Covalently-Crosslinked Mucin Biopolymer Hydrogels for Sustained Drug Delivery

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Original pdf file available at doi:10.1016/j.actbio.2015.03.024

Abstract

The sustained delivery of both hydrophobic and hydrophilic drugs from hydrogels has remained a challenge requiring the design and scalable production of complex multifunctional synthetic polymers. Here, we demonstrate that mucin glycoproteins, the gel-forming constituents of native mucus, are suitable for assembly into robust hydrogels capable of facilitating the sustained release of hydrophobic and hydrophilic drugs. Covalently-crosslinked mucin hydrogels were generated via exposure of methacrylated mucin to ultraviolet light in the presence of a free radical photoinitiator. The hydrogels exhibited an elastic modulus similar to that of soft mammalian tissue and were sensitive to proteolytic degradation by pronase. Paclitaxel, a hydrophobic anti-cancer drug, and polymyxin B, a positively-charged hydrophilic antibacterial drug, were retained in the hydrogels and released linearly with time over seven days. After four weeks of drug release, the hydrogels continued to release sufficient amounts of active paclitaxel to reduce HeLa cell viability and sufficient amounts of active polymyxin B to prevent bacterial proliferation. Along with previously-established anti-inflammatory, anti-viral, and hydrocarbon-solubilizing properties of mucin, the results of this study establish mucin as a readily-available, chemically-versatile, naturally-biocompatible alternative to complex multifunctional synthetic polymers as building blocks in the design of biomaterials for sustained drug delivery.

Keywords: mucin hydrogel, amphiphile, crosslinking, drug delivery, methacrylation, mucus

1. Introduction

Modern drug development relies inextricably on the concomitant development of appropriate drug delivery systems. The cost, safety, and bioavailability of drugs are greatly improved when the therapeutic molecules are delivered with precise control over delivery rates and localization. Hydrogels, hydrophilic polymer networks capable of absorbing thousands of times their mass in water, have shown significant promise as vehicles for controlled drug delivery [1,2]. Drug molecules are loaded into the porous hydrogel matrix, allowing for controlled release of the drug from the hydrogel over time with limited immunogenicity. The aqueous nature of hydrogels, however, presents a challenge because water-soluble molecules may simply diffuse out of the delivery material unhindered. Loading hydrophobic drugs into hydrogels presents another difficulty because of the incompatibility between the hydrophilic hydrogel polymer network and hydrophobic drug molecules. Insoluble in the aqueous hydrogel matrix, hydrophobic drugs tend to precipitate or simply release from the hydrogel in a rapid burst [1]. Though burst release patterns are useful for certain treatments, they are most often detrimental to the pharmacological and economical performance of the drug delivery system [3].

Chemical engineers have thus far addressed problems of drug delivery control in hydrogel-based drug delivery systems by designing nanodomain-structured materials, such as block copolymers, that bind to drug molecules through electrostatic [4,5] and covalent bonds [6,7] as well as hydrophobic interactions [8,9], delaying diffusion of the drug out of the material. More complex assemblies combining electrostatic and hydrophobic interactions have been developed to co-deliver hydrophilic and hydrophobic drugs [10–13] to thereby trigger drug synergy or suppress drug resistance. For example, co-delivery of paclitaxel with an interleukin-12-encoded plasmid

by nanoparticles suppresses cancer growth more effectively than the paclitaxel or the plasmid alone [13]. However, scaling up the production of such complex macromolecules is difficult and costly. Furthermore, the polymerization techniques used to create synthetic multifunctional hydrogel polymers frequently require cytotoxic catalysts, such as copper, that limit the hydrogels' biocompatibility and potential to reach the market [14]. In this work, we bypass the difficulty and expense of synthetic polymer engineering by exploiting a readily-available gel-forming biopolymer that naturally contains sufficiently various chemical moieties to bind diverse classes of molecules.

Mucin, a high-molecular mass glycoprotein, is the primary structural component of the mucus covering all wet epithelia, including within the nose, mouth, lungs, gastrointestinal tract, and female genital tract. Millions of years of evolution have endowed mucin the ability to bind a wide variety of molecules to protect organisms against viruses [15,16], bacteria [17,18], and small particles [19]. Mucin molecules are indeed equipped with an optimized and diverse chemistry: their protein backbone, rich in thiol groups, contains both hydrophobic and charged domains. Additionally, oligosaccharides attached to the mucin protein backbone provide intramolecular and intermolecular hydrogen bonding capabilities, high hydration and hydrophilicity, and carboxyl and sulfate groups that confer a net negative charge to mucin at neutral pH [20]. As a result of its unique chemistry, mucus constitutes a major biophysical barrier to the oral delivery of most drugs [21], binding and retaining the intended therapeutic molecules in its matrix [22]. Though the mucosal barrier presents a vexing problem for oral drug delivery, an opportunity emerges whereby mucin-drug interactions could be exploited to retain and release multiple types of drugs over long periods of time using mucin-based biomaterials.

To most effectively harness the properties of mucin to bind and release drugs, the mucin must first be assembled into a material that is easily handled and that leaves its biophysical properties intact. Mucin-based biomaterials in the form of thin films have been assembled layer-by-layer, complexing mucin with lysozymes [23], chitosan [24,25], or lectins [26,27]. Microparticles composed of mucin-alginate [28] or mucin-gelatin [29] complexes also have been created. However, the ability of solely mucin-based biomaterials to retain and release small drug molecules has not previously been investigated, nor has a robust macroscopic covalently-crosslinked mucin hydrogel previously been developed. In this work, we assemble methacrylated mucin into covalently-crosslinked hydrogels to study the drug binding and release capabilities of the mucin molecule. We characterize mucin hydrogels rheologically and investigate the loading and release from mucin hydrogels of polymyxin B, a positively-charged hydrophilic antibacterial drug, and paclitaxel, a hydrophobic anti-cancer drug. We demonstrate the sustained release from mucin hydrogels of both hydrophilic and hydrophobic model drugs, the absence of toxicity of mucin hydrogels on bacterial and mammalian cells, and the capacity of mucin hydrogels to load and retain sufficient quantities of active drug to maintain an antibacterial and anti-cancer effect over several weeks.

2. Materials and Methods

2.1. Materials

Bovine submaxillary mucin (BSM, Sigma-Aldrich, Lot SLBC2523V) was dissolved at 10 mg/mL in water, filtered through a 0.45 μm filter (Bottle-Top Vacuum Filter, Corning) to remove insoluble aggregates, then further purified by dialysis (100 kDa cutoff, Spectrum Labs)

against ultrapure water for 4 days to remove protein contaminants. BSM methacrylation was conducted with methacrylic anhydride (MA, Sigma-Aldrich) used as described below. Hydrogel formation was induced by free radical photoinitiator 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Sigma-Aldrich). Model drugs selected were paclitaxel (Invitrogen) and polymyxin B (Invitrogen) and their fluorescently-labeled versions, paclitaxel Oregon Green 488 conjugate (Invitrogen) and polymyxin B BODIPY FL conjugate (Invitrogen). Paclitaxel stock solution was prepared in dimethyl sulfoxide (DMSO) at 5 mg/mL, and polymyxin B stock solution was prepared in water at 5 mg/mL. Fluorescein isothiocyanate-labeled dextran of various molecular masses (5, 20, 40, 250, 2000 kDa) was obtained from Sigma-Aldrich. Polyglutamic acid (PGA, 25 kDa) was obtained from Sigma-Aldrich and fluorescently labeled by activating the carboxyl groups with *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (64.5 mM, Sigma-Aldrich) and *N*-hydroxysulfosuccinimide (4.6 mM, Sigma-Aldrich) for 30 minutes at room temperature, removing the reaction byproducts by centrifugation filtering (10 kDa cutoff, Spectrum Labs), and reacting with 5(6)-aminofluorescein (Sigma-Aldrich) in dimethylformamide solvent. After 1 hour at room temperature, excess 5(6)-aminofluorescein was removed by repeated centrifugation filtering. For degradation experiments, α-chymotrypsin from bovine pancreas (Sigma-Aldrich) and protease from *Streptomyces griseus* (Sigma-Aldrich) were used.

2.2. BSM-MA synthesis

BSM was dissolved at 10 mg/mL in ultrapure water. The solution was adjusted to pH 8 using sodium hydroxide (NaOH) and put on ice. Once cooled, a solution of MA was added to the solution at an MA to BSM mass ratio of 0.832%. The solution was gently stirred for 6 hours

while maintaining a pH of approximately 8 using a 5M NaOH solution, then placed overnight at 4ºC with gentle stirring. The solution was then centrifuged to remove excess MA and mucin precipitates and dialyzed for 2 days with 2 changes of water per day. The resulting methacrylated bovine submaxillary mucin (BSM-MA) was lyophilized and stored at -20ºC until use.

2.3. BSM-MA hydrogel formation

BSM-MA hydrogels were formed by dissolving BSM-MA at a concentration of 40 mg/mL in phosphate buffered saline (PBS) for 1 hour at 4ºC. Then, Irgacure 2959 photoinitiator dissolved in ethanol at a concentration of 100 mg/mL was added to the solution at a final concentration of 0.5 mg/mL, and the solution was immediately mixed by vortex before being placed in a mold. Molds used were wells of a 96-well plate or custom-made molds composed of 5 mm thick polydimethylsiloxane (PDMS) punctured with 8 mm diameter holes. The solution was then exposed to 365 nm wavelength ultraviolet light with exposure power of ~10 mW/cm^2 for 10 minutes to allow formation of the BSM-MA hydrogel. After formation, the hydrogels were unmolded and placed in fresh PBS solution.

2.4. Scanning electron microscopy (SEM)

BSM-MA hydrogel slabs, 8 mm in diameter, were dehydrated in baths of increasing ethanol concentration (50, 70, 80, 90, 95, 99, 100% ethanol, 10 min each) followed by baths of hexamethyldisilazane (2 baths of 10 min each). The resulting dehydrated hydrogels were further air-dried overnight, cut open, mounted on carbon tape, sputter coated with a 10 nm gold layer, and observed with a JOEL 6010LA scanning electron microscope.

2.5. BSM-MA hydrogel degradation

BSM-MA hydrogels (200 μL) were formed in 2 mL tubes, washed 3 times with 1 mL PBS, then immersed in 400 μL of either PBS (supplemented with 25 mM Ca^{2+}), α-chymotrypsin (400 μg/mL, 16 units/mL), or pronase (400 μg/mL, 1.4 units/mL) dissolved in PBS with 25 mM Ca^{2+}. The hydrogels were weighed each day after blotting to remove excess water. Images of the hydrogels were captured at day 7.

2.6. BSM-MA hydrogel rheology

BSM-MA hydrogels of 5 mm thickness and 8 mm diameter were formed using custom-made PDMS molds. The hydrogels were soaked in PBS at 37ºC for 24 hours before being placed between 2 steel plates of a rheometer (AR-G2, TA Instruments). The top and bottom plates were covered with sandpaper to prevent slippage. A frequency sweep was performed with constant strain of 1%. A strain sweep was performed with constant angular frequency of 1 rad/s. For stability assessment over several weeks, the shear modulus was measured for a constant strain (1%) and frequency (1 rad/s). After each measurement, the hydrogel was removed from the rheometer and stored in PBS at 37ºC.

2.7. Fourier transform infrared spectroscopy (FTIR)

BSM-MA or dialyzed BSM was dissolved at a concentration of 40 mg/mL in D_2O. D_2O, not H_2O, was selected as the solvent to avoid interference between the 1640 cm^{-1} absorption peak exhibited by H_2O and the protein amide I absorption peak in the same frequency region. The solution was placed on an ATR-FTIR and 128 spectra of each solution were recorded at 1 cm^{-1} resolution using a Thermo Fisher FTIR 6700 infrared spectrometer.

2.8. Drug release from BSM-MA hydrogels

Prior to hydrogel formation, either paclitaxel or polymyxin B was added to 40 mg/mL BSM-MA solution at a concentration of 200 μg/mL to form 50 μL BSM-MA hydrogels, each containing 10 μg of drug, in wells of a 96-well plate. By mass, 2.5% of the drug added was the fluorescently-labeled version of the drug. To measure drug release from the hydrogels over time, 150 μL of PBS was placed atop each hydrogel, and the hydrogel was left to release its drug payload at 37ºC. At each timepoint, a 50 μL sample was taken from the supernatant of each hydrogel and placed in a separate well plate. The entire supernatant was then replaced with fresh PBS. The fluorescence of each sample and of the hydrogels themselves was measured using a plate reader (Spectramax M3, Molecular Devices) to determine the quantity of drug that had been released from each hydrogel. Identical release experiments were also performed for fluorescently-labeled dextran and PGA loaded at 200 μg/mL in 50 μL BSM-MA hydrogels.

2.9. BSM purification by affinity chromatography

Drug release experiments were replicated using BSM-MA hydrogels formed with BSM further purified by lectin affinity chromatography prior to methacrylation and hydrogel formation. A jacalin-functionalized agarose bead matrix was loaded into a 10 mL column and washed with 175 mM tris (pH 7.4). BSM (20 mg/mL in 175 mM Tris, pH 7.4) was passed through the column, then washed with 20 mL 500 mM NaCl followed by 20 mL of melibiose (100 mM). The BSM was further purified by centrifugation filtering (20 kDa cutoff, Spectrum Labs) before methacrylation.

2.10. Bacterial culture and polymyxin B activity assay

Escherichia coli K12 bacteria expressing green fluorescent protein (GFP) and gentamicin resistance were used to assess the antimicrobial activity of BSM-MA hydrogels loaded with 10 μg of polymyxin B (200 μg/mL). From an overnight culture in Luria Broth (LB) supplemented with 25 μg/mL gentamicin (Sigma-Aldrich), the bacteria were diluted to OD_{600} 0.01 and cultured to the exponential growth phase. The culture solution was then diluted to OD_{600} 0.05 and 200 μL was placed atop each of the 50 μL hydrogels in wells of a 96-well plate. The 96-well plate with hydrogels was incubated at 37ºC and 5% CO_2 with shaking at 200 rpm.

To probe short-term bacterial growth kinetics on BSM-MA hydrogels loaded with polymyxin B, a 100 μL sample of the culture solution on each hydrogel was removed from the hydrogel each hour and placed in a well of a 96-well plate. The OD_{600} of the sample solution was immediately measured, and the sample solution was immediately returned to its respective hydrogel. After 24 hours, the hydrogels were observed by epifluorescence microscopy using an Observer Z1 inverted fluorescent microscope (Zeiss) with a 10X 0.3 NA objective (Zeiss) and the total fluorescence was quantified by whole-well measurements using a plate reader (Spectramax M3, Molecular Devices).

To assess the antibacterial effect of polymyxin B released from BSM-MA hydrogels while eliminating any effect of the presence of the loaded hydrogel itself, and to determine the time required for the hydrogels to release enough polymyxin B for pharmacological efficacy, 50 μL BSM-MA hydrogels loaded with polymyxin B were submerged in 100 μL of LB for various amounts of time (0-4 hours) in wells of a 96-well plate. Then, 90 μL of the resulting supernatant

of each hydrogel was transferred to wells of a separate 96-well plate and 10 μL of a concentrated solution (OD_{600} 0.5) of *E. coli* K12 bacteria was added to each well. After 4 hours of incubation, the OD_{600} of each culture solution was measured.

To evaluate the continuing antibacterial efficacy of polymyxin B-loaded BSM-MA hydrogels over several weeks, a fresh bacterial culture solution was incubated on each hydrogel approximately once every 5 days. After 4 hours of incubation, a 100 μL sample of the culture solution on each hydrogel was removed from the hydrogel, and the OD_{600} of the solution was immediately measured. Control conditions consisting of BSM-MA hydrogel without loaded polymyxin B, or of BSM-MA hydrogel topped with sterile LB, were also tested. Immediately after removal of the culture solution for OD_{600} measurement, the hydrogels were washed with PBS and sterilized with ultraviolet light (235 nm, 10 minutes). The hydrogels were then topped with PBS and stored at 37ºC for approximately 5 days until the next experiment following the same procedure.

2.11. HeLa cell culture and paclitaxel activity assay

The HeLa epithelial cancer cell line was maintained subconfluent in culture at 37ºC and 5% CO_2 in T25 flasks with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Invitrogen) and 1% antibiotic (25 units/mL penicillin, 25 μg/mL streptomycin) (Invitrogen). The cells were detached with trypsin-EDTA (Invitrogen) and resuspended in culture medium before use.

To assess the activity of paclitaxel released from BSM-MA hydrogels, 50 μL hydrogels loaded with 10 μg of paclitaxel were formed in permeable inserts (Transwell Permeable Supports, 0.8 μm pore size, Corning) that fit in 24-well plates. The hydrogels were sterilized with ultraviolet light (235 nm, 10 minutes) before and after each experiment. HeLa cells were plated at 30,000 cells/cm^2 and cultured for 24 hours without exposure to BSM-MA hydrogels or paclitaxel. The permeable inserts containing the BSM-MA hydrogels were then submerged in 1 mL of culture medium containing the cells and allowed to release paclitaxel into the culture medium for 24 hours. The metabolic activity of the HeLa cells was measured by adding concentrated alamar blue at a final concentration of 10% and letting the color develop for 4 hours. A 100 μL sample was taken and absorbance at 570 nm was measured using a plate reader (Spectramax M3, Molecular Devices). The hydrogels were washed with PBS, then stored in PBS containing penicillin and streptomycin (25 μg/mL) at 37ºC for 1 week before the next experiment following the same procedure.

2.12. Statistical Analysis

Statistical analysis was conducted using Prism 6 (GraphPad Software). All data are presented as the arithmetic mean ± standard deviation of at least 3 independent replicates. Where appropriate, statistical significance was evaluated using Student's *t*-test, assuming normal distribution of sample data, and differences with $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Generation of hydrogels from methacrylated mucin

To generate a mucin-based hydrogel suitable for drug delivery, we used mucin from bovine submaxillary glands (BSM) available commercially from Sigma-Aldrich. BSM was selected as the model mucin for its commercial availability and relatively low viscosity in liquid solution, which facilitated manipulation and mixture. Importantly, BSM contains both hydrophilic and hydrophobic domains conferring the amphiphilic properties necessary for dual binding of hydrophobic and hydrophilic drugs. Owing to its unique chemistry, BSM solubilizes hydrophobic dyes [30] as well as binds and solubilizes water-insoluble aromatic hydrocarbons, enhancing their bioavailability [31].

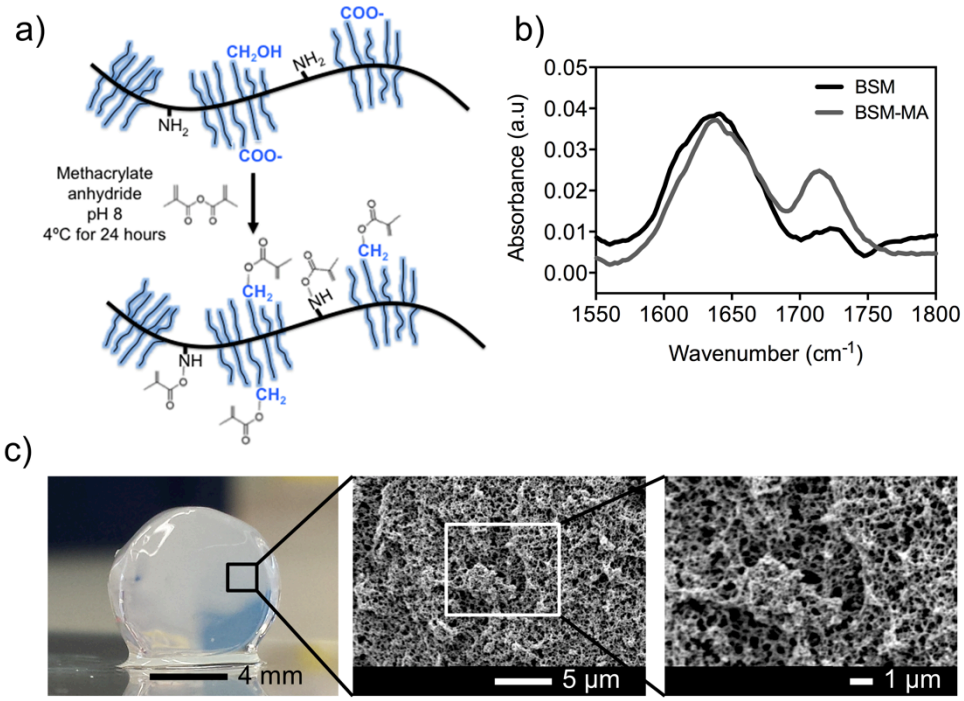

Figure 1. Methacrylation of BSM and assembly into BSM-MA hydrogels. a) Bovine submaxillary mucin (BSM) was methacrylated with methacrylic anhydride (MA) in aqueous medium. b) The appearance of an infrared absorption band at 1730 cm^{-1} confirmed the addition of methacrylate groups to BSM. c) In the presence of a free radical photoinitiator and ultraviolet

light, BSM-MA hydrogels formed; scanning electron microscopy revealed the hydrogel's porous, homogeneous nanostructure at the micron-range scale.

We methacrylated BSM following methacrylation protocols previously applied to other biopolymers, including gelatin [32,33], dextran [34], chitosan [35], chondroitin sulfate [36], and hyaluronic acid [37], but never before to mucin. Methacrylic anhydride (MA) reacts with a variety of functional groups, including amines present on the mucin protein core and hydroxyls present on both the protein core and attached glycans of the mucin molecule (**Figure 1a**). Fourier transform infrared spectroscopy performed on BSM and BSM-MA revealed typical protein peaks at 1550 cm^{-1} (Amide II) and the carboxyl group of sialic acid at 1730 cm^{-1} (**Figure 1b**) [38]. BSM-MA exhibited an additional peak at 1730 cm^{-1}, known to be the carbonyl group of methacrylate (1710–1730 cm^{-1}) [36,39]. The addition of a free radical photoinitiator and exposure to ultraviolet light resulted in the formation of a hydrogel, while the unmodified BSM solution remained liquid. Therefore, we concluded that the methacrylation of BSM was successful and that methacrylated BSM can be reliably assembled into moldable covalently-crosslinked hydrogels. **Figure 1c** shows a BSM-MA hydrogel after formation in a 5 mm thickness, 8 mm diameter PDMS mold. Scanning electron microscopy (SEM) was performed on a dehydrated BSM-MA hydrogel slab to elucidate its micron-range nanoscale structure. SEM images reveal an interconnected network of fibrils interspaced by pores of diameter in the tens to hundreds of nanometers. It is important to note, however, that these images may not indicate the pore size of the hydrated hydrogels, as even the most careful dehydration of hydrogels induces some structural change.

3.2. BSM-MA hydrogels exhibit solid-like behavior

The mechanical properties of an implantable material are essential to its biomedical applicability and are frequently responsible for its bioresorption mechanisms and kinetics [40]. Hydrogels must be robust enough to be manipulated and in application should match the mechanical properties of host tissue. Mechanical testing on a 5 mm thick, 8 mm diameter BSM-MA hydrogel was performed using a plate rheometer. A frequency sweep was performed with constant strain of 1% (**Figure 2a**). Results indicated that the mechanical behavior of BSM-MA hydrogels is independent of the angular frequency probed and, with a tensile storage modulus (G') consistently ten-fold higher than tensile loss modulus (G"), that BSM-MA hydrogels exhibit solid-like elastic properties. The shear modulus of the hydrogels over various strains was also tested, maintaining a constant angular frequency of 1 rad/s (**Figure 2b**). The BSM-MA hydrogels retained their elastic nature over a wide range of strains. Such mechanical behavior is typical of covalently-crosslinked materials that dissipate little elastic energy when sheared because their bonds are difficult to displace. The Young's modulus E of BSM-MA hydrogels, evaluated according to the relationship $E = 2G(1 + \nu)$, is approximately 3 kPa, using a Poisson's ratio ν of 0.5 as is expected for swollen hydrogels. Such a Young's modulus is comparable to that of soft mammalian tissue, including the liver and breast [41].

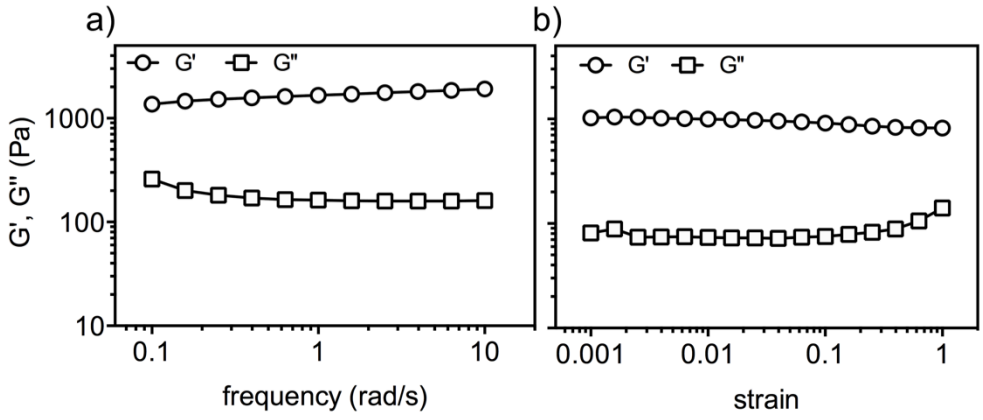

Figure 2. Mechanical testing of BSM-MA hydrogels. The mechanical behavior of the hydrogel was elucidated by shear rheometry testing, varying a) angular frequency (strain maintained at 1%) and b) strain (angular frequency maintained at 1 rad/s).

3.3. BSM-MA hydrogels are slowly degraded by a pronase protease mixture

The degradation behavior of an implantable material is critical because it influences both biocompatibility and efficacy of payload delivery. Premature drug release can occur if the degradation is too rapid; however, the material should resorb and disperse gradually over time to avoid massive fibrous encapsulation. BSM-MA hydrogels are composed of proteic and glycosidic structures, both of which are naturally present in mammalian tissue and can be digested by cell-secreted enzymes. To determine whether BSM-MA hydrogels are sensitive to proteases, BSM-MA hydrogels were kept for 7 days in PBS alone or supplemented with α-chymotrypsin or pronase proteases (**Figure 3**). A 19 ± 5% average decrease in mass was measured over the first 2 days for all BSM-MA hydrogels, likely due to the release and dispersal into solution of mucin that had not been properly crosslinked in the hydrogel matrix. Such initial mass loss did not affect the mechanical properties of the hydrogels, which remained constant for 4 weeks when stored in PBS (**Supplementary Figure 1**). However, to preclude any effect of initial hydrogel matrix rearrangement, all subsequent experiments were conducted after 2 days of storage in PBS. α-chymotrypsin had no measurable effect on the BSM-MA hydrogels, while pronase, a mixture of non-specific proteases, slowly degraded the hydrogels, frequently resulting in fractionated hydrogels by day 7. The relatively slow degradation in pronase and inactivity of α-chymotrypsin on BSM-MA hydrogels can be attributed to the protective effect of mucin-associated glycans [42,43]. The resistance of BSM-MA hydrogels to degradation by α-

chymotrypsin and mild susceptibility to pronase was further evidenced in a fluorescamine-based assay (**Supplementary Figure 2**). Upcoming investigations will shed light on the effect of crosslinking of mucin-bound glycans on the hydrogels' susceptibility to proteases and *in vivo* bioresorption.

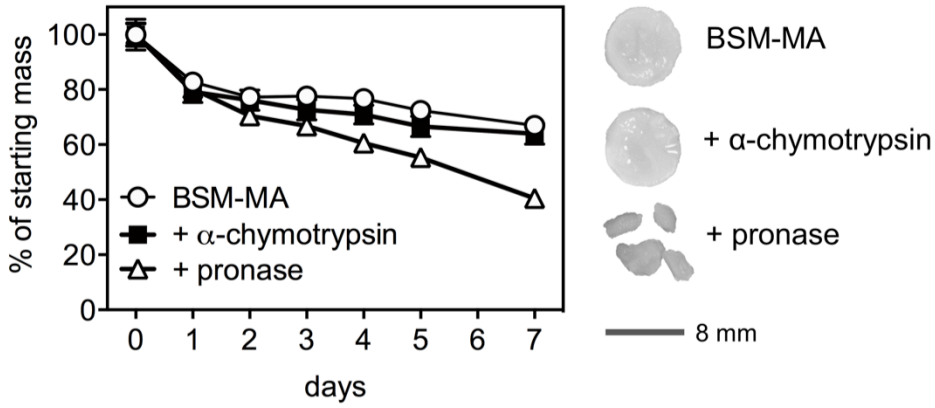

Figure 3. Protease-mediated degradation of BSM-MA hydrogels. Left: evolution of the mass of BSM-MA hydrogels was monitored over 7 days while subjecting the hydrogels to the action of α-chymotrypsin or pronase proteases. Error bars, difficult to resolve in the figure, indicate a standard deviation of less than 6% at each timepoint. Right: macroscopic scheme of the samples at day 7.

3.4. Sustained release of hydrophobic and positively-charged hydrophilic drugs

We investigated the capacity of BSM-MA hydrogels to retain and release over time both hydrophobic and hydrophilic drugs. As a model hydrophobic drug, we selected the anti-cancer drug paclitaxel, a small, uncharged molecule composed of multiple hydrophobic taxane rings that stabilizes microtubules of mammalian cells, blocking cell division [44]. As a model hydrophilic drug, we selected the antibacterial drug polymyxin B, a positively-charged peptide that perforates bacterial cell walls [45]. Chemical structures of paclitaxel and polymyxin B are

displayed in **Supplementary Figure 3**. A fluorescently-labeled version of each model drug was mixed with the BSM-MA solution prior to hydrogel formation. Drug release from each hydrogel into PBS supernatant was tracked over 7 days. Polymyxin B was strongly retained by the hydrogel, with about 2% released from the hydrogel after 7 days (**Figure 4**, insert). Paclitaxel was retained less parsimoniously, with 77 ± 5% of the loaded paclitaxel released over 7 days. Both drugs exhibited a nearly linear release from BSM-MA hydrogels over the 7 day period. Some dependency on initial drug concentration was observed, with a lesser fraction of the loaded drug released per unit time when the initial drug concentration in the hydrogel was increased from 200 μg/mL to 800 μg/mL (**Supplementary Figure 4**). Such observations suggest that drug molecules do not saturate the binding sites of the BSM-MA hydrogel system at the concentrations used in this study.

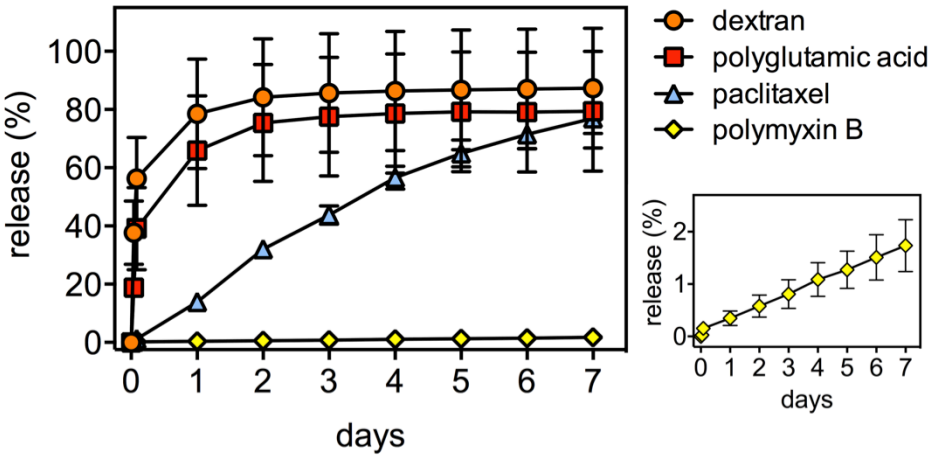

Figure 4. Drug and macromolecule release profiles from BSM-MA hydrogels. Dextran (5 kDa), polyglutamic acid (25 kDa), paclitaxel (0.854 kDa), and polymyxin B (1.3 kDa) were released from BSM-MA hydrogels over 7 days. The insert shows a zoomed-in view of the polymyxin B release profile.

Furthermore, we investigated whether the retarded release of the model drugs was due to mucin-drug interactions (mucin-specific physicochemical retention) or simply to the small pore size of the hydrogel (physico-structural steric retention). To do so, polyglutamic acid (PGA, 25 kDa) and dextran (5 kDa), both of greater molecular mass than the small drug molecules (0.854 kDa for paclitaxel and 1.3 kDa for polymyxin B), were loaded into and released from the BSM-MA hydrogels using the same procedure as for paclitaxel and polymyxin B. As shown in **Figure 4**, more than half of the PGA or dextran payload was released from the hydrogels within 24 hours. These results corroborate previous reports of relatively unhindered diffusion of PGA and dextran through mucus or mucin-based barriers [46,47], which occurs presumably due to the negative charge of PGA and the neutral, highly-hydrated structure of dextran. In contrast, hydrophobic interactions between mucin and paclitaxel and electrostatic interactions between mucin and polymyxin B are likely responsible for the observed physicochemical retention and sustained release of those molecules. Altogether, these findings confirm the cruciality of specific mucin-drug chemical interactions in the sustained release of both hydrophilic and hydrophobic drugs from BSM-MA hydrogels and open the door to the possibility of delivering two or more chemically dissimilar drugs simultaneously.

Pore size does, however, become limiting when the size of the molecular payload reaches or exceeds the order of magnitude of the hydrogel pore size, as we confirmed by loading and observing the release of fluorescently-labeled dextran of various sizes from BSM-MA hydrogels (**Figure 5**). A clear dependence on molecular mass was observed, with a higher fraction of the dextran trapped in the hydrogel for increasing dextran molecular mass. The hydrogel pore size

cutoff fell intermediate to the size of 40 kDa and 250 kDa dextran, corresponding to 4.78 nm and 11.46 nm hydrodynamic radii, respectively [48].

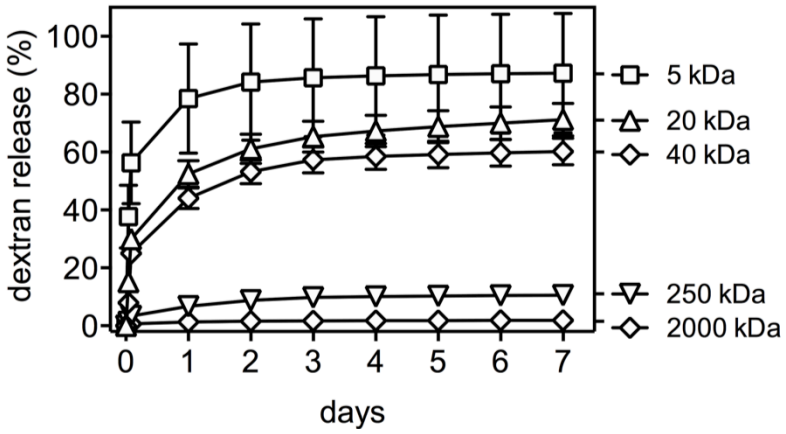

Figure 5. Release of dextran from BSM-MA hydrogels. Dextran of various molecular masses was released from BSM-MA hydrogels over 7 days. 250 kDa and 2000 kDa dextran were strongly retained by the hydrogels, suggesting a hydrogel pore size cutoff intermediate to the sizes of 40 kDa and 250 kDa dextran.

Because commercial BSM is frequently contaminated with other proteins such as albumin, the same drug release experiments were performed using BSM-MA hydrogels formed with BSM purified by lectin affinity chromatography (**Supplementary Figure 5**). The release profiles observed with purified BSM-MA hydrogels were similar to those observed with unpurified BSM-MA hydrogels, indicating that impurities in commercial BSM do not play a major role in drug retention or release (**Supplementary Figure 6**).

3.5. Sustained release of active paclitaxel and polymyxin B for 4 weeks

Sustenance of drug release over several days does not indicate *per se* that the BSM-MA hydrogel is continuing to release sufficient amounts of active drug to produce the desired effect on target

cells. Drug molecules could be deactivated during the crosslinking process or due to their interactions with each other or with mucin. Thus, the amount of active drug released could be too low for pharmacological relevance. We assessed the continuing bioactivity of the released therapeutics over several weeks by exposing *E. coli* bacteria to polymyxin B-loaded BSM-MA hydrogels or HeLa epithelial cancer cells to paclitaxel-loaded BSM-MA hydrogels.

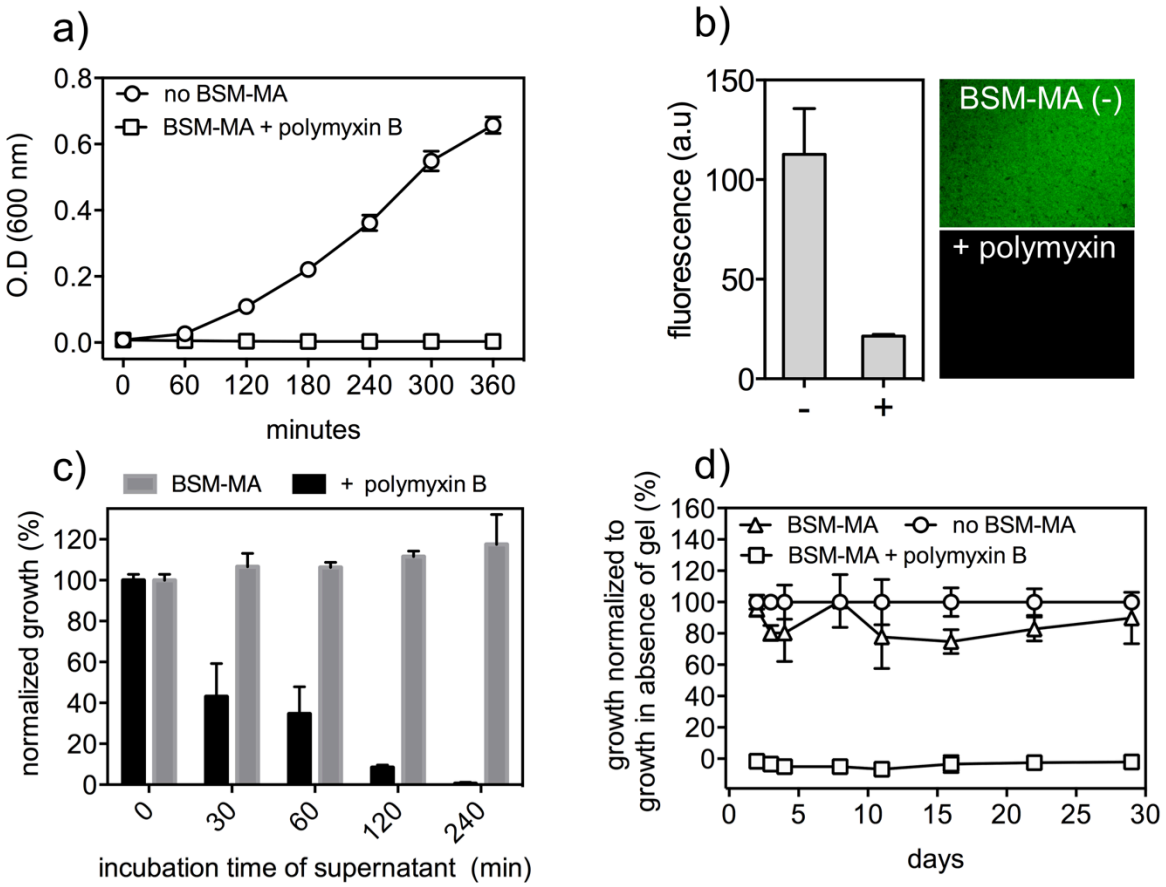

Figure 6. Activity of polymyxin B released from BSM-MA hydrogels. a) Short-term growth curves for *E. coli* in solution placed atop BSM-MA hydrogels loaded or not loaded with polymyxin B. b) Images of GFP-expressing *E. coli* biofilm on the surface of BSM-MA hydrogels after 24 hours of culture and quantification of fluorescence. c) Bacteriostatic effect of culture medium infused with PBS supernatant that had been incubated atop polymyxin B-loaded BSM-

MA hydrogels for increasing amounts of time. Differences in bacterial growth were statistically significant at 30 minutes ($p = 0.012$), 60 minutes ($p = 0.011$), and thereafter ($p < 0.01$). d) Continuing long-term antibacterial efficacy of polymyxin B-loaded BSM-MA hydrogels. Differences in bacterial growth were statistically significant ($p < 0.01$) at each timepoint.

E. coli K12 bacteria in solution did not proliferate when exposed to a polymyxin B-loaded BSM-MA hydrogel over 6 hours, indicating that sufficient quantities of active polymyxin B were present to preclude bacterial growth (**Figure 6a**). The GFP-expressing *E. coli* K12 strain used in this study forms biofilms, tightly-adherent surface communities, which can be observed and quantified by fluorescence microscopy. After 24 hours, a thick biofilm covered the surface of the hydrogel not loaded with polymyxin B, while no bacteria nor biofilm was observed on the hydrogel loaded with polymyxin B **(Figure 6b)**. The observed antibacterial effect of the loaded hydrogel could be due either to surface-bound polymyxin B or to the release into solution of polymyxin B. To determine the mechanism responsible, PBS was placed atop polymyxin B-loaded BSM-MA hydrogels for different amounts of time (0-4 hours), then the resulting supernatant was added to LB and used to grow *E. coli*. **Figure 6c** shows complete inhibition of bacterial proliferation by supernatant from a 4-hour release period, confirming that polymyxin B released from BSM-MA hydrogels retains its activity and indicating that the release of polymyxin B from BSM-MA hydrogels into solution is alone sufficient to inhibit bacterial growth. This does not, however, preclude the possibility that surface-bound polymyxin B molecules also contribute to the antibacterial action of the loaded hydrogels. We probed the longevity of the antibacterial activity of polymyxin B-loaded BSM-MA hydrogels by repeatedly challenging the same hydrogel with bacterial solution over the course of several weeks. The

hydrogel retained its antibacterial efficacy for more than 4 weeks (**Figure 6d**). Negative controls indicated that the BSM-MA hydrogel itself was not toxic to bacteria.

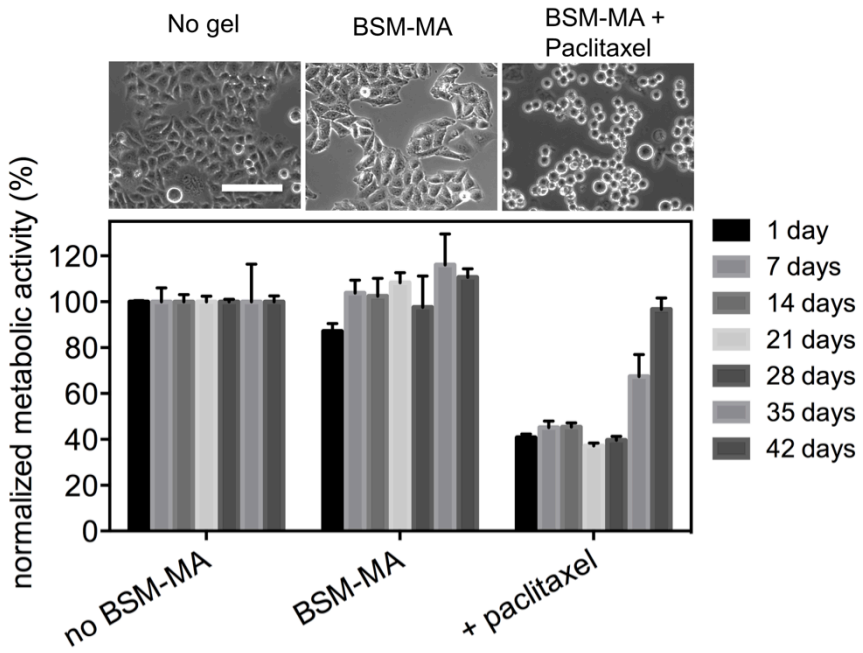

Figure 7. Activity of paclitaxel released from BSM-MA hydrogels. Effect of paclitaxel released from BSM-MA hydrogels on HeLa epithelial cancer cell line morphology (images) and metabolic activity (graph). The observed decrease in metabolic activity was statistically significant from day 1 through day 28 ($p < 0.01$) and on day 35 ($p = 0.024$), but not on day 42 ($p = 0.34$). Scale bar is 40 μm.

The activity of paclitaxel released from BSM-MA hydrogels was evaluated on the HeLa human epithelial cancer cell line. BSM-MA hydrogels loaded with paclitaxel were placed in the same culture medium as, but not in contact with, HeLa cells growing on a polystyrene surface. After a 24-hour incubation and drug release period, the HeLa cells exposed to BSM-MA hydrogels loaded with paclitaxel exhibited reduced metabolic activity relative to HeLa cells exposed to hydrogels not loaded with paclitaxel. Morphological changes consistent with paclitaxel-induced

stress and apoptosis [49] were also observed (**Figure 7**). These experiments confirm that paclitaxel released from BSM-MA hydrogels retains its activity and that pharmacologically sufficient amounts of active paclitaxel are released from BSM-MA hydrogels to impede growth of HeLa cells by inhibiting metabolic activity and blocking cell division. We probed the longevity of the anti-cancer activity of paclitaxel-loaded BSM-MA hydrogels over several weeks by repeating the experiment with the same hydrogels once every 7 days. Significant reduction in HeLa cell metabolic activity was observed until day 28, after which the anti-cancer effect of the paclitaxel-loaded hydrogels decreased progressively.

4. Conclusions

We demonstrate here, for the first time, the assembly of mucin biopolymers into robust macroscopic hydrogels capable of retaining and releasing both hydrophobic and hydrophilic therapeutic molecules in a sustained manner. Using methacrylated bovine submaxillary mucin along with a free radical photoinitiator in the presence of ultraviolet light, we covalently crosslinked mucin into a mechanically-stable hydrogel matrix with rheological properties favorable for physiologic implantation. We demonstrated that through specific physicochemical interactions with the mucin fraction of the hydrogel, small therapeutic molecules, either hydrophobic or positively-charged hydrophilic, can be delivered via mucin hydrogels over long periods of time and produce intended effects on target cells for more than 4 weeks. The results of this study reveal the chemical versatility and natural multifunctionality of the mucin biopolymer and open the door to immunocompatible mucin-based biomaterials that obviate the need for complex multifunctional synthetic polymers.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are deeply grateful to Prof. Katharina Ribbeck for insightful discussions and for supporting this study, conception to completion, in her laboratory. The authors also thank Dr. Gulden Camci-Unal for valuable discussions regarding methacrylation of mucin and Ms. Alice Galais for generating the chemical structures in Supplementary Figure 3. Finally, the authors gratefully acknowledge the Research Science Institute for support to C.V.D and the Marie Curie International Outgoing Fellowship (BIOMUC) for support to T.C.

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Covalently-crosslinked mucin biopolymer hydrogels for sustained drug delivery

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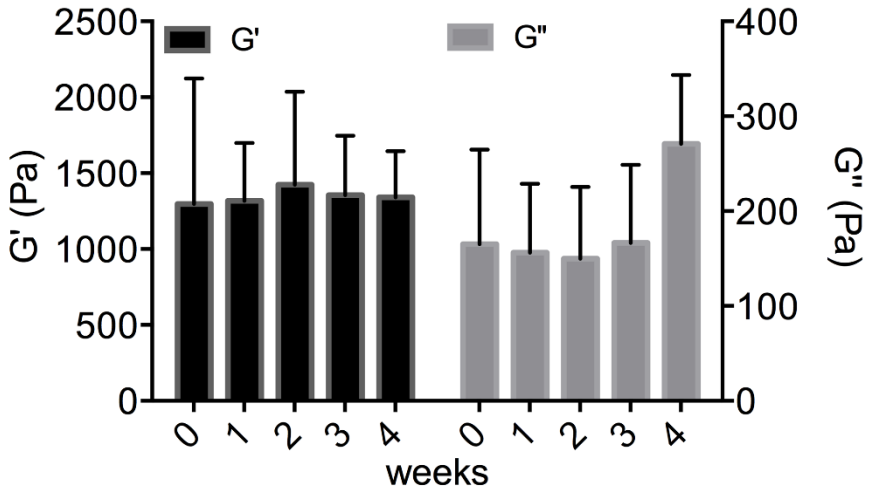
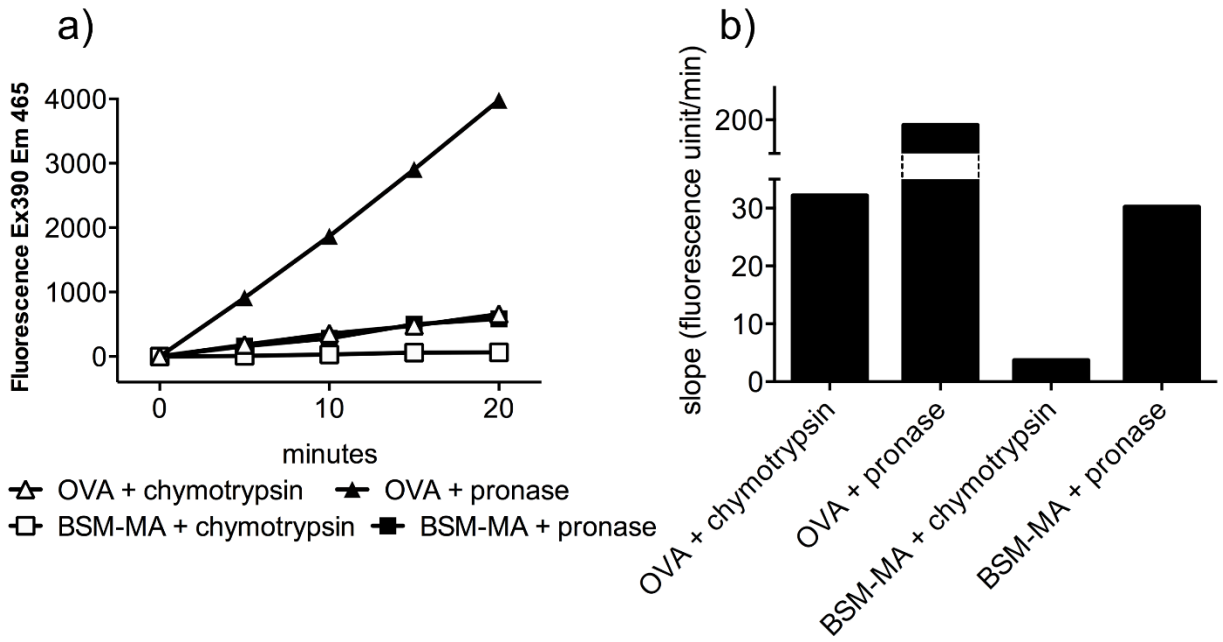
Acta Biomaterialia (2015)
DOI: 10.1016/j.actbio.2015.03.024

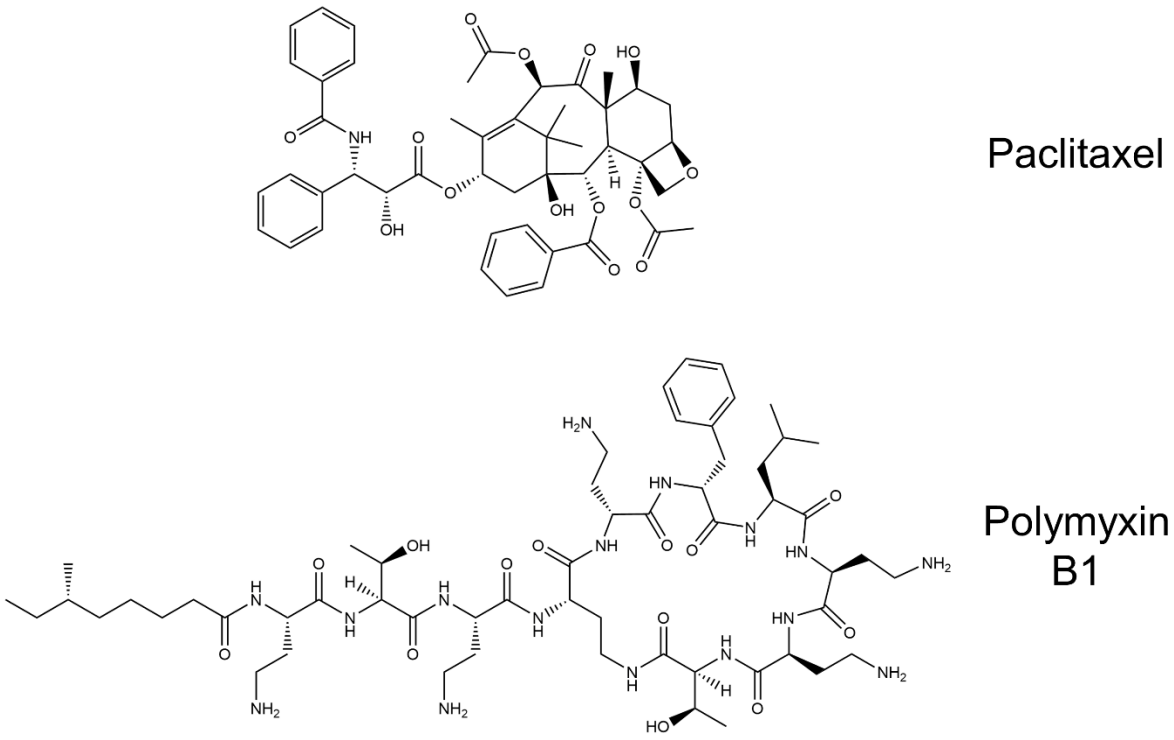
Supplementary Figures



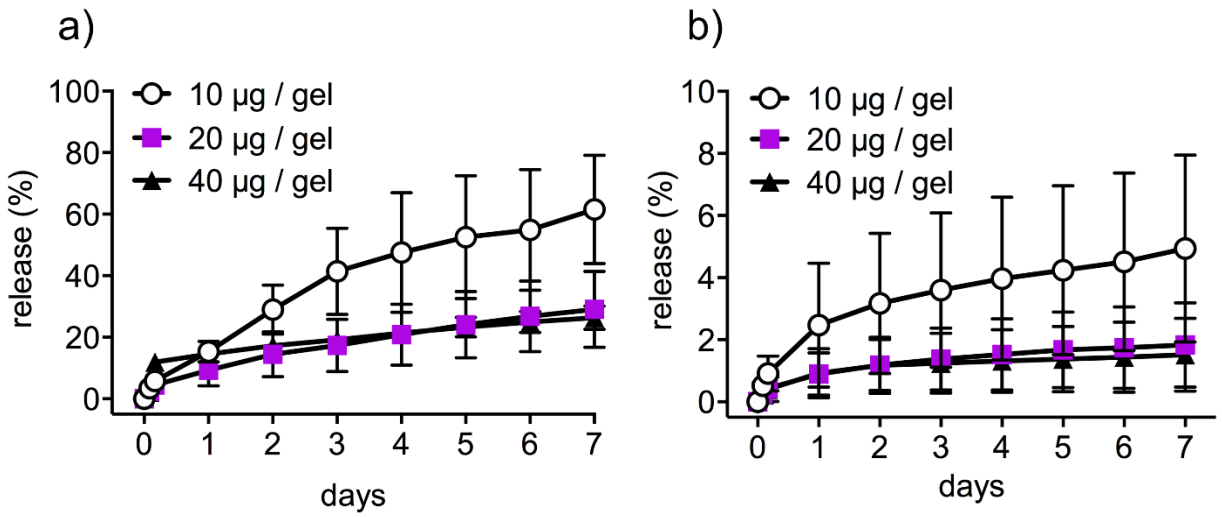
Supplementary Fig. 1. Mechanical stability of BSM-MA hydrogels in PBS. We investigated the resistance of BSM-MA hydrogels to spontaneous degradation at 37 ºC in non-sterile PBS. The shear moduli of the hydrogels did not change significantly over 4 weeks, suggesting that water and small quantities of bacteria and enzymes present in the environment do not affect the mechanical properties of BSM-MA hydrogels over 4 weeks and do not influence *in vitro* release kinetics.



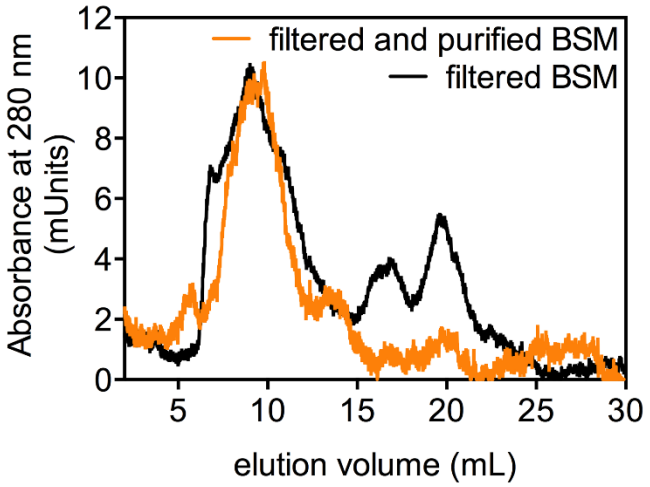
Supplementary Fig. 2. Susceptibility of BSM-MA to α-chymotrypsin- or pronase-mediated degradation. We subjected solutions of BSM-MA (375 µg/mL final concentration in PBS with 25 mM Ca^{2+}) or ovalbumin (OVA, used as a control) to the action of α-chymotrypsin or pronase (both at 200 µg/mL final concentration in PBS with 25 mM Ca^{2+}). The action of protease on proteins exposes free amines to which the bicyclic organic molecule fluorescamine binds and fluoresces [S1]. Fluorescamine (Sigma-Aldrich) dissolved at 5 mg/mL in acetone was used at a final concentration of 150 µg/mL to measure the presence of free amines in each solution. Supplementary Fig. 2a displays the increase of fluorescence of each solution over time, and Supplementary Fig. 2b displays corresponding slopes representing the change in fluorescence per unit time for each solution. All values were normalized for the contribution of fluorescence from the proteases themselves. OVA is sensitive to both proteases, with a more pronounced response to pronase, while BSM-MA is only slightly sensitive to pronase and not sensitive to α-chymotrypsin.



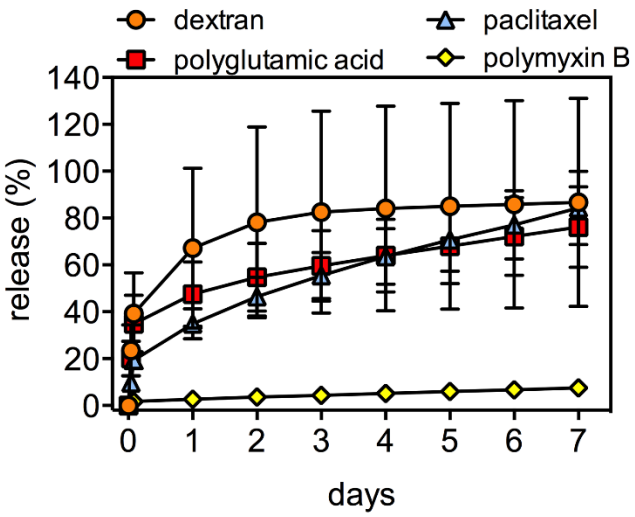
Supplementary Fig. 3. Chemical structures of model drugs paclitaxel and polymyxin B. Paclitaxel is a small, uncharged molecule composed of multiple hydrophobic taxane rings that stabilizes microtubules of mammalian cells, blocking cell division [S2]. Polymyxin B is a positively-charged, hydrophilic peptide that perforates bacterial cell walls [S3].



Supplementary Fig. 4. Concentration dependency of drug release from BSM-MA hydrogels. The concentration of paclitaxel (a) or polymyxin B (b) loaded into the hydrogels was varied from 200 µg/mL (10 µg in 50 µL) to 400 µg/mL and 800 µg/mL (20 µg and 40 µg in 50 µL, respectively). The release profiles indicated a reduction in the percentage of released drug per unit time for higher initial drug concentrations, suggesting that the loading capacities of the 50 µL BSM-MA hydrogels were not reached with a drug concentration of 200 µg/mL.



Supplementary Fig. 5. Size-exclusion chromatography of unpurified BSM and purified BSM. To assess the purity of BSM purified by lectin affinity chromatography, analytical size-exclusion chromatography (Superose 6, GE Healthcare) was performed for unpurified BSM solution (5 mg/mL in PBS, filtered through a 0.45 µm filter) and for purified BSM solution (5 mg/mL in PBS). Elution was performed with PBS, and proteins were detected by ultraviolet absorbance at 280 nm. Unpurified BSM solutions presented a peak in the early fractions, corresponding to high molecular mass mucin molecules, and another peak in the later fractions, corresponding to lower molecular mass protein contaminations, most likely albumin. The purified BSM was eluted exclusively in the early fractions, confirming its purity.



Supplementary Fig. 6. Drug release from purified BSM-MA hydrogels. BSM-MA hydrogels were generated using BSM purified by lectin affinity chromatography. Paclitaxel, polymyxin B, dextran, or PGA was loaded into and allowed to release from the purified hydrogels. The release profiles observed with purified BSM-MA hydrogels were similar to those observed with unpurified BSM-MA hydrogels, confirming that the sustained release of paclitaxel and polymyxin B from BSM-MA hydrogels is facilitated primarily by the mucin component of the hydrogel.

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