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Layer-by-layers of Polymeric Micelles as a Biomimetic Drug-Releasing Network¹

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Abstract

Mucin networks are lubricous coatings formed through the continuous deposition and complexation of mucin glycoproteins. This nanoporous mesh covers many epithelial surfaces, providing surface hydration and tissue protection. Previously, we demonstrated the synthesis of a biomimetic mucin using biotinylated-filomicelles crosslinked via streptavidin using a layer-by-layer (LBL) approach. These networks recreate the fibrous nature of mucin and can serve as a drug release network, due to its formation from drug carrier building blocks. In this work, the ability to vary the network properties by blending filomicelles with spherical micelles is demonstrated. In addition, the deposition of a dense polymer coating on the mucin network was shown to act as a barrier to control diffusion and contributed to improved structural stability under simulated oral chemical conditions. These biomimetic coatings can be utilized as a drug delivery system, providing a localized and tunable release of payload for oral applications.

Keywords

biomimetic; biotin-streptavidin; filomicelles; mucin; oral drug delivery

¹**Supporting Information** is available online from the Wiley Online Library or from the author.

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1. Introduction

Mucins are polymeric glycoproteins that are secreted from epithelial goblet cells in the gastrointestinal and respiratory tracts, and salivary gland acinar cells in the oral cavity.^[1] These glycoproteins are key constituents in forming lubricious coatings on biological surfaces and help provide selective permeability and tissue protection.^[2–4] Mucin possesses a bottle brush-like molecular structure, which upon a complex self-organization process, forms a 3-dimensional biofunctional network that resembles a randomly crosslinked, viscoelastic polymer.^[5] In our earlier work,^[6] an artificial mucin network was formed by crosslinking polymeric filamentous micelles. These synthetic networks were created using a simple layer-by-layer (LBL) deposition of complementary biotinylated filomicelles (FM) and streptavidin (Scheme 1), recapitulating the microstructural characteristics (e.g., growth pattern, thickness, and pore size) of natural mucin. By utilizing FM as the building block, network formation was facilitated through the lateral spanning of the FM.^[6] In comparison, networks formed using spherical micelles (SM) resulted in “island formation” with large gaps between domains, requiring the addition of more layers before full coverage was observed. Here, we evaluate the ability to control the porous nature of the resulting network through blending of spherical and filamentous micelles. In addition, as micelles can be loaded with a variety of small molecules, the ability of these networks to serve as a drug release vehicle is also investigated. To control the rate of release, the micelle networks were coated with an additional polymeric barrier using a previously described LBL system of biotin-poly(acrylic acid) and streptavidin (Scheme 1).^[7] These functional coatings can be integrated with non-invasive delivery systems (e.g., thin films and patches) to facilitate localized, sustained release coupled with localized tissue protection (e.g. in buccal cavity). Overall, this system has potential applications in the field of regenerative medicine, especially among subjects with either diseased or compromised mucosal epithelium.

2. Results and Discussion

2.1. Layer-by-layer Micelle Networks and their Structural Similarity to Natural Mucin

In our previous work,^[6] the synthesis of polymeric filamentous and spherical micelles from amphiphilic diblock copolymers was demonstrated. Methoxy-poly(ethylene glycol)-b-poly(lactic acid) diblock copolymers were synthesized with varying block lengths of the poly(lactic acid) chains to adjust the critical packing parameters and thereby shift the micellar morphology from spherical to filaments (Figure 1).^[8] These micelles were functionalized with biotin and, when deposited onto synthetic/biological surfaces, demonstrated mucin-like structures.^[6]

In natural mucin networks, the structural properties (e.g., pore size, thickness, viscosity, and anisotropy) vary across different epithelial regions, primarily dictated by the local pH and ionic environment. Interestingly, these physical properties can be highly deterministic of its biological function. In general, the structure of human and pig gastric mucins are similar to a beaded necklace.^[9, 10] Hong et al (2005) examined the mucin aggregation tendency as a function of pH and suggested that it bears a striking similarity to micellar aggregates.^[9] Importantly, the FM networks fabricated in our present study possessed a striking similarity to natural mucin glycoproteins, which form a continuous reticulated structure (Figure 2 and

3). After network deposition onto a polystyrene surface, the substrate height topography and texture was modified (Figure 3 and Figure S1 in Supporting information), where a significant increase (165 nm) in the height topography was observed. The observed phase-shift intensity was due to the hard hydrophobic polystyrene surface being replaced by the more compliant, hydrophilic PEG coating of the FM micelles (Figure S1 in Supporting information). Substrate-network confluency was observed, with a near complete coverage (~100%).

In general, the topographical features of nanoparticle-based multilayered systems possess high surface roughness owing to their distinctive lateral-growth pattern.^[11, 12] However, in FM networks, it was determined that the topographical irregularities were only at nano length scales (Figure S2 in Supporting information).^[6] The surface roughness factor can play an important role in the performance of biomaterials (e.g., implants and biofunctional coatings) by significantly altering biological responses (e.g., bacterial adherence or macrophage invasion). For instance, with intraoral implant materials, it is documented in literature that above a threshold surface roughness (R_a) of 200 nm, significant bacterial adhesion and retention is observed.^[13] For implant coatings, reducing the surface roughness can improve wear resistance, where the recommended root mean squared (RMS) roughness is <50 nm.^[14] Although the current FM network is suggested as a topical delivery system, its substrate growth tendency can also be extended to surface coating applications (e.g., as implant or stent coatings). These FM networks possess a surface roughness (R_a) of 55 nm and a RMS value of 70.9 nm, which suggests a relatively smooth surface topography.

2.2. Synthetic Networks from Micellar Compositional Blends

2.2.1. Morphological Characteristics of Networks from Blends of Spherical-Filamentous Micelles—Successful functioning of mucin networks is determined by their morphology. Key variables that determine morphology include mucin chain length, polydispersity, degree of glycosylation, and pH.^[10, 15] For instance, Menarguez et al. (2003)^[16] observed four distinct structural nanomorphologies in cervical mucin networks. Subsequent studies by Brunelli et al. (2007)^[17] further demonstrated that structural transitions in cervical mucus occur in response to changes in the menstrual cycle, where during ovulation the mucin network is highly porous, highly permeable, and contains less continuous spherical aggregates. In the preovulatory stage, however, compact, less permeable, continuous fibrillar (pearl-like) networks are observed, which hinder spermatozoa migration. In our previous work,^[6] the network formation mechanism from spherical and filamentous structures was studied. Comparable to cervical mucus, the filomicelle networks were aided by a high aspect ratio (micelle length to diameter ratio, L/D), which enabled more ready bridging to form a continuous, cross-linked, fibrillar network. Spherical micelles, on the other hand, with a relatively low aspect ratio showed poor network formation tendency and resulted in a non-continuous spherical aggregate.^[6]

In the current work, this shape-based dependence was further studied by forming networks using different FM-SM micelle blends. The micelles were synthesized to contain a small molecule, curcumin (2.65% w/w), which is a fluorophore that facilitates visualization. Additionally, curcumin is a well-known natural curuminoid with a versatile therapeutic

capability (e.g., antiinflammatory, antimicrobial, anticancer, and antiarthritic). The synthesized SM possessed an average particle diameter of 240 ± 5.5 nm, while the predominant length distribution of FM ranged from 5–35 μ m (Figure 1).

Utilizing these synthesized micelles, as a proof of concept, 7-layered networks were developed from different SM/FM blends (0, 20, 40, 60, and 100 wt% FM) and visualized for their network formation ability and lateral growth (homogeneity of coverage) (Figure 4, and Figure S3 in Supporting information). SEM and fluorescence imaging of blends having increasing percentage of FM showed a logarithmic improvement in lateral network growth (Figure 5). The networks formed from predominant spheres (20% FM) possessed a network-substrate coverage of 50–70%, while micelle-LBL networks formed from mixtures containing 40–60% FM had significant improvement in lateral growth, with a substrate coverage of 80–90%. For the high FM containing networks, filomicelles likely functioned as a structural scaffold facilitating an easier bridging of spherical aggregates. As expected from these observations, the networks formed from 100% FM showed a near complete confluency in network-substrate coverage (~100%) (Figure 4, and Figure S3 in Supporting information).

Jeon et al. (2006)^[11] performed molecular dynamics simulations in studying independently the LBL growth mechanism from particulate systems (e.g., nanoparticles (NP), polyelectrolytes (PE), and systems). Driven by charge-based interactions, a lateral expansion growth pattern was observed due to electrostatic repulsive forces. These results were consistent with the experimental work performed by Ostrander et al. (2001)^[12]. In those systems, the extent of surface coverage during multilayer depositions improved in the following order: NP-NP < NP-PE < PE-NP < PE-PE.

Interestingly, the substrate coverage improvement with an increasing percentage of FM is strikingly similar to the trends observed in the particulate systems described above. Here, the FM serves the role of the flexible polyelectrolyte with better structural aspect ratio, while NP size is comparable to the SM. In affinity-based multilayer systems, the lateral growth improves in the following order: 0% FM (or spherical-spherical) < 20–40% FM (or predominant spheres) < 60% FM (predominant filomicelle) < 100% FM (filomicelle-filomicelle) (Figure 4 and 5). These results suggest that the relative spherical to filomicelle ratios plays a significant role in determining network structure and substrate surface coverage. For synthetically developing well-formed mucin networks, it is recommended for micellar population to possess at least 40% FM during LBL development.

2.2.2. Bacterial Adherence Studies on Networks Formed from Micellar Blends

—As the epithelium lines many exterior surfaces, it can be populated with a wide variety of bacterial flora whose diversity varies based upon the anatomical regions and physiological conditions (e.g., normal vs diseased state). For instance, nearly 500 different bacterial species (e.g., *Streptococcus*, *Gemella*, *Granulicatella*, *Veillonella*) can be found on the human oral epithelium.^[18–20] The role of natural mucin networks is to serve as a protective barrier and reduce bacterial accessibility to the underlying tissue. In general, the mucin structure can be demarcated into two zones: 1) the outermost loosely adherent mucin layers that function as a sacrificial trap in agglutinating bacteria^[5, 6] and 2) the innermost tightly bound mucin layers that act as a barrier. The capture mechanism of mucins can be primarily

attributed to the mucoadhesive interactions from their glycoprotein chains, while the barrier effect can emanate from size differences between bacteria and network pore size, which provides a selective exclusion effect.

In the previous work, bacterial growth on filamentous and spherical networks was studied independently.^[6] It was established that the growth inhibition effect resulted from release of antibacterial curcumin payloads from the micellar structures. In the present work, synthetic networks formed from different spherical–filomicelle blends were studied for their bacterial inhibition capacity. As the network formation ability improved with increasing filomicelle content, curcumin drug content within the bulk structure also increased in proportion to the percentage of FM. Therefore, the networks formed from micelle mixtures with predominant spheres (<40% filomicelle) possessed low overall drug content of ~10 µg/ml, and demonstrated a weak bacterial inhibition effect of $11.55 \pm 7.24\%$. This was followed by a relatively moderate growth inhibition of $18.86 \pm 7.38\%$ from networks formed from 40 to 60% filomicelle population possessing drug content of ~19.5 µg/ml. Consistent with our earlier findings, the networks formed from predominant filomicelles (~80% FM) (Figure 6) contained higher curcumin drug content (~22 µg/ml) within their networks, and resulted in higher bacterial growth inhibition ($34.7 \pm 7.88\%$). These antibacterial studies exemplify the biofunctional properties of synthetic network coatings.

2.3. Polymeric Layer-by-Layer Capping Barriers over Synthetic Networks

2.3.1. Developing Polymeric Capping Barriers over Synthetic Networks—As a means of further enhancing stability and network properties, capping layers were formed on top of 7-layered FM networks possessing complete substrate-surface confluency. A previously published polymeric layer-by-layer (polymeric-LBL) deposition process was adopted for this purpose.^[7] This process involves multilayer depositions of alternating streptavidin and biotinylated poly(acrylic acid) (Figure 7a).

Poly(acrylic acid) (PAA) chains (MW ~225,000 Da) containing ~40 biotins/PAA chain were synthesized (Supporting information and Figure S5) and used to develop a robust capping barrier. LBL capping growth was monitored by tracking radiolabeled streptavidin (¹²⁵I-streptavidin) for increasing number of capping layer additions (NoLcapping). Due to the lack of specific affinity linkages, the non-biotinylated poly(acrylic acid) (Biotin-0/PAA225k) was unable to develop any capping layers over FM networks. However, the biotinylated polymer resulted in a clear linear increase in streptavidin mass with increasing number of capping layer depositions (NoLcapping= 1, 3, 5, and 7) (Figure 7b), due to self-organization driven by biotin-streptavidin crosslinkers. Therefore, the 7-layered capping layer additions from these biotinylated polymers showed nearly 7.75-fold higher protein mass increase than that of the non-biotinylated polymers, demonstrating the ability to form capping barriers.

2.3.2. Stability of Polymeric Capping Barriers—The barrier stability was studied by tracking ¹²⁵I-streptavidin loss from the capping layers. Since capping barriers were formed over 7-layered FM networks using streptavidin based cross-links, its stability was evaluated under proteolytic (pronase) conditions.

Capping layers formed from non-biotinylated polymer (Biotin-0/PAA225k) possessed a weak barrier (Figure 7c). The unconjugated polymers lacked affinity-based functional moieties, which resulted in a weakly held polymeric layer over synthetic networks via charge based polymer poly(acrylic acid)-protein streptavidin interactions. Due to this non-specific barrier, resistance to protein loss did in fact increase with increasing the number of capping layers (NoLcapping= 3, 5, and 7).

However, with biotinylated polymer (Biotin-40/PAA225k), barriers with more than 3 capping layers demonstrated a more substantial reduction in protein loss, where nearly 60–65% of streptavidin remained intact after 24 hr of exposure to proteolytic enzyme, suggesting considerable capping robustness due to the affinity interactions (Figure 7c).

2.3.3. Effect of Polymeric Capping Barriers on Network Chemical Stability—

The chemical stability of uncapped FM networks (NoLcapping=0) was previously demonstrated.^[6] In the present work, to study the effect of polymeric barriers on chemically induced network destabilization, 7-layered FM network with variable number of capping layers (NoLcapping= 3, 7 and 15) were formed. These chemical stability tests were performed under proteolytic pronase and simulated salivary environment due to its high relevancy as intra-oral conditions. The extent of protein destabilization from those capped-FM networks was independently tracked from its network interlayers and base layer. By tracking the protein loss from network interlayers, the impact of capping on bulk synthetic mucin structures could be evaluated.

In our earlier work, it was shown that a 7-layered FM network (NoLcapping=0) acts as a barrier to protect its base streptavidin layer. The diffusion of a large proteolytic enzyme (MW ~20,000 Da) was greatly hindered due to the thick FM networks and thus was unable to readily reach the base streptavidin layer. In the current work, addition of a soluble polymeric capping layer over the FM network created a smaller network mesh size. These additional polymeric capping barriers further reduced the accessibility into the base streptavidin layer. Hence, such polymer shielding from chemical effects contributed to a reduced base streptavidin destabilization, improving overall network intactness. Thus, under both protease and salivary environment, a reduction in base streptavidin layer destabilization was observed with increasing capping layer, suggesting reduced network disassembly. This trend can be readily noted by comparing streptavidin loss with and without capping layers (Figure 8a),^[6] where the 7-layer capped networks showed nearly 29% lesser protein loss than the uncapped structures after 26 hr incubation with protease. Such structural stability improvement due to cappings was even more pronounced under simulated salivary environment, where nearly 45% less mass loss from no-capped structures was observed at 26 hr.

Similarly, in FM network layers that immediately underlay the capping layers, the effect of this polymer barrier effect was pronounced under the simulated salivary environment. For instance, the 7-layered capped networks showed nearly 43% less protein loss than uncapped structures at 26 hr.^[6] A reduced streptavidin destabilization was observed with increasing number of capping layers. Interestingly, the capping effect was relatively less pronounced under protease exposure compared to salivary environment (Figure 8b). For instance, in a

proteolytic environment the 7-layered cappings showed only 12% less protein loss from uncapped structures at 26 hr. The proteolytic environment resulted in a relatively higher destabilization effect on protein-based network cross-links in comparison to simulated salivary environment. Due to this high destabilizing potential, the barrier effects were relatively less pronounced in the FM layers directly underlying the capping layers as opposed to the base layer.

2.4. Drug Release from Synthetic Mucin Networks and the Impact of Affinity based Polymeric Capping Barriers

The commonly adopted strategies used for fabricating LBL delivery platforms with tunable drug release can be broadly categorized as: 1) stimuli triggered release (e.g., temperature and pH), 2) release dependent on inherent properties of the building blocks (e.g., biodegradability), and 3) incorporating barriers for controlling release (e.g., post-modifications such as covalent/thermal crosslinking). For instance, a recent study by Zhu *et al.* (2013)^[21] demonstrated stimulus induced on-demand drug release using thermo-responsive spherical micelles with anticancer drug loadings. In conventional multilayered systems, the drugs were loaded from complementary charge compensations or via passive loading within the self-assembled structure. Therefore, to control drug diffusion from relatively weak loading, a more rigorous post-modification process is commonly employed (e.g., covalent crosslinking via thermal or chemical methods). For instance, Wood *et al.* (2006) used thermal crosslinking methods to demonstrate the ability to control interlayer drug diffusion within a polyelectrolyte multilayer (PEM) network.^[22]

However, these post-modification methods may be not recommended when functional coatings require milder processing conditions (e.g., in protein/peptide-based coatings). Such processing difficulty can be avoided in the present system through formation of robust capping barriers via highly specific biotin-streptavidin non-covalent linkage. Additionally, controlling polymeric molecular properties or altering the thickness of capping barriers helps tune the drug release from the network.

Unlike the traditional PEM-based networks, in the current systems the interlayer drug diffusional issue can be regulated by controlling the release kinetics from the micellar core. Here, the PEG chains form an external barrier which along with curcumin drug-PLA core interactions help form the major (primary) diffusional barrier. To further modify release kinetics, a secondary diffusional barrier was created over the primary barrier via polymeric capping.

2.4.1. Drug Release from Synthetic Mucin Networks Without Capping Barriers

—The release of curcumin from FM networks remained unhindered despite increasing the network thickness ($NoL_{FM-LBL} = 1, 3, 5$ and 7). In nanoporous FM networks without a secondary diffusional barrier, the overlaying micellar structures did not impact diffusional curcumin release. As such, no observable difference was observed under both salivary (Figure 9a) and protease environments (Figure 9b).

Combining the recent advancements in drug carrier formulation or release strategies, for instance by modifying the primary barrier through crosslinked shell or using polymer-drug

conjugate, can significantly alter the drug release kinetics of the network.^[23–25] These changes in primary barrier properties can be incorporated for developing a biofunctional scaffold or an implant coating that requires a sustained delivery (e.g., weeks to months).^[26, 27]

2.4.2. Tunable Drug Release from Networks With Capping Barriers—To study the effects of the secondary barriers on drug release without significantly overshadowing the effects of the primary barrier, 7-layered FM networks with increasing number of capping barrier depositions (NoLcapping= 1, 3, 5, 7, 10, and 15) were formed separately. Interestingly, even with the low number of polymeric capping layers, a significantly modified drug release profile was observed from FM networks, suggesting an added diffusional resistance that delayed release. This capping layer effect was observed under both salivary (Figure 9c) and proteolytic conditions (Figure 9d). Also, more importantly, the self-assemblies showed significant robustness under both chemical environments (Figure 10), where the drug release primarily remained a diffusion controlled process as opposed to network destabilization based drug release.

3. Conclusions

The present studies demonstrated the ability to modify surfaces by forming affinity driven layer-by-layer networks of polymeric micelles, and their physical and biofunctional similarity to natural mucin structures was highlighted. In addition, deposition of additional polymeric capping barriers was shown further to tune drug release. These polymeric barriers demonstrated significant stability in proteolytic and simulated salivary conditions. Utilizing the facile network formation dynamics through affinity linkages, these polymeric networks provide exciting possibility as drug eluting biofunctional coatings.

4. Experimental Section

4.1. Materials

2-(4-hydroxyphenylazo) benzoic acid (HABA), crystal violet, curcumin (from curcuma longa), 2-(N-morpholino)ethanesulfonic acid (MES), glucose, mucin (from bovine submaxillary glands), and protease derived from *Streptomyces griseus* (pronase) were purchased from Sigma Aldrich (St.Louis, MO). Iodogen[®] iodination reagent (1,3,4,6-tetrachloro-3 α -6 α -diphenylglycouril) and pentylamine-biotin were purchased from Thermo Scientific (Rockfort, IL). Poly(acrylic acid sodium salt) (MW ~225,000 Da) was purchased from Polysciences, Inc (Warrington, PA) and was lyophilized prior to use. Recombinant streptavidin (produced in *E.Coli*) was purchased from Prospec-Tany (East Brunswick, NJ). N-hydroxysuccinimide (NHS) was purchased from Acros Organics (Waltham, MA). *Staphylococcus aureus* subspecies Rosenbach was purchased from ATCC (Manassas, VA). Avidin (from egg whites) was purchased from Life Technologies[™] (Grand Island, NJ).

4.2. Biotin-Micelle Synthesis

Biotin functionalized spherical (SM) and filamentous micelles (FM) were synthesized with curcumin drug loading using cosolvent/evaporation method, by adopting a previously

established procedure.^[6, 28] Custom-synthesized poly(ethylene glycol)-block-poly(lactic acid) based diblock copolymers were used. The synthesis and characterization of the amphiphilic diblock copolymers (methoxy-poly(ethylene glycol)-b-poly(lactic acid) (mPEG-b-PLA) and biotin-poly(ethylene glycol)-b-poly(lactic acid) (biotin-PEG-PLA)) are detailed in the earlier work.^[6] For synthesizing spherical micelles, the mPEG-PLA diblock containing low PLA chain length (PLA ~68% w/w) was used, while relatively higher PLA chain length (PLA ~80% w/w) was used in forming filamentous micelles.

To synthesize biotin-functionalized micelles, the diblock copolymers mPEG-PLA (50 mg, ~91% w/w) and biotin-PEG-PLA (5 mg, ~9% w/w) were dissolved in chloroform solvent (oil phase). DI water (aqueous phase) was added in large excess to the oil phase containing polymeric blend (water:oil ~25:1 (vol. basis)), and was dispersed into a homogeneous suspension using ultrasonic homogenizer (Fisher Scientific, Sonic dismembrator Model 100). This oil/water emulsion was stirred vigorously (~16 hr) at room temperature until the oil phase is completely evaporated to form hardened biotin-micelles. Micelles were loaded with curcumin (1.5 mg, ~2.65% w/w) during synthesis. The larger sized particulate fraction was separated using centrifugation (1000 g for 10 minutes). While, the desired nanoparticle fraction was isolated by spinning down at 7500 rpm for 45 minutes.

4.3. Micelle-LBL Network Formation

In vitro micelle-LBL networks were developed on polystyrene plates at room temperature, adopting the procedure from our earlier work.^[6] In forming micelle-LBL networks, the streptavidin base layer addition was provided (1 mg/ml in phosphate buffered saline ((PBS, pH 7.4), 100 µl/well, and 1 min incubation). To this base layer, complementary biotin-micelles (~5 mg/ml in DI water, 100 µl/well, and 1 min incubation) and streptavidin additions (~1 mg/ml in PBS, 100 µl/well, and 1 min incubation) was provided sequentially. This rinsing cycle was repeated until a desired number of layer (NoL) additions were achieved. Excess biotin-micelles or streptavidin during each LBL addition steps was removed using a PBS rinse followed by a DI water (0.2 µm filtered) rinse post-network development.

In forming natural mucin depositions, glycoproteins (from bovine submaxillary glands) were allowed to adsorb onto the polystyrene substrates (0.1 wt% in DI water, 1 h incubation). The excess mucin was gently rinsed off using DI water rinses (0.2 µm filtered), and the networks were allowed to dry off at room temperature. The 7-layered FM network and natural mucin depositions were visualized under a scanning electron microscope (SEM, S-4300, Hitachi), and images were taken at randomly selected locations at different magnifications.

4.4. Atomic Force Microscopy of Micelle-LBL Networks

Filamentous micelles (FM) were synthesized as described earlier under biotin-micelle synthesis. These FM were used in formation of 7-layered filomicelle network over polystyrene substrate, adopting the same procedure as described above under micelle-LBL network formation. The topography and surface roughness were imaged in a non-contact (tapping) mode AFM (Agilent Technologies, Series 4500), before and after network formation over the substrate. The networks were imaged in air medium (non-liquid mode)

using a long cantilever (T190) (VISTAprobes® Phoenix, AZ) with a radius of curvature less than 10 nm (Spring constant- 48 N/m, resonant frequency-190 kHz, length- 225 μm , width- 40 μm , tip height- 14 μm). To visualize network morphologies at different length scales, the scans were performed using large and small area scanners. Under larger area scans 10 $\mu\text{m} \times 10 \mu\text{m}$, and using smaller area scans 2 $\mu\text{m} \times 2 \mu\text{m}$ and 0.5 $\mu\text{m} \times 0.5 \mu\text{m}$ networks surface area were visualized. For image visualization and analysis, Gwyddion software was used.

4.5. Network Formation from Spherical-Filamentous Micelle Compositional blends

Spherical and filamentous micelles were synthesized as described above under biotin-micelle synthesis. The micelle formulations were blended to form different compositional ratios (0, 20, 40, 60, 80 and 100% w/w FM). These micelle blends were used in the formation of synthetic networks, adopting the procedure as described above under micelle-LBL network formation. 7-layered micelle-networks were deposited on polystyrene 12-well microplates under similar conditions and visualized under epifluorescence microscopy (Nikon Eclipse LV100, FITC filter, under 10 $\times$ /20 $\times$ objectives, NIS Elements).

4.6. Scanning Electron Microscopy Imaging of Networks Formed from Spherical-Filomicelle Blend

7-layered networks were developed over polystyrene substrate adopting the same procedure as described above under network formation from compositional blends. During LBL network formation process, the excess micelles and streptavidin were removed by PBS rinses between each layer, and finally rinsed off with DI water (0.2 μm filtered) post-network development. The procedure is repeated for different micelle blends, till a desired number of layer addition (NoL=7) was achieved. These networks were visualized under a scanning electron microscope (SEM, S-4300, Hitachi), and images were collected from randomly selected locations at different levels of magnification.

4.7. Bacterial Growth on Networks Formed from Micelle Compositional Blends

7-layered networks were formed from different spherical-filomicelle blend ratios (0%, 20%, 40%, 60%, 80% and 100% of filomicelles) on a polystyrene 48-well microplates (Becton Dickinson Biosciences, San Jose, CA). The biotin-micelles used for the studies contained ~2.6% w/w curcumin drug encapsulation within the micellar core. Bacterial growth and subsequent evaluation on extent of network-bacterial adhesion was performed as described in our earlier work.^[6, 29] In brief, suspensions of *Staphylococcus aureus* Rosenbach strain was taken in Bacto Brain Heart Infusion (BHI) broth and was cultured overnight. Prior to introducing the culture to networks, a 1% w/v D-glucose and 2% w/v sodium chloride was added to the broth to improve biofilm formation over networks. The culture was diluted and added to the networks (200 μL , 10^5 cell forming units/200 μL), covered, and incubated at 37 $^{\circ}\text{C}$. After 24 hr, in quantifying the extent of bacterial growth over networks, a crystal violet solution (0.1% w/v in DI water, 40 μL) was added to each well and incubated for 10 minutes at room temperature. Subsequently, the supernatant was removed, and the biofilms are rinsed at least thrice with PBS. To disperse the crystal violet retained within bacterial cell walls, acetic acid (200 μL , 30 vol%) was added and incubated for 10 minutes at room temperature. The solution was then mixed and absorbance values were area scanned at $\lambda=$

600nm (BioTek Synergy Mx, Gen5 2.0, Winooski, VT). The extent of bacterial growth over networks formed from micelle mixture was calculated as per the equation given below:

$$\% \text{Bacterial Growth} = \frac{(\text{Abs}_{\text{LBL(bacteria)}} - \text{Abs}_{\text{LBL(no- bacteria)}})}{(\text{Abs}_{\text{Substrate(bacteria)}} - \text{Abs}_{\text{Substrate(no- bacteria)}})} \times 100$$

4.8. Polymeric-LBL Capping Barriers over FM Networks (Capped-FM networks)

In vitro 7-layered FM networks were deposited on biotin-coated polystyrene plates (Thermo Scientific Pierce, Rockford, IL, preblocked 96-well strip plates), by providing alternating additions of biotin-micelles and streptavidin, adopting the same procedure as described above under micelle-LBL networks formation. To these preassembled structures, capping layer additions were provided by alternative rinsing from biotinylated poly(acrylic acid) (biotin-PAA) (~1 mg/ml, 100 µl/well, 1 min incubation) and streptavidin (~1mg/ml, 100 µl/well, 1 minute incubation). This rinsing cycle was repeated until a desired number of polymer capping layers were achieved. Excess biotin-PAA and streptavidin were removed by rinses with PBS between each additions. Here, the number of capping layer (NoLcapping) represents the number of polymeric PAA layer additions provided during polymeric-LBL deposition process.

4.9. Demonstrating Polymeric-LBL Capping Growth over FM networks

A modified capped-FM network formation procedure was used. 7-layered FM networks were deposited on biotin-coated polystyrene plates from alternating additions of biotin-micelles and non-radioactive streptavidin. This was followed by capping barrier growth from complementary rinses of biotinylated poly(acrylic acid) and ¹²⁵I-streptavidin, till a desired number of capping layer (1, 3, 5 and 7) additions was achieved over the networks. Excess biotin-PAA and ¹²⁵I-streptavidin were removed by rinses with PBS between each addition. Streptavidin mass in capping layers was tracked using a gamma counter (Perkin Elmer 2470 Wizard²) with polymeric-LBL additions.

4.10. Evaluating Stability of Capping Barriers

The capping barriers were selectively formed using ¹²⁵I-streptavidin, using the above described procedure. The capped-FM networks (3, 5, and 7) are formed over the preassembled 7-layered FM network. These structures were incubated under proteolytic pronase (0.01% w/v, 100 µl) environment, at 37 °C for different times. The capping layer stability in FM networks was quantified from extent of radioactive streptavidin mass loss from its layers, which was tracked using a gamma counter (Perkin Elmer 2470 Wizard²).

4.11. Effect of Capping Barrier on Chemical Stability of FM Networks

Capped-FM networks were developed using a modified formation procedure, as described below. The effect of capping barrier on network destabilization was studied by tracking independently the loss of ¹²⁵I-streptavidin from the network interlayers and the base streptavidin layer.

For measuring the network interlayers, a non-radiolabeled streptavidin layer was first added to the base biotin layer (~1 mg/ml in PBS, 100 µl/well, and 2 min incubation), followed by alternating biotin-FM (~5 mg/ml in DI water, 100 µl/well, and 1 min incubation) and ¹²⁵I-streptavidin additions (~1 mg/ml in PBS, 100 µl/well, and 1 min incubation) until the desired NoL (=7) were deposited. To these modified FM-network, the capping layers were developed from complementary biotinylated poly(acrylic acid) (~1 mg/ml in DI water, 100 µl/well, and 1 min incubation) and non-radioactive streptavidin additions (~1 mg/ml in PBS, 100 µl/well, and 1 min incubation), till a desired number of capping layers (NoLcapping= 3, 7, and 15) were achieved. For measuring the base layer loss, a similar procedure was adopted, except that ¹²⁵I-streptavidin was added to the base biotin layer, while the overlaying network interlayers and capping layers were deposited using non-radiolabeled streptavidin.

These capped-FM networks were incubated in simulated saliva (100 µl of 1.3 mM KH₂PO₄, 16 mM Na₂HPO₄, 136.9 mM NaCl, pH= 6.75)^[6, 30] or pronase (0.01% w/v, 100 µl) at 37 °C for different durations. Streptavidin mass remaining in the networks was measured using a gamma counter (Perkin Elmer 2470 Wizard²).

4.12. Drug Release Studies from FM Networks

FM networks with- and without polymeric-LBL capping barriers were deposited on biotin-coated polystyrene plates separately, adopting the procedure as described in the earlier sections. For drug release studies, the FM and capped-FM networks were developed from non-radioactive streptavidin.

In evaluating drug release without capping layers, FM networks with varying number of LBL depositions (1, 3, 5, and 7) were formed. For assessing drug release with capping layers, 7-layered FM network with varying number of polymeric capping addition (NoLcapping= 1, 3, 5, 7, 10, and 15) were formed separately.

Drug release from the synthetic mucin networks was carried out under *in vitro* oral chemical conditions, either by incubating at 37 °C in simulated saliva (100 µl of 1.3 mM KH₂PO₄, 16 mM Na₂HPO₄, 136.9 mM NaCl, pH= 6.75) or proteolytic solution (0.01% w/v pronase, 100 µl) for different times. To determine the curcumin mass remaining within the networks after drug release, the entire surface area was mapped using fluorescence area scans using a microplate spectrophotometer (Ex/Em: 420/500 nm, top/bottom read, BioTek Synergy Mx, Gen5 2.0, Winooski, VT).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix/Nomenclature/Abbreviations

NP	nanoparticles
PE	polyelectrolytes
PEG	poly(ethylene glycol)
PLA	poly(lactic acid)
PEM	polyelectrolyte multilayer
PAA	poly(acrylic acid)
biotin-PAA	biotinylated PAA
Biotin-0/PAA225k	non-biotinylated PAA (MW ~225 kDa)
Biotin-40/PAA225k	biotinylated PAA (#40 Biotin molecule per PAA chain)
FM	filomicelle
SM	spherical micelle
LBL	layer-by-layer
Micelle-LBL	LBL network of micelles
FM-LBL	filomicelle LBL network
SM-LBL	spherical micelle layer-by-layer network
¹²⁵I-streptavidin	radiolabeled streptavidin
NoL	number of micelle addition cycles in micelle networks
NoLcapping	number of polymer addition cycles in capping layers
Capped-FM	FM network with cappings

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a) Histogram: Filomicelle length distribution (based on microscopic imaging)

b) Spherical micelle Size distribution by intensity

Figure 1.

Size distribution of filamentous and spherical micelles. Length distribution of filomicelles (FM) are estimated from ImageJ post-processing on FM fluorescence micrographs. Size distribution of spherical micelles (SM) measured using dynamic light scattering (DLS), which showed an average particle diameter of ~245 nm.

Figure 2.

a) SEM micrographs of *in vitro* natural mucin network depositions recapitulated under similar conditions as synthetic mucin networks formation over the polystyrene substrate. b) Synthetic mucin networks formations were visualized under different levels of magnification in demonstrating porous network formation.

Figure 3. FM networks are visualized under tapping mode atomic force microscopy (AFM) at different levels of magnification. a) Topographic images of 7-layered network projecting surface height features b) AFM phase imaging showing finer surface features of the 7-layered FM network via measurement of subtle differences in material property.

Figure 4. Scanning electron micrographs of 7-layered micelle networks made from different spherical-filomicelle compositional blend ratios (% filomicelles = ~0, 20, 40, 60, 80 and 100). Inset shows those micelle-LBL networks at an higher magnification level (Scale bar = 5 μm).

Figure 5. Percent substrate coverage from 7-layered micelle networks formed from different micelle mixture (% filomicelle or sphere:filomicelle ratio). With increasing % filomicelle in the micellar blends better network-substrate coverage was achieved. (Refer to Figure 4 and Supplementary figure S3)

Figure 6. Bacterial growth on curcumin-loaded synthetic networks measured using crystal violet stain, after 24 h bacterial exposure. 7-layered networks were deposited using varying micellar compositional blends (filomicelle: sphere ratio). As FM content increased, there was an observable decrease in bacterial development. A 2-sample t test was performed comparing bacterial growth in substrate control with different micelle-commixture networks. The * represents significant difference from the substrate (control) with $p < 0.05$, while ** represents $p < 0.1$.

Figure 7.

Generation of a LBL capping barrier on FM mucin construct. a) Simplified scheme of filomicelle networks with different numbers of polymeric capping layers (1, 3, 5, and 7). b) Polymeric capping layer growth over synthetic networks. Biotinylated polymer (Biotin-40/ PAA225k) was able to exhibit capping layer growth on top of the FM networks (7-layers). The non-biotinylated polymer (Biotin-0/PAA225k) due to lack of biotin-streptavidin affinity linkages was unable form capping layers with increasing polymeric-LBL addition cycles. c) Evaluating stability of capping layers. The capping layer loss was tracked to evaluate its stability under destabilizing harsh protease. The capping barrier coatings from biotinylated polymer (Biotin-40/PAA225) demonstrated better barrier stability, whereas weak charge based non-biotinylated polymers due to lack of affinity interactions demonstrated a relatively poor capping barrier intactness. The barrier tendency offered by biotinylated capping layers was expected to shield the underlying protein-based synthetic network via its polymeric properties (e.g. chain length or molecular conformation) and was expected to improve its bulk structural stability.

Figure 8.

In vitro chemical stability of 7-layered filomicelle networks with polymeric capping barriers under effects of simulated oral chemical environment. a) Network destabilization was tracked independently from base streptavidin layer under *in vitro* salivary and protease environment. b) Network destabilization was tracked independently from streptavidin loss from FM network interlayers under *in vitro* salivary and protease environment. The effect of different number of polymeric capping layers on network destabilization under chemical effects was illustrated.

Figure 9.

Drug release studies from filomicelle networks and effect of polymeric capping barrier in tuning its drug release under simulated oral environment. FM networks were developed over the polystyrene substrate with increasing number of layer additions (or network thickness) (No. of micelle layer additions = 1, 3, 5, and 7). The curcumin drug release from those networks without incorporating capping barriers was studied under a) simulated salivary environment b) protease environment. Also, drug release from 7-layered FM network was studied after incorporating polymeric capping barriers (No. of polymeric capping layer

additions = 1, 3, 5, 7, 10, and 15) under c) simulated salivary environment and d) protease environment.

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Figure 10. Scanning electron micrographs of 7-layered FM networks after subjecting to destabilizing chemical effects of protease environment a) at T= 0 hr b) at T= 6 hr and c) at T= 24 hr.

Scheme 1.

Scheme showing hierarchical self-assembly approach adopted to develop a synthetic biomimetic network. By utilizing highly specific biotin-streptavidin interactions, the drug loaded biotinylated-micelles are cross-linked to form networks that mimic natural oral mucin coatings. This synthetic mucin is proposed to function as a drug releasing network in oral delivery. By incorporating polymeric capping barriers, the synthetic networks are expected to offer a tunable drug release.