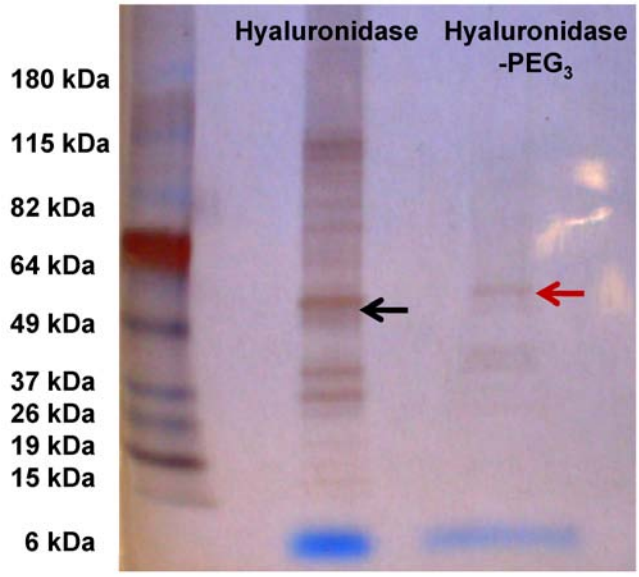


Supplementary Figure 1. SDS-PAGE analysis of the expression and purification of the putative bacterial hyaluronidase (Hase) TDE0471 (black arrow) and Hase-PEG_3 conjugates (red arrow).

Methods and Materials:

Protein Expression

The putative bacterial hyaluronidase TDE0471 gene with a poly(histadine) tag was supplied in the pET30 plasmid (a kind gift from Dr. J. Christopher Fenno, University of Michigan) [1]. The plasmid was purified using the Midi-Prep kit (Qiagen, Valencia, CA). Then, the plasmid was transfected into a competent Rosetta E. coli (DE3) strain using standard molecular biology techniques. A single colony was selected and expanded overnight in 80ml LB media (Invitrogen, Carlsbad, CA) with kanamycin and chloramphenicol (Sigma-Aldrich, St. Louis, MO). Then, the E. coli were centrifuged, suspended in 30ml LB media, and 10ml of the concentrated E. coli was transferred into 1L LB media with kanamycin and chloramphenicol. Once the cells reached an OD_{600nm} of 0.35, 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific, Fairlawn, NJ) was added to the media. After 2.5 hours of exposure to IPTG, 1mM phenylmethanesulfonylfluoride (PMSF) (Pierce, Rockford, IL) was added to the media, the cells were centrifuged to form a pellet that was stored at -80°C.

Protein Purification:

Cell pellets were suspended in pH 8.0 Bugbuster Protein Extraction Reagent (Novagen, Darmstadt, Germany) with 1mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) and 0.6mM PMSF. Then, the lysate was centrifuged at 28,000 rpm for 60 minutes using a Thermo Sorvall WX Ultracentrifuge (Fisher Scientific, Fairlawn, NJ, USA). The supernatant was transferred onto a Hislink™ column (Promega, Madison, WI, USA), rinsed with wash buffer (10mM HEPES, 10mM imidazole, pH 8.0), and eluted with elution buffer (10mM HEPES, 1M imidazole, pH 8.0). The eluted protein was placed in 10kDa molecular weight cutoff (MWCO) dialysis tubing (Fisher Scientific, Fairlawn, NJ) and dialyzed three times against 4L of wash buffer.

Synthesis, Purification, and Characterization of Hase-PEG$_3$ conjugates

The dialyzed protein was reacted with a 60X molar excess of poly(ethylene glycol)-diacrylate (PEGDA) via a Michael-type addition reaction at pH 8.0 and 37°C for 2.5 hours. Then, the Hase-PEG$_3$ conjugates were purified using the previously described Hislink™ column chromatography process. The purified Hase-PEG$_3$ conjugates were placed in 10kDa MWCO dialysis tubing and dialyzed three times against 4L of ultrapure H_20. Finally, the Hase-PEG$_3$ conjugates were frozen in a -80°C freezer, lyophilized, and stored at -20°C. SDS-PAGE gel electrophoresis was used to analyze the molecular weight of the Hase-PEG$_3$ conjugates using standard molecular biology techniques.

Calculation of TDE0471 Hase isoelectric point

The isoelectric point of TDE0471 Hase was calculated using the following equation and amino acid sequence:

Equation: $$Z = \sum_x N_x \frac{10^{pKa_x}}{10^{pH} + 10^{pKa_x}} - \sum_y N_y \frac{10^{pH}}{10^{pH} + 10^{pKa_y}}$$

Where N_x was the number of the N-terminus and the side chains of Lysine, Histidine, Arginine, and pKa_x was their respective pKa values. The N_y corresponded to the C-terminus and the

number of the Cysteine, Glutamic Acid, Aspartic Acid, and Tyrosine amino acids, and pKa_x was their respective pKa values.

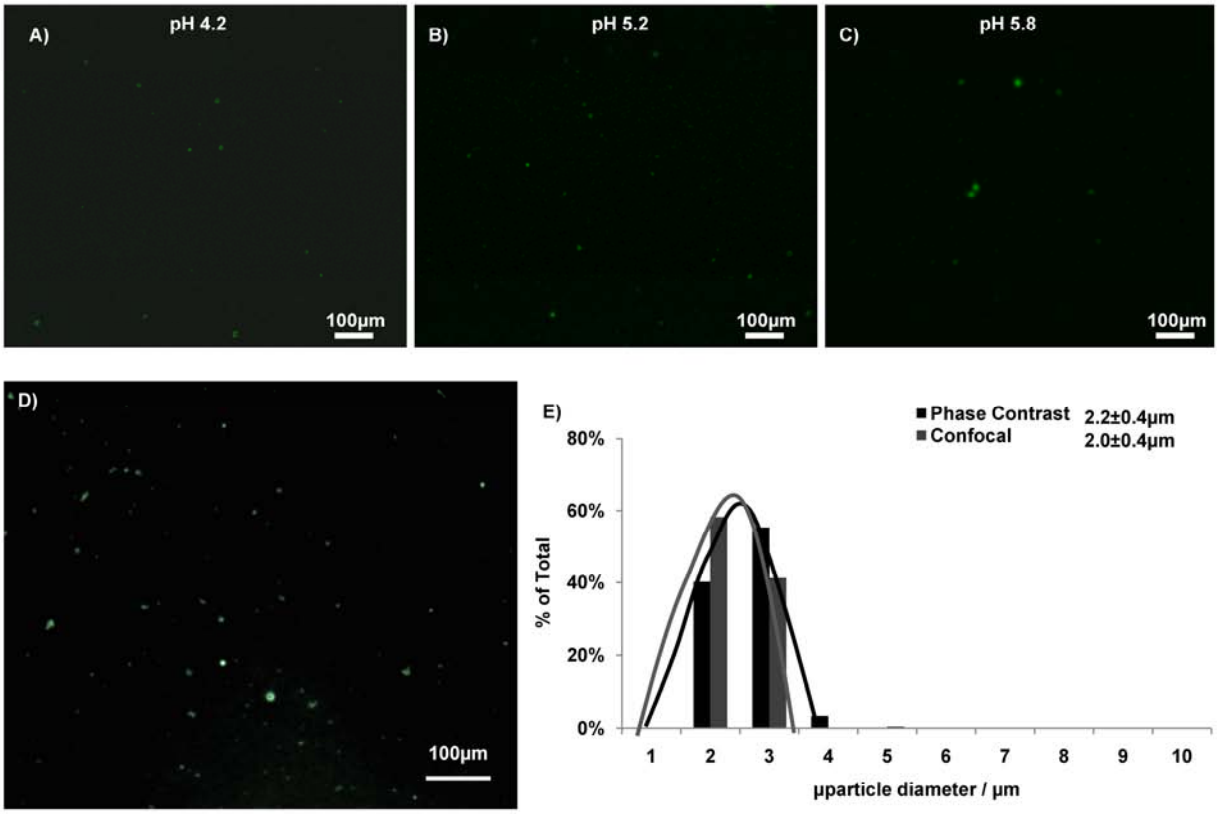
Sequence:

M H H H H H H K N S I S V F L L V V A A L V L L A G C Q Q K A N K K D G G N Q Q G
G L N P P S P G T P T F T V T F D V Q G R G K T P A A L T V P K D S L L T A A Q T P P
L E F S G W E F G G W Y K D A F K T H E W N N A S D T V T E N T T L Y A R W T H T
Y P P A V Q D L W Q S K T D R P E D F Y R I P A L A V T K D G T L L A V T D L R Y K
N N S D L G N N H R I D L L I K R S E D N G K A W S E A V N I T K T L P T D Q T G Y G
D A A I V A D R E S D D V L I L C V H G N V T Y Q A G N A S N H L K V I Q F V S H D
G G K T F P E K K D I S N T I F G F N H S W F S L F F G S G R I M Q S R Y I K A G S H Y
R I Y S A L L S K R F I H S N D H H D N A V V Y S D D F G S T W H V L G D A S T S P I
P D G N E A K V E E L P D G S V I L S S R N G T A N G R L I N I F T Y S D P D T G A G S
W S S K Q F L N L G S G S G T N G E I L I L K A R K T D T K D P V Y L A F Q S L P D G
P G R S K V T I H W R E L T N N T I T A H D F V S A A T W N S H S Y V V Q T G D S A
Y S T M D V Q R D G G I G F L Y E R N T R G L E Y D I A Y K N L P I D V I T N G A Y E
A I F L G T G S V Q C P Y T D L E G K P V D P S V K E Y Y K N E K L H W K E

Formation of Hase-PEG_3 hydrogel microspheres

5mM hyaluronidase-PEG_3 conjugates were prepared in pH 6.2 buffer (20mM MES and 150mM NaCl) with 0.1% Irgacure 2022 (Ciba, Basil, Switzerland). 5µl of the Hase-PEG_3 conjugate containing prepolymer solutions were cast between two glass slides treated with Sigmacote (Sigma-Aldrich, St. Louis, MO, USA) and exposed to 4mW/cm^2 UV light for 0.5 minutes. The resulting microspheres were transferred into pH 7.4 phosphate buffered saline (PBS). Photomicrographs were acquired using a Nikon Eclipse TE-2000-S Microscope (Nikon, Melville, NY, USA).

1. G. Seshadri, G. S. A. Myers, H. Tettelin, J. A. Eisen, J. F. Heidelberg, R. J. Dodson, T. M. Davidsen, R. T. DeBoy, D. E. Fouts, D. H. Haft, J. Selengut, Q. H. Ren, L. M. Brinkac, R. Madupu, J. Kolonay, S. A. Durkin, S. C. Daugherty, J. Shetty, A. Shvartsbeyn, E. Gebregeorgis, K. Geer, G. Tsegaye, J. Malek, B. Ayodeji, S. Shatsman, M. P. McLeod, D. Smajs, J. K. Howell, S. Pal, A. Amin, P. Vashisth, T. Z. McNeill, Q. Xiang, E. Sodergren, E. Baca, G. M. Weinstock, S. J. Norris, C. M. Fraser and I. T. Paulsen, *P Natl Acad Sci USA*, 2004, **101**, 5646-5651.

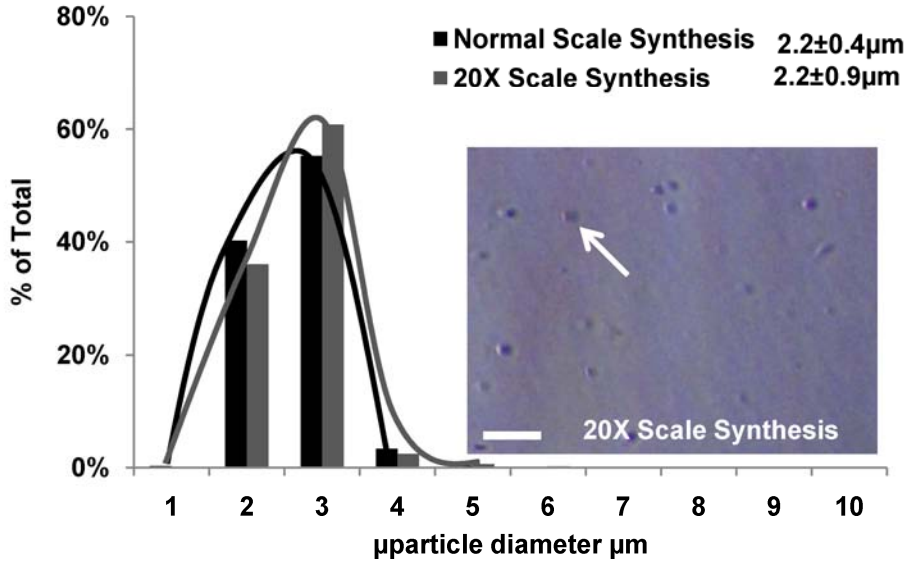


Supplementary Figure 2. Representative confocal micrographs of PEG-CaM-PEG microspheres formed at pH A) 4.2, B) 5.2, and C) 5.8 acquired with a 20X air objective. D) Representative high magnification confocal micrograph of PEG-CaM-PEG microspheres formed at pH 4.2 acquired with a 60X oil objective. E) Size distribution of PEG-CaM-PEG microspheres formed at pH 4.2 as measured from micrographs acquired using bright field and confocal imaging.

Methods and Materials: PEG-CaM-PEG microspheres were formed at pH 4.2, 5.2, and 5.8 were placed in pH 8.0 buffer (20mM HEPES, 150mM NaCl, 10mM $CaCl_2$). Microspheres were reacted with NHS-fluorescein (Pierce, Rockford, IL, USA) for 2 hours. Then, unreacted NHS-fluorescein was removed by extensively washing the microspheres on a 0.22µm Steriflip membrane (Millipore, Billerica, MA, USA) with pH 8.0 buffer (20mM HEPES, 150mM NaCl, 10mM $CaCl_2$). The membranes were rinsed with pH 6.3 buffer (20mM MES, 150mM NaCl, 10M $CaCl_2$) and microsphere containing buffer was transferred into 24 well plates. 20X confocal micrographs were acquired using a Nikon Eclipse Ti confocal microscope (Nikon, Melville, NY,

USA). 60X confocal micrographs were acquired using a BD Pathway confocal microscope (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Photomicrographs were acquired using a Nikon Eclipse TE-2000-S Microscope (Nikon, Melville, NY, USA). The size of the microspheres was quantified using ImageJ (Freeware, NIH, Bethesda, MD) by measuring >200 microspheres.

Supplementary Movie 1. 3D rendering of fluorescein-labeled PEG-CaM-PEG microspheres formed at pH 4.2.



Supplementary Figure 3. Comparison of the size distribution of normal scale and 20X scale syntheses of PEG-CaM-PEG microspheres formed at pH 4.2. Inlayed is a representative micrograph of PEG-CaM-PEG microspheres formed from a 20X scale synthesis. Scalebar denotes 10µm.

Method: 10 mM PEG-CaM-PEG solutions were formed at pH 4.2 using Citric Acid buffer (20mM Citric Acid, 150mM NaCl, and 10mM $CaCl_2$). 0.05% Irgacure 2959 (Ciba, Basel, Switzerland) was included as a polymerization photoinitiator. Normal scale (5µl) or 20X scale (100µl) prepolymer solutions were cast between two glass slides separated by a 1mm spacer, and exposed to UV light (4mW/cm^2) for 0.5 minutes to initiate polymerization. The resulting hydrogels were transfered to pH 6.3 MES buffer. Photomicrographs were acquired using a Nikon Eclipse TE-2000-S Microscope (Nikon, Melville, NY, USA). The diameter of

>500 microspheres per experimental condition was measured using ImageJ software (Freeware, NIH, Bethesda, MD).

Supplementary Figure 4. Representative photograph of a free standing PEG-CaM-PEG hydrogel formed from a 10mM PEG-CaM-PEG hydrogel prepolymer solution at pH 6.3. A coin was included for size reference.