Pulsatile Release of Insulin from Layer-by-Layer Assembled Microgel Thin Films

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Summary: We describe studies concerning the construction and characterization of insulin-impregnated poly(*N*-isopropylacrylamide-*co*-acrylic acid) microgel thin films prepared by Layer-by-Layer (LbL) polyelectrolyte assembly. These films can be built up in a highly uniform fashion and display linear buildup dependence even up to 30 layers. Thermoresponsivity of these drug loaded films can be utilized to obtain extended pulsatile release of insulin over many cycles. Continuous thermal pulsing allows solubilization of the embedded peptide and subsequent diffusion through the film layers. The magnitude of release can be tuned based on film thickness. This type of microgel thin film construct proves to be extremely robust and can potentially pulse out constant bursts of peptide for more than one month at a time.

Keywords: insulin; macromolecule drug delivery; microgels; polyelectrolyte assembly; pulsatile release

Introduction

The field of polyelectrolyte multilayer thin films has recently found great popularity in biotechnology. The buildup of these films, first introduced by Decher,[1] is commonly achieved through a Layer-by-Layer (LbL) protocol where oppositely charged polyelectrolytes are deposited based on alternating electrostatics.[2] There are many advantages to this type of technology and entail precise manipulation at the molecular level as well as three dimensional control over the surface functionality and constitution of these systems. Furthermore, this process is simple and quite adaptable.[3] Thus, many applications in the biosciences have been explored including loading and release of small model drug compounds within polyelectrolyte thin films.[4,5] Incorporation of biologically relevant molecules into multilayer thin films has also been successfully explored for the field of biosensing.[6-10] Caruso and coworkers have made significant advances in the development of hollow polyelectrolyte capsules that could potentially be utilized for drug release applications.[11] Sukhorukov et al. expanded these investigations and encapsulated macromolecular species into polyelectrolyte shells based on pH dependent tunability of the shell porosity.[12-14] Enhancing the functionality of these systems is highly attractive and has been successfully explored using metal and inorganic colloidal nano-objects.[15-19]

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Integration of stimuli responsive soft materials such as hydrogel networks presents another compelling direction to be explored.

Hydrogels are water swollen polymeric networks that have been vigorously investigated as vehicles for controlled drug release systems due to their high water content, soft tissue-like consistency and, potential biocompatibility.[20-24] Poly(*N*-isopropylacrylamide) (pNIPAm), one of the most widely explored temperature sensitive hydrogels, displays a reversible volume phase transition at a lower critical solution temperature (LCST) of 31 °C, where the hydrogel network hydrophobically collapses upon itself, expelling water in an entropically favored fashion.[25] In recent years, significant work has focused on utilizing this reversible deswelling event for controlled peptide release, among which, many studies have focused on hydrogel insulin delivery systems.[26-31] Work done previously in our group has shown that inclusion of discrete thermoresponsive microgels within multilayer thin films can be achieved in order to build up functionally enhanced thermoresponsive polymeric thin films.[32] We wanted to extend these studies to the investigate controlled macromolecule release from thermoresponsive multilayer thin films. In this work, we present results dealing with the construction and characterization of insulin-impregnated microgel thin films for controlled pulsatile release applications. Our aim was to effectively load a model peptide, insulin, into stimuli responsive microgels and then build up multilayer thin films in a uniform manner. We hoped to achieve extended, cyclical release of the peptide while tunability over the quantity of macromolecule released was explored by controlling the release temperature and film thickness. These highly robust films show potential in future macromolecule drug release systems.

Experimental

Materials and measurements

All chemicals were obtained from Sigma Aldrich unless otherwise stated. *N*-isopropylacrylamide (NIPAm) was recrystallized from hexane (J. T. Baker) prior to use. *N,N'*-Methylenebis(acrylamide) (BIS), ammonium persulfate (APS), anhydrous acrylic acid (Fluka), 95 % ethanol, 200 proof anhydrous ethanol, KOH, formic acid (J.T. Baker), 70,000 MW polyallylamine hydrochloride (PAH), FITC-insulin (5,800 MW; monomeric) from bovine pancreas, sodium chloride and potassium dihydrogenphosphate (KH_2PO_4) were used as received. 3-aminopropyltrimethoxysilane (APTMS) was from United Chemical Technologies. Anhydrous dibasic sodium phosphate (Na_2HPO_4) was purchased

from EM Science. Glass microscope coverslips (22 x 22 mm) were purchased from Fisher Scientific. 0.2 μm nylon membrane discs and Specta/Por 10, 000 MWCO dialysis membrane were purchased from VWR. Water used in all experiments was distilled and then purified using a Barnstead E-Pure system operating at a resistance of 18 MΩ. A 0.2 μm filter was incorporated into this system to remove particulate matter.

1 mol % BIS crosslinked p(NIPAm-*co*-AAc) (9:1) microgels were synthesized via free radical precipitation polymerization as described previously, with minor modifications.[33] The total monomer concentration was 100 mM, APS was used as the radical initiator and no surfactant was used. The NIPAm monomer and BIS crosslinker were dissolved in 100 mL of nanopure water and then continuously stirred in a three-neck, 200 mL round-bottom flask. This mixture was heated to 70 °C while being purged with N_2 gas. Once the solution was stable at 70 °C, the acrylic acid comonomer was added. Fifteen minutes later, the reaction was initiated by adding a hot (70 °C) 35 mg/mL APS solution (1 mM total concentration). The reaction mixture turned turbid within 10-15 minutes, denoting effective initiation. Polymerization proceeded for 4-6 hours under a constant stream of nitrogen. Following synthesis, the microgels were cleaned via filtration with a P2 Whatman filter paper and then dialysis (using 10,000 MWCO) for 2 weeks against nanopure water with a daily exchange of fresh nanopure water.

Photon correlation spectroscopy (PCS, Protein Solutions, Inc.) was used to obtain hydrodynamic radii and light scattering intensities of the microgel solutions. Solutions of pH 3.5 and 6.5, 10 mM, were first prepared using the appropriate buffer systems (formate and phosphate). Prior to analysis, the cleaned microgels were diluted in filtered media (using 0.2 μm filters) until a count rate of 250 KCt/sec was obtained. The suspensions were then equilibrated at each temperature for 10 minutes before measurements were taken. Longer equilibration times did not result in variations of particle radius, polydispersity or light scattering intensity. The data points presented here are an average of 25 measurements with a 5 second acquisition time and a S/N threshold of 2.5. Hydrodynamic radii were calculated from the measured diffusion coefficients using the Stokes-Einstein equation. All correlogram analyses were performed with manufacturer-supplied software (Dynamics v.5.25.44, Protein Solutions, Inc.).

To load the microgels with insulin, a stock solution of FITC-insulin was first prepared (3 mg FITC-insulin/1.5 mL HCl) since insulin is most soluble at low pH values.[29] This concentrated solution was then mixed with 10 mL of the 1 mol % BIS p(NIPAm-*co*-AAc)

(9:1) microgels, and the pH was adjusted to 7.4 by slow addition of base (0.1 M NaOH). This solution was allowed to stir in the refrigerator overnight in the dark.

The spin coating technique was employed to quickly build up the multilayer thin films. Glass microscope slides were used as the substrates and were first cleaned by placing in a plasma cleaner using Argon gas (Harrick Plasma Cleaner/Sterilizer PDC-32G) for 10-15 minutes. After this treatment the slides were rinsed vigorously with 200 proof anhydrous ethyl alcohol. APTMS was then used to yield positively charged amine-functionalized glass slides. The slides were bathed in a 0.4% APTMS solution (in 200 proof ethanol) for two hours at room temperature, and were then rinsed with 95% ethanol to remove any unreacted silane. The slides were stored in 95% ethanol until use. Prior to spin coating, an APTMS-functionalized slide was rinsed copiously with nanopure water and then dried under nitrogen gas. The slide was positioned on the spin coater (Speedline Technologies, Spincoater, P6700 Series) vacuum chuck. During film deposition, the rotor speed was maintained at 3000 rpm. Deposition of one bilayer consisted of depositing 5 drops of the FITC-insulin impregnated microgels, rinsing well with nanopure water, depositing 5 drops of a 4.0 x 10^{-5} M PAH solution and then rinsing with water. After each step, adequate removal of excess water was ensured by allowing 20 seconds to pass between steps. To monitor the buildup of these multilayer thin films, UV-vis spectroscopy was employed. After each bilayer of a film was deposited, the absorbance of the film was taken in the wavelength range of 400-600 nm (fluorescein absorption) using a bare glass slide as a reference.

To probe the direct release capabilities of these films, a rectangular piece of an insulin-impregnated microgel thin film (10 x 22 mm) was cut and then mounted to the side of the cuvette that was filled with 1.35 mL of 0.02 M PBS release medium. The fluorescence emission of the release medium was monitored under constant stirring at 220 rpm using an excitation wavelength of 473 nm and an emission wavelength of 512 nm. Thermally induced pulsatile release was monitored at 25, 37 and 40 °C. Extended release invesitgations of the insulin loaded films were also performed by placing the films in 4 mL of 0.02 M PBS. These films were left in cold medium (25 °C) for one hour, the medium was then fully replaced and the films were placed in a 40 °C hot bath for the same length of time and the cycle was then repeated every hour for one month. The release medium samples were then analyzed via fluorescence spectroscopy using an excitation wavelength of 473 nm and an emission wavelength of 512 nm, to obtain cumulative release profiles.

Results and Discussion

Particle Synthesis and Characterization

Dually responsive 1 mole % BIS crosslinked p(NIPAm-*co*-AAc) (9:1) microgels were selected for the fabrication of insulin-impregnated multilayer thin films. The principal monomer, NIPAm, lends thermosensitivity to these systems, while the comonomer, acrylic acid, allows for pH tunability. Characterization of particle size as a function of temperature and pH was ascertained using PCS. Panel a of Figure 1 displays the volume phase transition behavior of these microgels at two different pH values.

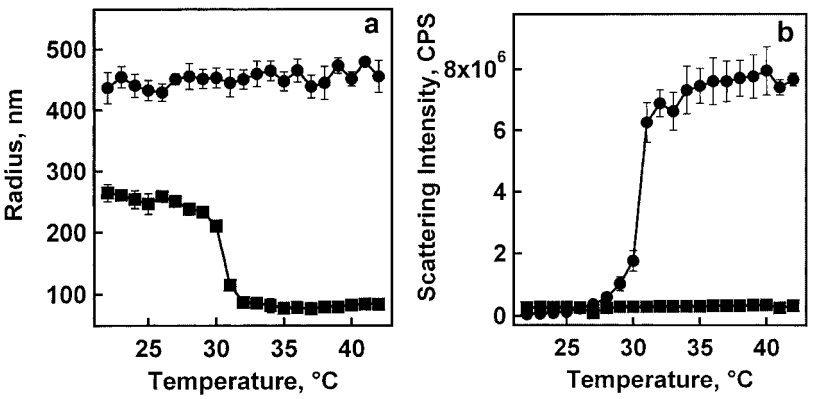

Figure 1. (a) Volume phase transition curves for 1 mole % BIS p(NIPAm-*co*-AAc) (9:1) microgels and (b) light scattering profiles in pH 3.22 (closed squares) and pH 6.15 (closed circles) 10 mM media.

The microgels exhibit an average hydrodynamic radius of approximately 280 nm under low pH conditions of 3.22 (closed squares) at low temperatures. At approximately 31 °C, the microgels undergo a volume change and deswell to an average hydrodynamic radius of 80 nm. This transition temperature correlates well with the typically observed LCST of pNIPAm microgels.[34] Under these pH conditions, almost all of the acid groups within the microgels are protonated and thus not charged. At pH 6.15 (closed circles), however, the system is above the pKa (~4.3) value, resulting in a majority of the acid groups being deprotonated and hence negatively charged. This causes a significant increase in microgel size to 450 nm due to tremendous osmotic swelling and Coulombic repulsion between the negatively charged AAc moieties. Under these pH conditions, no volume phase transition within the temperature range of 22–42° C is observed. This is mainly a result of the

enhanced hydrophilicity of the system, resulting from an increased amount of negatively charged acid groups that facilitate greater water solvation.[35-38] Similar findings of increased swelling ratios resulting from enchanced hydrophilicity of hydrogel networks has been previously reported.[35-38] The corresponding light scattering profiles of the same systems are illustrated in Figure 1b. At both low and high pH values, the microgels display a minute amount of scattered light intensity at low temperatures. This results from the fact that these soft microgel networks are extremely water swollen (approximately 95% by volume) and are thus, highly index matched to their environment. When the systems deswell into dense hydrophobic networks at temperatures above the LCST under low pH conditions, however, the microgels exhibit an elevated scattering cross section. This yields a pronounced increase in scattered light intensity to be observed.

Insulin Loading and Thin Film Buildup Confirmation

As described above, FITC-labeled insulin was chosen as a model macromolecule for the construct of these peptide loaded microgel thin films. The p(NIPAm-*co*-AAc) (9:1) microgels were first loaded with a concentrated solution of this therapeutic agent by taking advantage of the pH responsivity of these systems, as well as the pH-dependent solubility of insulin itself. Since insulin is most soluble under acidic pH conditions, it was first dissolved in acid to prepare the stock solution.[26] This solution was then incorporated into the microgel solution via stirring. Slow addition of NaOH was used to gradually increase the pH to 7.4. In doing this, the size of the microgels increases due to osmotic swelling and Coulombic repulsion between negatively acid groups. This pH switch allows for the microgels to become more porous as well as the insulin solubility in water to decrease. The overall effects that supposedly propel insulin partitioning into the microgel networks are an amalgamation of its low water solubility[26] as well as hydrophobic effects and electrostatic interactions (at pH 7 insulin is zwitterionic).[39]

For thin film deposition, the well known LbL assembly technique was used, as described in detail above (see Experimental section). Previous work done in our lab has shown that p(NIPAm-*co*-AAc) (9:1) microgels can be utilized as a polyanion for the construction of thermoresponsive thin films.[32] Here, the insulin impregnated microgels at pH 7.4 are overall negatively charged and thus, can serve as the anionic component in thin film assembly. Polyallylamine hydrochloride (PAH) was then deposited as the polycationic layer. A detailed description of the construction of such films has been previously

reported.[40] In this work, we chose to investigate the possibility of building up thicker films uniformly that could possibly release more effective amounts of insulin. The linearity of buildup for a thick 30-layer film was probed using UV/vis spectroscopy. Figure 2a shows a monotonic growth in film absorbance as the layer number increases. Panel (b) verifies that this increase is linear as a function of film layer number. These findings suggest that the FITC-insulin loaded microgel thin films are being built up in a uniform manner.

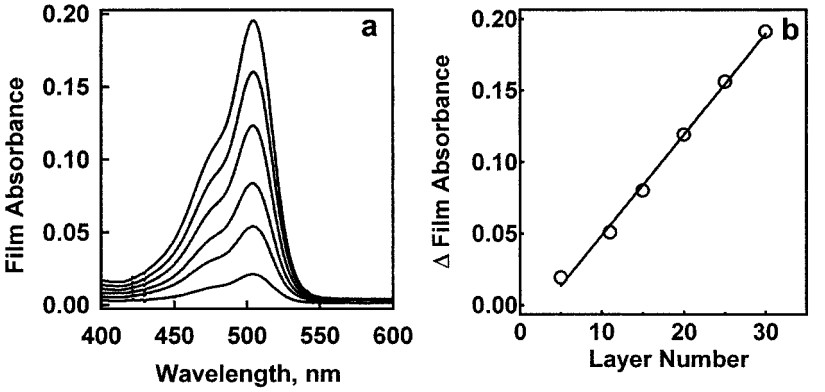

Figure 2. FITC-insulin loaded microgel thin film buildup confirmation for a 30-layer film. (a) Increase in film absorbance with increasing layer number. (b) Plot of film absorbance versus layer number.

Pulsatile and Extended Release Studies

Thermoresponsivity of these films under physiological pH conditions is key for pulsatile drug delivery investigations. While the microgels themselves in solution do not display a PT within the temperature range measured (see Figure 1), the film construct is such that the PAH counterlayer serves as a polycation to compensate the negatively charged insulin-impregnated microgels. This charge compensation is what presumably allows these films to be built up in a uniform manner. Previous work has shown that these polyanionic microgels can be deposited within electrostatic based thin films using PAH as the polycation that do display thermoresponsivity[32] as well as controlled pulsatile release of insulin over many cycles.[40] In this work, these properties were directly probed for a thicker 30-layer film where the fluorescence emission of the release medium was monitored at 512 nm as a function of time and temperature. Figure 3 illustrates the thermally induced direct release profiles obtained from this thicker peptide loaded film.

Panel (a) represents the thermal pulses that the film is subjected to while Panel (b) illustrates the actual oscillations in release. When the highly loaded film is initially placed into the cold release medium, not an insignificant amount of peptide is released presumably from

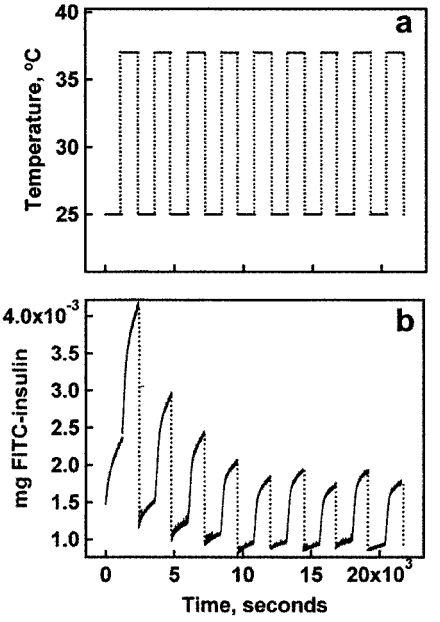

Figure 3. Thermally induced direct pulsatile release profiles of FITC-insulin from a 30-layer microgel thin film into 0.02 M PBS. Panel a illustrates the cyclical temperature pulsing from 25 to 37 and back to 25 °C. In panel b, the dashed lines indicate full media replacement.

weakly adsorbed insulin molecules at the film exterior. After ramping the temperature to 37 °C, the amount of peptide released increases significantly. Upon full media replacement (as indicated by dashed lines), the film displays significantly reduced insulin release upon reswelling at 25 °C. But, upon duplicating the thermal pulse, a second burst of insulin is quickly expelled from the film. The peptide can be repeatedly pulsed in response to thermal modulation over cycle times while the quantity released stays roughly consistent. This feature of thermally tuning the magnitude of insulin released makes these model thin film constructs highly attractive for macromolecule drug delivery applications. To test the robustness of these thin films, extended release studies were performed for one month, as described in detail above (see Experimental section). Cumulative release

profiles for 3-, 6- and 9-layer films are illustrated in Figure 4a. These release curves indicate a strong relationship between the quantity of insulin released and the film layer number. Thus, these films are also tunable based on their thickness. It is interesting to note that the profile for the 3-layer film seems to have reached a plateau region towards the end of the month (perhaps indicating film depletion). But, the 6- and the 9-layer curves still display an upward slope, thus indicating that they may still be capable of releasing insulin for much longer periods of time. As illustrated with the direct release experiments, Figure 4b indicates again thermal tunability of release. This panel focuses on cycles 10–22 for the 3-layer film curve shown in Figure 4a where the even numbers represent hot cycles and the odd numbers represent cold cycles. This plot confirms that the films can repeatedly turn on release under hot conditions and turn off release under cold conditions.

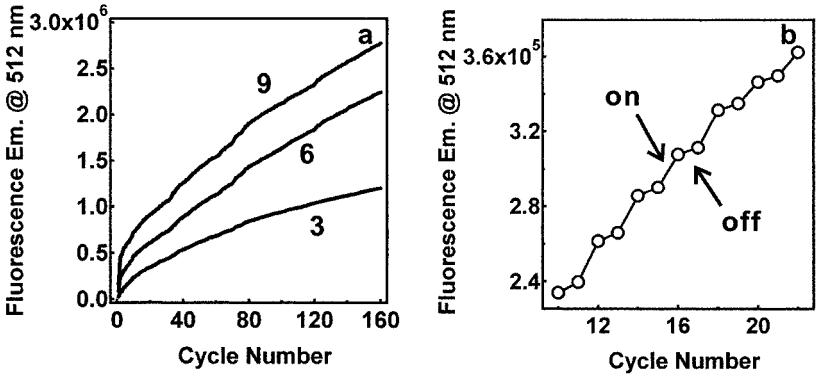

Figure 4. (a) Cumulative thermally induced release profiles for FITC-insulin loaded microgel thin films over 1 month (numbers indicate film layer number). (b) Expanded view of cycles 10-22 for the 3-layer film.

Conclusions

Dual-responsive microgels have been effectively loaded with a therapeutic macromolecule and have been successfully integrated into LbL assembled thin films that exhibit regulated release characteristics. These insulin impregnated thin films can be built up in a uniform manner even up to 30 layers. Thermal tunability of these films allows for solubilization and partitioning of encapsulated peptide. These films can repeatedly pulse out fast bursts of FITC-insulin over many cycles and prove to be remarkably robust. These novel films show promise for future macromolecule release systems.

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