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Structurally stable N-*t*-butylacrylamide hydrogel particles for the capture of peptides

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ABSTRACT

Hydrogel particles have proven to be powerful tools for the capture and concentration of low abundance, low molecular weight peptides and proteins from complex biofluids, such as plasma. The primary means of recovering and washing the particles following harvesting is through centrifugation, which can be a very time-consuming process depending on harvest conditions. To improve the process of particle recovery, washing, and elution we have developed new particle formulations: incorporating N-*t*-butylacrylamide (tBA) in the polymer backbone with monomers bearing more acidic functional groups and higher degrees of cross-linking. These particle formulations produce a stable architecture that does not significantly respond to changes in environmental conditions, such as pH and temperature. These two new formulations impart structural stability to the particle, control swelling, and improve pelleting through centrifugation, even at high pH values. These structurally stable microparticles yield improved particle recovery while maintaining the peptide capture properties of the particle.

1. Introduction

The spread of antibiotic resistant bacterial pathogens poses a tangible threat to public health and incurs significant economic costs. [1,2] Unfortunately, the antibiotic development pipeline, which has focused primarily on a few known classes of antibiotic drugs, has not been able to address this challenge. [3] Accordingly, there is need for new therapeutic strategies and new antibiotics to combat the growing challenge of bacterial resistance. Cationic antimicrobial peptides (CAMPs), which are essential elements of innate immunity in higher organisms, exhibit broad-spectrum antimicrobial activity, including both Gram-positive and Gram-negative bacteria, coupled with an absence of widespread bacterial resistance. Hence, they have garnered interest as a potential resource for the development of new antibacterial therapeutics. The host-defense peptidomes of evolutionarily ancient species with reputations for having robust immunity, such as the American alligator whose blood has been shown to have antimicrobial properties, [4,5] represent promising sources of novel potent CAMPs and are of particular interest.

Functionalized hydrogel particles have been used to capture peptides and protein biomarkers from complex biological samples, such as human sera. [6–8] We employed a bioprospecting approach to CAMP discovery that used custom-developed functionalized hydrogel particles to capture and enrich peptides with physicochemical properties similar to those associated with CAMPs. [9,10] The harvested peptides were subjected to analysis using tandem mass spectrometry (electron transfer dissociation, ETD) and the peptide sequences were determined based on their ETD spectra. This process allowed for the direct identification of intact individual CAMP and CAMP-like peptides in their native forms from 100 μL of alligator plasma, which resulted in the detection and sequencing of >100 peptides. Of these 100 peptides, 45 were identified as potential novel CAMPs based on the results from web-based prediction algorithms and rational analyses of the physicochemical properties of the peptide sequences. [10]

While the analyses described above were successful in identifying novel potential CAMPs, they also revealed that the alligator peptidome contained a complex mixture of CAMPs and CAMP-like peptides which were not fully captured in the initial investigation due to the large number of peptides and the presence of non-CAMP peptides. The study employed hydrogel particles that were based on cross-linked copolymers of N-isopropylacrylamide (NiPAm) with monomers bearing negatively-charged carboxylate and sulfonate groups to complement the cationic character of the targeted

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CAMPs. However, the physicochemical properties of hydrogel particles and harvesting conditions can be altered to more effectively preferentially harvest CAMP and CAMP-like peptides from complex mixtures such as plasma while excluding undesired peptides and proteins. For example, the monomer feed can be adjusted to include a greater number of negatively charged functional groups, and the particle architecture adjusted to fine-tune the peptide harvesting properties. Additional harvest conditions, such as pH, can then be adjusted to further enhance the preferential capture of CAMPs and CAMP-like peptides.

The present study focuses on how environmental conditions (pH) and alterations in the polymer scaffold and architecture of the hydrogel particles influence their harvesting and physical properties. The hydrogel particles that have been used to date for capturing proteins and peptides incorporated cross-linked NiPAm copolymers with bait-containing functional monomers. Polymers containing NiPAm and acidic/basic monomers are generally environmentally responsive, shrinking and swelling in response to changes in temperature and pH, which has contributed to their utility for biomedical applications, such as drug-delivery. However, this behavior does not contribute to the utility of hydrogel particles for capturing and enriching low abundance proteins and peptide from biofluids. The present harvests will be performed from plasma solutions that have been titrated to either pH 7.4 or pH 9, in order to enhance the preference for capturing cationic peptides with high pI values. Elevated pH conditions are accompanied by increasing the hydrophilicity of NiPAm-based particles that contain acidic monomers, such as acrylic acid, which makes the particles more difficult to pellet from solution and complicates recovery of the particles following harvesting. To reduce sensitivity to environmental conditions, particles have been prepared that incorporate t-butylacrylamide (tBA) within the NiPAm copolymer backbone. Polymers containing tBA do not demonstrate the same sensitivity to temperature or pH as do those based on NiPAm. In addition to the inclusion of tBA, another variation on the standard harvesting hydrogel particles has been explored, one in which an inert core is encapsulated within a highly cross-linked and acrylic acid (AAc)-rich shell (Fig. 1).

2. Experimental

2.1. Materials

N,N'-methylenebisacrylamide (BIS), acrylic acid (AAc), methyl acrylate (MA), potassium persulfate (KPS), N-t-butylacrylamide (tBA), 2-acrylamido-2-methyl-propanesulfonic acid (AMPS), and lithium hydroxide were purchased from Sigma Aldrich, Corp. and used as received. N-isopropylacrylamide (NiPAm) was purchased from Sigma Aldrich, Corp. and recrystallized from hexanes. Pierce Coomassie Plus was purchased from ThermoFisher Scientific and used as received. Trifluoroethanol was purchased from Acros and trifluoacetic acid was purchased from Thermo Scientific and used as received.

2.2. Instrumentation

Photon correlation spectroscopy (PCS) was obtained with a Beckman Coulter N5 Submicron Particle Size Analyzer. 1H NMR spectra in D_2O and $CDCl_3$ were obtained using a Bruker DRX 400 MHz instrument. 1H NMR spectra were used to verify monomer purity and incorporation of monomers into the polymer. Initial assessment of harvest performance was performed on the Thermo Orbitrap XL. Sequencing of peptides was run on Thermo Orbitrap Elite with ETD and PEAKS sequencing software.

2.3. Methods

2.3.1. PNiPAm-co-tBA-co-MA synthesis

The pNiPAm-co-tBA-co-MA particles were synthesized via free radical precipitation polymerization using a one-pot process. The core monomer solution was prepared by dissolving NiPAm (5.6 mmol), BIS (0.18 mmol), and MA (1.35 mmol) in 30 mL dH_2O. The solution was then vacuum filtered through a 0.45 μm nylon membrane into a 3-neck round bottom flask and then purged with N_2 with stirring for 15 min. While the solution was being degassed, it was heated in an oil bath to 72–75 °C. Solid tBA (1.87 mmol) was then added to the reaction mixture, and the solution continued degassing for an additional 15 min. Polymerization was then initiated by the addition of 0.0043 mmol KPS dissolved in 1 mL H_2O, with full opalescence occurring within two minutes of initiation. The core reaction was allowed to continue with continuous stirring under N_2 for 3 h at 75 °C.

The shell monomer solution was prepared by dissolving NiPAm (7.92 mmol) and BIS (0.18 mmol) in 30 mL dH_2O. The solution was vacuum filtered through a 0.45 μm nylon membrane and sparged with nitrogen and moderate stirring for 3 h. The shell solution was then added in a drop-wise fashion at a rate of 1 drop per second to the hot core solution using an addition funnel. Following addition of the shell solution, the reaction was allowed to continue stirring for 3 h under nitrogen at 75 °C. After three hours, the solution was cooled to room temperature under nitrogen overnight.

2.3.2. Saponification of pNiPAm-co-tBA-co-MA particles

Particles formulated to contain methyl acrylate (MA) were saponified, according to literature protocols [9], using lithium hydroxide in aqueous methanol. Particles (60 mL, approximately 800–1000 mg) were pelleted and resuspended in a 75% methanol solution. The particle solution was added to a round bottom flask and sparged with nitrogen for 20 min. Lithium hydroxide (0.6600 g) was suspended in 6 mL 75% methanol and added to the particles dropwise with a wide tip plastic pipette. The mixture was stirred overnight under nitrogen. The saponified particles were dialyzed against 2 L dH2O with dialysate changed two times a day for three days. Washed particles were pelleted with the supernatant discarded to concentrate the particles before a final resuspension in dH2O.

2.3.3. PNiPAm-co-tBA core pAAc shell synthesis

The pNiPAm-co-tBA core particles were also synthesized via free radical precipitation polymerization utilizing a one-pot process. The core solution was prepared by dissolving NiPAm (6.62 mmol, 0.75 g) and BIS (0.18 mmol, 0.03 g) in 30 mL dH_2O. After vacuum filtration through a 0.45 μm membrane, the solution was degassed with stirring under N_2 for 15 min while heating to 72–75 °C. To the hot solution, tBA (2.21 mmol, 0.28 g) was added. The monomers were then degassed an additional 15 min before polymerization was initiated with 0.04 mmol KPS dissolved in 1 mL dH_2O. Full opalescence was achieved within two minutes of initiation and the core reaction was allowed to proceed for 3 h at 75 °C.

With minor alterations, the highly cross-linked shell solution was prepared as previously described by dissolving NiPAm (5 mmol, 0.57 g) and BIS (2.5 mmol, 0.39 g) in 30 mL dH_2O. Once fully dissolved, the solution was vacuum filtered through 0.45 μm membrane. Acrylic acid (1.25 mmol, 85.7 μL) was added to the filtered solution and degassed for 3 h before dropwise addition to the core solution with high stir rate. The core-shell mixture was maintained at 75 °C for 3hr. Particles were washed via dialysis against dH_2O for three days.