ (Supplementary Figure 3A). These results suggested that differently sized microsphere populations could be formed from a single pre-polymer solution. This capability could be useful in applications that require control over hydrogel microsphere geometry [44]. Also, the raffinose concentration in the pre-polymer solution influenced the partitioning of PEG-CaM-PEG between the top and bottom phases, which could influence the size of the resulting microspheres (Figure 4). However, this mechanism to tailor microsphere size may not be ideal in future embodiments of this approach, as the ratio of protein to excipient sugar will also need to be optimized to maximize protein stability during processing.

CaM's conformation in the pre-polymer solution influenced the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres (Figure 4). This may be explained in part by the previous observation that CaM is more hydrophobic in its Ca²⁺ bound "extended dumbbell" conformation than in its Ca²⁺ free "apo conformation" (Figure 4, inset crystal structures) [45]. Specifically, Ca²⁺-bound CaM's increased hydrophobicity could have facilitated closer packed PEG₃₄₀₀-CaM-PEG₃₄₀₀ conjugates in the dispersed phase due to interactions between PEG and hydrophobic protein domains [46], and CaM's low affinity dimerization in concentrated solutions [47]. The increased PEG₃₄₀₀-CaM-PEG₃₄₀₀ packing density in the dispersed phase could have also increased the probability of terminal acrylate groups encountering reactive acrylate groups during photopolymerization, thereby increasing the microsphere formation rate. Indeed, increasing the microspheres' formation rate, by increasing the photoinitiator concentration, restricted the microspheres' ability to coarsen and decreased their average diameter (Figure 4 and 5A). Nichols *et al.* observed a similar trend in which the mean diameter of PEG microspheres decreased with increasing temperatures during microsphere formation. Their study hypothesized that PEG's solubility decreased with increasing temperatures and, in combination with the temperature-enhanced radical initiation rate, this resulted in microspheres with smaller mean diameters than microspheres formed at lower temperatures [43]. Many proteins change their solubility when they undergo conformational changes [48]. Therefore, allosteric protein conformational changes may provide a new, general mechanism to control the size of hydrogel microspheres during processing.

Increasing the photopolymerization time decreased the magnitude of hydrogel swelling (Figure 6). This result is consistent with established Flory-Rehner hydrogel network theory [49] and the findings in previous studies of PEG₃₄₀₀-CaM-PEG₃₄₀₀ hydrogel disks, in which hydrogels with greater crosslinking densities had smaller equilibrium swelling ratios [14]. Therefore, photopolymerization could provide an adaptable mechanism to tune the hydrogel network properties of PEG-CaM-PEG hydrogels for a wide variety of biomaterial applications.

CaM's nanometer scale conformational change translated to rapid, high magnitude hydrogel microsphere volume changes (Figure 7). Microspheres with larger magnitude swelling changes underwent greater magnitude ligand-induced volume changes (Figure 6 and 7A). This trend has been observed in previous studies, in which hydrogel disks with greater equilibrium swelling ratios underwent greater magnitude ligand-induced volume changes [14]. The magnitude of the microspheres' ligand induced volume changes was tuned by varying the identity of the CaM-specific ligand. CPZ produced larger magnitude ligand-induced volume changes than TFP. This observation could be attributed to CPZ's higher affinity for CaM compared to TFP (Figure 7B) [50]. This trend mirrors the results previously observed for PEG-CaM-PEG microspheres that were formed with a water-in-oil

suspension polymerization, in which CPZ induced larger magnitude hydrogel volume decreases than TFP [15]. Also, CPZ triggered faster ligand-induced volume changes than TFP. CPZ has a lower molecular weight than TFP. Therefore CPZ could diffuse faster than TFP through PEG₃₄₀₀-CaM-PEG₃₄₀₀ hydrogels, which would produce more rapid volume changes. Therefore, designed variations in ligand-protein affinity and molecular weight may ultimately provide a more broadly adaptable mechanism to control the properties of dynamic hydrogels.

The growth factor encapsulation technique is also a critical regulator of growth factor release from hydrogel networks, as the encapsulation technique controls the mass of growth factors originally incorporated within the hydrogel network. Growth factors have been encapsulated in hydrogels either by infusing them into existing hydrogels in an “uptake and release” approach, or by including them in the pre-polymer solution prior to crosslinking. The uptake and release approach has maintained growth factor bioactivity, but has not facilitated high growth factor encapsulation efficiencies since the growth factors must diffuse from a concentrated growth factor solution into a pre-formed hydrogel. For example, less than 15% of the total mass of VEGF in solution was encapsulated into gelatin hydrogels over the course of 24 hours using an uptake and release approach [51]. Encapsulating growth factors during hydrogel crosslinking has enabled greater growth factor encapsulation efficiencies compared to the uptake and release approach and has facilitated the rapid formation of the growth factor laden hydrogels. For example, Gu *et al.* encapsulated 67% of the initial VEGF in the pre-polymer solution into alginate microspheres in less than one hour [34]. High growth factor encapsulation efficiencies are critically important for clinical growth factor-releasing devices, in which milligram quantities of growth factors are often delivered for therapeutic benefit [52]. Therefore, the development of biomaterial processing techniques that facilitate high growth factor encapsulation efficiencies are critically needed for many biomedical applications.

In this study, the VEGF encapsulation efficiency was $65.8 \pm 4.8\%$ (Figure 8A), which was similar to the protein encapsulation efficiencies previously observed with other aqueous two phase suspension polymerizations. For example, dextran microspheres had a 98% immunoglobulin G (IgG) encapsulation efficiency when 0.22 M KCl was included in the aqueous two phase suspension to induce IgG partitioning into the dispersed phase [53–54]. In a separate study, De Groot *et al.* obtained greater than 90% encapsulation efficiencies of interleukin-2 into hydroxyethyl methacrylated dextran (HEMA-dextran) microspheres if the water content of the aqueous two phase suspension was below 70% [55]. Recently, Vlugt-Wensink *et al.* characterized the effect of common industrial excipients on the encapsulation efficiency of human growth hormone (hGH) into HEMA-dextran microspheres during an aqueous two phase suspension polymerization process. hGH encapsulation efficiencies of 47–68% were achieved in the absence of excipients, but only 27% of the total hGH was encapsulated in the microspheres in the presence of a common excipient, sucrose. The decreased encapsulation efficiency of hGH in the presence of sucrose was attributed to sucrose inducing a greater fraction of hGH to partition into the continuous phase [56]. Taken together, these studies suggest that an emulsion’s physiochemical properties strongly influence the partitioning of proteins into hydrogel microspheres and, in turn, their encapsulation efficiency. It is noteworthy that VEGF encapsulation during photopolymerization greatly increased the encapsulation efficiency (66% of total VEGF encapsulated/5mg microspheres) when compared to a previously used uptake and release approach [15] (10% of total VEGF encapsulated/50mg microspheres) (Figure 8A). Therefore, aqueous two phase suspension polymerizations may enable the use of dynamic hydrogels in applications where high growth factor loading efficiencies are necessary.

In this study, the ligand-induced volume decrease produced fluid exclusion and triggered VEGF and BMP-2 release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. This mechanism is distinct from the mechanisms that control protein release from traditional dynamic hydrogels that undergo phase-transition-induced network collapse. In particular, protein release is typically restricted during phase transition-induced volume changes due to the hydrogels' decreased mesh size and the protein's interaction with hydrophobic polymer chains [10]. In contrast, PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres rapidly released 7-fold more VEGF upon ligand-induced collapsed compared to microspheres maintained in Ca²⁺ buffer (Figure 8C). This ligand-induced increase in the mass of VEGF released is similar to the 8-fold increase in VEGF released from PEG-CaM-PEG microspheres formed with a water-in-oil suspension polymerization in a previous study [15].

Hydrogels that were crosslinked for longer periods of time released less VEGF when triggered with a CaM-specific ligand than microspheres that were crosslinked for shorter periods of time (Figure 8D–E). The decreased mass of VEGF released from more highly crosslinked microspheres could be attributed to their smaller magnitude ligand-induced volume changes (Figure 7A), and the possibility that VEGF was covalently incorporated into the hydrogel network during photopolymerization. This covalent protein incorporation during encapsulation into hydrogels was previously observed by Lin and Metters in PEG hydrogel networks. They decreased the level of protein conjugation into hydrogel networks by conducting photopolymerization in the presence of Cu²⁺, Zn²⁺, and Ni²⁺ [57]. Therefore, including these divalent cations in the pre-polymer solution may facilitate the complete release of encapsulated growth factors in future studies.

In agreement with previous reports, the aqueous two phase suspension polymerization decreased the fraction of VEGF in its native conformation compared to the VEGF that had never been encapsulated into or released from hydrogel microspheres (Figure 8F). For example, Gu *et al.* demonstrated that VEGF maintained 57% of its bioactivity after encapsulation in a acrylated *star*-poly(ϵ -caprolactone-*co*-d,l-lactide) photocrosslinked material [58]. Protein unfolding has been mechanistically explored by Lin *et al.* by varying the polymerization initiator solubility, polymer concentration, and crosslinking time. In general, a protein's native folding was decreased during the photocrosslinking process by increasing the initiator concentration, decreasing the polymer concentration, and increasing the initiator hydrophobicity [59]. Together, these results suggest there are several approaches that could be used in future studies to increase VEGF's activity after encapsulation and release from PEG-CaM-PEG hydrogel microspheres. However, in this study we were unable to directly test VEGF's bioactivity in a cell-based assay after triggered release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ hydrogel microspheres, as the TFP and CPZ concentrations used were cytotoxic to endothelial cells. To address this concern we are currently developing cell impermeable CaM binding ligands that will potentially not affect cell viability for future studies.

Electrostatic attraction plays a key role in controlling the encapsulation and release of growth factors from dynamic, protein-based hydrogels. Previously, increasing the concentration of negatively charged CaM in hydrogel networks increased the mass of positively charged VEGF absorbed into the hydrogel disks and decreased VEGF's release rate from the disks [14]. Similar trends were observed in this study when we compared the encapsulation and release of VEGF (isoelectric point=8.5) and the more positively charged BMP-2 (isoelectric point=9.0) from PEG-CaM-PEG hydrogel microspheres (Figure 9). For example, BMP-2 had a higher encapsulation efficiency than VEGF (79.5 $\pm$ 3.0% and 65.8 $\pm$ 4.8% for BMP-2 and VEGF, respectively) which could be attributed to the potentially increased electrostatic attraction between BMP-2 and CaM (Figure 9A–B). However, many factors control protein partitioning and encapsulation in aqueous two phase systems,

including polymer charge, polymer concentration, polymer molecular weight, protein concentration, salt concentration, salt identity, and temperature [60–61]. Therefore, other factors may play an important role in controlling the encapsulation efficiency of therapeutic proteins into dynamic hydrogel microspheres. The increased electrostatic attraction between BMP-2 and CaM also likely played a significant role in tuning the growth factor release rate. For example, PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres released 66.8±8.7% of their encapsulated VEGF, but only 27.0±3.5% of their encapsulated BMP-2, when exposed to TFP for 5 minutes (Figure 9C–D). Many growth factors are positively charged at physiological pH [62], therefore tailoring the electrostatic attraction between the hydrogel and growth factors may be useful to tune protein encapsulation and release from future dynamic, protein-based materials.

Significantly, the electrostatic attraction between CaM and BMP-2 facilitated the triggered release of BMP-2 from PEG₃₄₀₀-CaM-PEG₃₄₀₀ hydrogels after 24 hours in ligand free buffer (Figure 9C). This temporal control over BMP-2 release, in combination with the previously demonstrated temporal control over VEGF release, suggested that dynamic, protein-based materials could be used to control the release of other growth factors. This control over growth factor release could be important during processes where the temporal release of growth factors, over the course of hours to days, directs the progression of important biological processes including wound healing, tissue regeneration, and stem cell differentiation [1–4].

5. Conclusion

We have developed an adaptable approach to form dynamic, protein-based microspheres using an aqueous two phase suspension polymerization in the presence of a common pharmaceutical excipient, raffinose. These microspheres formed without the use of an organic phase, surfactants, mechanical strain, or toxic photoinitiators. Significantly, these hydrogel microspheres underwent ligand-induced volume changes in response to specific biochemical ligands, which affords increased specificity over traditional dynamic hydrogel microspheres that respond to physiochemical stimuli. The aqueous two phase suspension polymerization approach produced microspheres with a high encapsulation efficiency of therapeutically significant proteins, VEGF and BMP-2. Temporal control over VEGF and BMP-2 release was achieved by varying the timing of CaM's ligand-induced conformational change. The approach described here to form protein-based hydrogel microspheres may be broadly adaptable for applications that require high protein encapsulation efficiencies, require temporally-triggered release, and/or prohibit the use of organic solvents, surfactants, or mechanical strain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of the pre-polymer solution conditions that induce microsphere formation. Photomicrographs of hydrogels formed from pre-polymer solutions with varying ϕ s of PEG-CaM-PEG + raffinose. The white lines demarcate the edges of macroscopic hydrogel disks (A-D). Photomicrograph of a hydrogel formed from a 20% PEG-CaM-PEG pre-polymer solution without raffinose (E) (Hydrogel disks in A, B, and E are delineated by dashed white circle). Schematic representation of PEG-CaM-PEG hydrogel microsphere formation via an aqueous two phase suspension polymerization in the presence of raffinose, and macroscopic hydrogel disk formation in the absence of raffinose (F).

Figure 2.

Characterization of the effect of aqueous two phase suspension stability on the size distribution of hydrogel microspheres. Photographs of the pre-polymer solution A) 0 minutes, B) 30 minutes, C) 60 minutes, D) 90 minutes, E) 120 minutes, and F) 150 minutes after mixing. Representative bright field micrographs of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres formed from the bottom phase of pre-polymer solutions G) 0 minutes, H) 30 minutes, I) 60 minutes, J) 90 minutes, K) 120 minutes, and L) 150 minutes after mixing. Scalebar denotes 30μm. M) Size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres formed from pre-polymer solutions acquired from the bottom of the sample tubes. * Denotes significant difference from fraction of initially formed microspheres, t test, $p < 0.05$.

Figure 3. Fraction of PEG₃₄₀₀-CaM-PEG₃₄₀₀ in the top or bottom phases of pre-polymer solutions composed of 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀ and varying initial raffinose concentrations.

Figure 4.

Characterization of the effect of CaM's conformation on the size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. Representative photomicrographs of microspheres formed with A) Ca²⁺ buffer, B) EGTA buffer, and C) Mg²⁺ buffer, with inlayed crystal structures of CaM's likely conformation in each pre-polymer solution. The red hydrophobic methionine residues are solvent-exposed in Ca²⁺-bound CaM, and solvent-buried in Ca²⁺-free CaM (PDB, 3CLN and 1CDF). D) Size distribution of 20% PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres that were formed with Ca²⁺ buffer, EGTA buffer, or Mg²⁺ buffer.

Figure 5. Effect of simply tunable microsphere processing conditions on the size of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. A) Size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres formed with varying photoinitiator concentrations. * Denotes significant difference from microspheres formed with 0.05% Irgacure 2959, t test, $p < 0.05$. Scalebar denotes $15 \mu\text{m}$. B) Size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres formed with varying mixing times. * Denotes significant difference from microspheres formed with 10 seconds of mixing, t test, $p < 0.05$.

Figure 6.

Characterization of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microsphere swelling. Size distribution of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres that were crosslinked for A) 0.5 minutes, B) 1 minute, C) 2 minutes, and D) 3 minutes before and after swelling.

Figure 7.

Characterization of the ligand-induced volume change of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. A) TFP-induced volume change of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres that were photocrosslinked for (■) 0.5 minutes, (■) 1 minute, or (■) 3 minutes with representative micrographs of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres photocrosslinked for 0.5 minutes before and after exposure to TFP. Scalebar represents 50μm. B) Ligand-induced volume changes of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres that were exposed to TFP (■) or CPZ (■) with representative micrographs of PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres photocrosslinked for 0.5 minutes before and after exposure to CPZ. Scalebar represents 50μm. C) Schematic representation of CaM's nanometer scale conformational change translating to hydrogel microsphere volume changes (PDB, 3CLN and 1LIN).

Figure 8.

VEGF encapsulation and temporally triggered release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. A) The fraction of VEGF incorporated into PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres after 0.5 or 3 minutes of UV photocrosslinking. B) The mass of VEGF released from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres continuously exposed to Ca²⁺ buffer (■), exposed to TFP after 0 and 2 hours (■), or maintained in Ca²⁺ buffer then exposed to TFP after 24 and 26 hours (■). C) The mass of VEGF released from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres exposed to Ca²⁺ buffer (■) or multiple cycles of TFP exposure and Ca²⁺ buffer washes (■). D) The mass of VEGF released from microspheres crosslinked with for 0.5 minutes (■) or 3 minutes (■). E) The fraction of VEGF released from microspheres crosslinked for 0.5 minutes and continuously exposed to Ca²⁺ buffer (■), crosslinked for 0.5 minutes and subjected to multiple cycles of TFP exposure and Ca²⁺ buffer washes (■), or crosslinked for 3 minutes and subjected to multiple cycles of TFP exposure and Ca²⁺ buffer washes (■). F) The fraction of VEGF that maintained its native conformation after encapsulation and release from microspheres in the presence of TFP, or VEGF that was never encapsulated into microspheres. Buffer exposed to microspheres that did not contain VEGF was also measured.

Figure 9.

BMP-2 and VEGF encapsulation and release from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres. A) Crystal structures of negatively charged CaM (PDB, 3CLN) in which its acidic residues are blue and positively charged VEGF (PDB, 2VPF) and BMP-2 (PDB, 3BMP) in which their basic residues are red. B) The fraction of BMP-2 and VEGF incorporated into PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres after 0.5 minutes of UV photocrosslinking. * Denotes significant difference from fraction of BMP-2 incorporated, t test, $p < 0.05$. C) The mass of BMP-2 released from PEG₃₄₀₀-CaM-PEG₃₄₀₀ microspheres exposed to TFP after 0 and 2 hours (■), or maintained in Ca²⁺ buffer then exposed to TFP after 24 and 26 hours (■). D) The fractions of VEGF released from microspheres continuously exposed to Ca²⁺ buffer (--■--), VEGF released from microspheres subjected to multiple cycles of TFP exposure and Ca²⁺ buffer washes (-■-), BMP-2 released from microspheres continuously exposed to Ca²⁺ buffer (--■--), or BMP-2 released from microspheres crosslinked for 0.5 minutes and subjected to multiple cycles of TFP exposure and Ca²⁺ buffer washes (-■-).