Published in final edited form as:

Adv Healthc Mater. 2013 July ; 2(7): 983–992. doi:10.1002/adhm.201200375.

Affinity Based Multilayered Polymeric Self-assemblies for Oral Wound Applications

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Abstract

Oral mucositis, a painful and debilitating ulcerative wound condition, is a frequently occurring complication following chemo- and/or radiotherapy. While the current standards of therapy (e.g., gels and mouth rinses) provide temporary relief, there is still an unmet need for a robust, long acting barrier that can provide lubricating protection in oral wounds, thereby enhancing the wound healing response. It is proposed that an affinity based layer-by-layer (LBL) self-assembly that can be administered as a series of mouth rinses could permit the formation of protective barriers, providing a modular approach to regenerative oral therapy. In this study, biotinylated poly(acrylic acid) was synthesized for developing LBL assemblies using biotin-streptavidin affinity linkages. To explore the ability of developed LBL assemblies to potentially resist the harsh intraoral environment, *in vitro* chemical and *ex vivo* mechanical tests were performed. The stability results demonstrated significant LBL barrier stability with wear resistance. From statistical analyses, it was deduced that polymer MW and the number of LBL layers contributed significantly to chemical barrier stability. Also, the extent of biotin conjugation played a key role for LBL development and in mechanical barrier stability. Thus, the proposed affinity based LBLs with their excellent barrier properties offer a modular treatment approach in oral mucosal injuries.

Keywords

anti-adhesion; barrier stability; biotin-streptavidin; layer-by-layer; oral mucositis

1. Introduction

Oral mucositis (OM) is a commonly occurring side-effect resulting from chemo- and/or radiotherapy in the treatment of cancer, especially in head and neck cancers.^[1] This debilitating acute oral wound condition is characterized by epithelial denudation, leading to ulcerous oral lesions that consequently result in dysphasia and pain, which negatively affect the patient's overall quality of life.^[2–4] Every year in the United States, approximately 132,000 patients subjected to anti-cancer therapies develop OM (as per 2003 data).^[5–6] More than 90% of patients undergoing cytotoxic therapies for head and neck cancer (HNC) have increased risk of developing OM, with the estimated per patient cost exceeding \$17,000 (USD).^[4, 7] Current treatment strategies for OM are similar to methods used in dermatological conditions, including gel formulations, or lubricating mouth rinses, which provide a very brief palliative relief, requiring multiple applications (~10/day) spread throughout the day with poor patient compliance.^[6, 8]

Due to the complex environment, including continual salivary flushing, fluid exuding from wounds, mastication, bacterial flora and digestive enzymes, the oral cavity represents a significant treatment challenge for creating an effective oral mucosal barrier for periodontal barrier treatment and potential drug delivery.^[9] Indeed, there has been very exciting work focusing on the use of oral bioadhesive films for localized drug delivery.^[10] Unfortunately, patients suffering from OM invariably develop a highly irregular mucosal surface from extensive ulcerous sores (occupying >50% of the oral cavity), making application of bulky films and gels difficult, especially along the gingival.^[3]

As an alternative to these approaches, *in situ* multilayered barriers can be developed as a series of non-viscous mouth rinses, allowing for full coverage of all oral mucosal tissues. By providing a series of alternating streptavidin (or non-immunogenic neutravidin) and biotinylated polymer mouth rinses, multilayered polymeric barrier systems can be grown from the buccal surface in a layer-by-layer (LBL) process, as shown in Figure 1. Furthermore, this film formation process may provide an opportunity for specific targeting to wound surfaces through fibrin and may also be used as a drug delivery system.^[11] Yet, in order to realize this system, a LBL system must both be chemically and mechanically stable and create a lubricating surface that reduces adhesion to opposing tissue surfaces mitigating oral pain.

In this work, LBL barriers were formed and evaluated for their *in vitro* stability in proteolytic and salivary fluids. Further, to demonstrate their ability to decrease surface interaction with adjacent tissues, such as the tongue and cheeks, *ex vivo* adhesion studies were performed. From results of repeated contact barrier fatigue model tests, it was shown that biotinylated polymers with more biotin conjugation, higher MW and number of assembly layers performed well without any barrier loss on repeated loading. Thus, the tunable affinity based LBL polymeric self-assemblies with durable barrier properties offer a potential treatment strategy as an oral drug carrier.

2. Results and Discussion

2.1. Layer-by-Layer Assembly formation

To create multi-layered polymeric barriers, biotin-streptavidin crosslinking was used. Polymers were functionalized with biotin through diimide chemistry using a zero-length N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) crosslinker.^[12] Poly(acrylic acid) (PAA) of different molecular weights (MW 10,000/ 50,000/ 90,000 Da) with varying extents of biotin conjugation (Biotin+, Biotin++) were synthesized. See supporting information Figure S1 for characterization of biotinylated polymers used in this study.

2.1.1. In vitro LBL—In developing *in vitro* LBL assemblies on radioimmunoassay plates (RIA), an initial base layer (Layer 1) of streptavidin was formed (See supporting information Figure S2). Protein coverage in the base layer was fixed below monolayer saturation, ensuring that developed assemblies were anchored directly to the hydrophobic polystyrene surface.^[13] Unoccupied sites in base layer were blocked using BSA to minimize non-specific adhesion effects.^[14] LBL systems with the desired number of streptavidin layers (NoL= 1, 3, 5 and 7) were developed over this protein base layer (scheme shown in Figure 2(a)).

LBL growth, as analyzed by increasing mass of radiolabeled streptavidin in the assemblies (fractional streptavidin mass from base layer " M_L/M_1 "), demonstrated significantly better LBL formation with high MW biotinylated polymers (Biotin-5.55/PAA 90, Biotin-7.14/PAA 90) (Figure 2(b)). In 7-layered assemblies of these biotinylated polymers (Biotin-PAA 90), the protein mass increase was nearly 3 fold higher than that for the unconjugated polymer (Biotin-0/PAA 90), indicating the significant contribution from affinity linkages. Interestingly, the high MW unconjugated polymer (Biotin-0/PAA 90) was able to form an additional protein layer, likely a result of weak charge-based interactions. Yet, these structures were not stable enough to form multilayers. Low MW polymers (PAA 10) were unable to form LBL assemblies. This poor self-assembly formation was likely due to their polymeric tendency to destabilize the adsorbed protein base layer owing to surfactant like effects (Figure 2(d)).^[15] As expected, LBLs developed using intermediate MW biotinylated polymers (Biotin-1.23/PAA 50, Biotin-2.75/PAA 50) possessed moderate tendency to form assemblies (Figure 2(c)).

In examining the growth properties of traditional polyelectrolyte multilayers (PEM), it can be deduced that PEMs with an exponential layer growth were common in polypeptides or with biological components.^[16–17] Hence, it was hypothesized that the weak non-linear increase found for unconjugated high MW polymers (Biotin-0/PAA 90) was likely a result of weak polyanion (PAA) interactions with protein (streptavidin). PEMs of highly charged polymers typically result in linear growth; it was likely that the better linear assembly tendency found in LBLs of high MW weak polyanion PAA (Biotin-5.55/PAA 90, Biotin-7.14/PAA 90) was due to the significant contributions by a more stable affinity based biotin-streptavidin linkages.^[16]

2.1.2. Multivariate analysis on in vitro LBL assemblies—Principal component regression (PCR) on *in vitro* LBL was performed to analyze the interdependencies of

polymer/layer properties on self-assembly growth. LBL factors that can influence the response variable, M_L/M_1 , were polymer MW, extent of biotin conjugation and number of layers (NoL). The results of PCR analysis demonstrated significant main effects from all LBL factors with interactions (Figure 2(e)). The significant main effects substantiate that all LBL factors contributed to self-assembly growth. From the interactions, a significant resultant positive influence caused by interdependence of various LBL factors in affecting assembly growth was observed. The contribution of interactions was shown through significant 2-way interactions (MW*Conj, Conj*NoL and MW*NoL) and squared interactions (Conjugation*Conjugation and MW*MW). This demonstrates the synergistic role of factor interactions involved in barrier formation.

2.2. LBL Chemical Stability

In vitro LBL systems developed for various synthesized Biotin-PAA materials (of different MW/conjugation/NoL) were subjected to chemical stability tests, with an aim of studying harsh intra oral chemical effects on LBL assemblies. To this end, both bacterial protease effects and salivary effects on layer stability were assessed.

2.2.1. Protease Stability—In studies of LBL stability under exposure to the proteolytic enzyme pronase, the unconjugated polymers (Biotin-0/PAA 90, Biotin-0/PAA 10) were readily degraded, reflecting poor chemical stability (Figure 3(a, d)). The stability of Biotin-0/PAA 90 material was found to be indistinguishable from the streptavidin base layer (Layer 1), which supports the weak association hypothesis for the unconjugated polymers. Biotinylated polymers (Biotin-5.55/PAA 90, Biotin-7.14/PAA 90) were found to be more resistant to protease-induced digestion, as shown in Figure 3(a). In studying layer effects, LBLs with an increased number of layers (NoL), i.e., Biotin-5.55/PAA 90, Biotin-7.14/PAA 90, yielded better assembly stability (Figure 3(b)). This improved stability resulting from more assembly layers can be attributed to decreased accessibility of the streptavidin molecules to enzyme due to increased layer thickness/crosslinking. LBLs of high MW polymer (PAA 90) had distinctly better barrier stability when compared to those for lower MW polymer (PAA 10), supporting the importance of PAA shielding of streptavidin from proteolytic degradation (Figure 3(c)). Stability of barriers composed of low MW polymers (Biotin-1.21/PAA 10, Biotin-4.91/PAA 10) was comparable to that for the streptavidin base layer (Layer 1).

Multivariate analysis (PCR) was performed on the protease stability response factor, mass loss at $t_{1/2}$. This was selected as an indicator of the extent of LBL degradation in studying the main factors (MW, conjugation and NoL). Interestingly, all the effects resulted from direct influence of each main factor with an absence of any factor interactions (Figure 3(e)). In studying factors controlling the rate of LBL degradation ($t_{1/2}$), polymer MW and number of assembly layers (NoL) played important roles as indicated by their significant main effect contributions. Conjugation was found to insignificantly affect LBL degradation rate, thereby demonstrates weak influence.

2.2.2. Salivary Stability—Saliva contains a variety of components with surfactant properties that are capable of destabilizing protein-based barriers. To evaluate this effect,

layers were studied in the presence of UWS. In LBLs exposed to UWS, biotin conjugation did not seem to be a strong effecter of barrier stability (Figure 4(a)). However, an increased number of LBL layers decreased mass loss from assemblies (Figure 4(b)). This layer effect was likely due to the increased crosslinking and network formation that created more resilient barriers. Polymer molecular weight effects in UWS were similar to those observed in the protease studies, where higher MW polymers resulted in better stability, further substantiating the role of polymer MW (Figure 4(c)). Interestingly, PAA 10 materials resulted in enhanced protein loss from assemblies, as also observed in protease studies, reflecting the earlier described inherent surfactant effects of low MW PAA (Figure 4(d)).

PCR analysis of salivary stability as measured by mass loss at $t_{1/2}$ and $t_{1/2}$ demonstrated significant contributions from polymer MW and number of assembly layers (NoL) (Figure 4(e)). Interestingly, the degree of conjugation for the range of conjugations tested was not a strong factor in determining LBL stability in saliva.

2.2.3. Mechanism of LBL degradation—By evaluating mass loss as a result of incubation in saliva, it was not initially clear whether surface layers eroded away with time or if there was a full thickness loss or a random loss occurring in pockets throughout the layer. To test which of these modes of barrier degradation dominated, 5-layered assemblies with a single radiolabeled streptavidin layer (innermost, middle or outermost) were subjected to salivary degradation as shown in Figure 5(a).

Each individual layer of biotinylated polymer (Biotin-7.1/PAA 90) in LBLs performed better than its respective layer of unconjugated polymer (Biotin-0/PAA 90) (Figure 5(b)). This notable increase in stability reinforces the role of multilayered structures in providing chemical barrier effects as described earlier. The rapid loss of the innermost layer from unconjugated materials verifies their inability to develop LBL assemblies and suggests a full thickness loss of adsorbed constituents.

In biotinylated LBL systems, the outermost layer (Layer 5) was found to be the most susceptible to mass loss. A higher protein loss from outer layer was likely due to the direct salivary degradation in the absence of a capping polymeric layer, suggesting an erosion type mass loss effect. The innermost layer (Layer 1/Base layer) with its overlaid polymeric layers resulted in a highly effective barrier, as indicated by its reduced mass loss (Figure 5(b)). As expected, the middle layer (Layer 3) possessed better stability when compared to the outer layer, but it experienced more protein loss in comparison to the innermost layers. This barrier tendency exhibited by slowed degradation of the middle and innermost layers was absent in unconjugated materials. Overall, assemblies of biotinylated polymers followed a top to bottom progression in chemical degradation, suggesting a surface erosion mechanism and an operative polymeric barrier effect from multilayered structures.

2.3. *Ex vivo* LBL

While layers were effectively formed in *in vitro* plates, it was important to verify that this same barrier effect can occur directly on tissue surfaces. Because porcine dermis has a structurally similar collagen arrangement to human dermis, LBLs were formed on the subdermal surface of porcine tissue as a mimic of the oral mucosal surface.^[18] Tissue

patches of equal dimensions were used during the studies to maintain a consistent surface area. LBL assemblies were developed utilizing the non-specific protein interactions with tissue binding sites to form tissue protein complexes in base layer.^[19] As seen in Figure 6(a), the *ex vivo* LBL developed resulted in the characteristic mass increase associated with LBL self-assembly formation. In 5-layered LBL assemblies for biotinylated PAA materials (Biotin-1.23/PAA 50, Biotin-2.75/PAA 50), the streptavidin mass per tissue surface area was 3.18 mg/m² and 3.62 mg/m², respectively, which was an increase of 31–49 % protein mass from unconjugated PAA material (Biotin-0/PAA 50). For unconjugated polymer (Biotin-0/PAA 50), there was a relatively weak streptavidin mass increase, suggesting a non-specific binding during assembly growth that was verified through control comparison with extent of protein adsorption in tissue without any polymer addition. The *ex vivo* studies suggest the ability to form and thereby to distinguish tissue-LBL assemblies even with relatively low assembly layers (NoL=5) from conjugated polymers.

2.4. LBL Mechanical Stability

With mandibular or tongue motions or abrasion from food, oral mucosal surfaces are continually exposed to wear conditions. These motions result in repeated tissue-tissue contacts. With exposed ulcerative surfaces, these contacts result in high friction and increased force of separation that results in extreme pain during talking and eating. It was hypothesized that the present LBL assemblies could reduce tissue/tissue adhesion and thereby potentially mitigate the associated pain. Hence, to study LBL durability and ability to reduce tissue adhesion, adhesion tests were performed on developed *ex vivo* LBL assemblies (Figure 6(b)). Based on the performance trends observed in the *in vitro* studies, *ex vivo* LBLs of higher polymer MW (50,000 / 90,000 Da) and increased assembly layers (NoL=14) were used for mechanical testing. In analyzing LBL barrier durability, a repeated contact barrier fatigue model was used, where the developed *ex vivo* assemblies was subjected to repeated mechanical loading cycles (load pull-offs). In these experiments, for each load pull-off cycle, a work of separation (WoS) was found by integrating the area under the curve in the load vs. displacement plot (inset of Figure 6(c)). Between each cycle, the tissue was washed with saline to mimic the salivary flushing one may expect in the oral cavity, which would remove detached LBL films. By performing repeated loading cycles on tissues prior to assembly development, a “tissue trend” was obtained to account for possible tissue variations during repeated loadings. On developing *ex vivo* LBL over this studied tissue, repeated contact loading was provided to study LBL wear resistance (Figure 6(c)).

From results of the adhesion tests, LBLs developed with biotinylated polymers (Biotin- 7.1 / PAA 90 & Biotin- 2.75/ PAA 90) were able to significantly reduce tissue-tissue adhesion by their barrier properties as reflected from a decreased peak area (Figure 6(c)). In analyses from wear tests, the biotinylated polymers also were able to maintain their physical barrier effect by remaining non-adhesive to surrounding tissues, even on higher numbers of contact cycles. The physical barrier effect from biotinylated materials (Biotin- 7.1 / PAA 90 & Biotin- 2.75/ PAA 90) is shown in Figure 6(d) by a decreased WoS and a minimal increase in WoS on repeated loading cycles (expressed in terms of tissue normalized WoS).

Surprisingly, LBLs formed from non-biotinylated PAA (Biotin-0/PAA 90) not only resulted in poor barrier stability but also increased WoS. The enhanced tissue-tissue adhesion observed with unconjugated polymers was likely a result of protein/polymer charge-based interactions caused by weakly formed layers, lending to a “glue” type effect. With LBLs of increased biotin conjugation, better physical barrier were formed (Biotin-7.1 > Biotin-2.71 > Biotin-0), thereby demonstrating the effect of biotin conjugation in formulating durable barriers. Thus, biotinylated polymers of higher MW and NoL yielded better barrier stability.

3. Conclusion

In conclusion, the ability to develop affinity based multilayered polymeric assemblies was demonstrated for different synthesized biotinylated PAA materials. Such multilayered polymeric barriers greatly reduced the adhesive properties of exposed tissue surfaces, reducing adhesion. Because these LBL assemblies possess good chemical stability in salivary and proteolytic media, they provide an exciting solution that merits further investigation for potential treatment to different oral wound applications.

4. Experimental Section

Materials

Poly(acrylic acid sodium salt) (MW 10,000 and 50,000 Da) and PAA (MW 90,000 Da) were purchased from Polysciences, Inc. (Warrington, PA) and were lyophilized prior to use. All other chemicals were used as purchased without any further purification unless stated. N-hydroxysuccinimide (NHS) was purchased from Acros Organics (NJ). Streptavidin was purchased from Thermoscientific (Rockford, IL). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl), protease derived from *Streptomyces griseus* (pronase), 2-(N-morpholino)ethanesulfonic acid (MES) and 2-(4-hydroxyphenylazo) benzoic acid (HABA) were purchased from Sigma Aldrich (St. Louis, MO). Pentylamine-biotin and avidin were purchased from Thermoscientific (Rockford, IL).

Porcine dermal tissue, used as a model of mucosa, was prepared by excising skin (dorsal region) with the subdermal subcutaneous fat, placed in a towel soaked in saline and stored in a sealed container at -20°C . The stored pig tissue was thawed prior to use.

Polymer biotinylation synthesis

PAA biotinylation was carried out with modification in previously published procedure and by using instructions from biotinylation product kit (Thermo Scientific).^[20] In polymeric biotinylation by EDC-NHS coupling chemistry, the molar ratio of acrylic acid repeating unit: EDC: NHS (1:10:20) was fixed. The extent of biotin conjugation on PAA was altered based on molar ratio of pentylamine-biotin to carboxylic acid repeat in PAA. To obtain a low extent of biotin conjugation [Biotin+], a molar ratio of Pentylamine-biotin to PAA (1:5) was used, whereas for increased extent of conjugation [Biotin++] the molar ratio used was 1:2.5. All the materials were weighed out to meet the desired molar ratios. PAA, EDC and NHS were dissolved in the MES buffer, taken in a sealed flask at room temperature with a regulated pH of 5.0–6.0. To the reaction mixture, pentylamine-biotin was added immediately and pH was regulated to 7.0–8.0 in order to enhance the reaction of carboxylic

acid with amine to form a stable amide bond. The reaction was carried out for a period of 24 hrs.

Purification and Characterization

The biotinylation reaction mixture was filtered off to remove any precipitate suspensions. And further purification was carried out using ultrafiltration (Millipore, USA), removing excess unreacted pentylamine-biotin, EDC and NHS from various synthesized biotinylated PAA (Biotin-PAA) product. RP-HPLC (Shimadzu Prominence) was carried out on ultra-filtered biotinylation reaction samples. An isocratic mode with a mobile phase of acetonitrile (organic phase) and water+1% trifluoroacetic acid (aqueous phase) in (15:85) % volume ratio was used in RP-HPLC analysis. All purified reaction samples were subjected to HABA-avidin assay. HABA stock solution and HABA/Avidin working solution was prepared as outlined by Shuvaev et al.^[21] To the HABA/Avidin working solution (red/orange colored), purified reaction samples from polymer biotinylation synthesis was added. This addition resulted in color change (pale orange/ yellow) which was measured from the decrease in the absorbance at 500 nm using a UV-vis spectrophotometer (Cary WinUV). From comparison with calibration made from known biotin addition to HABA/avidin working solution, the estimate of extent of biotinylation in various synthesized biotinylated PAA molecules was determined.

Base Layer Adsorption

For various radiolabeled streptavidin concentration (100 μ l, 1–1000 nM) added to RIA plates (1 minute incubation), the amount of protein adsorbed was measured using gamma counter (Perkin Elmer). The excess unbound streptavidin was washed off by providing PBS rinses prior to gamma counter measurements.

Atomic Force Microscopy (AFM)

A non-treated hydrophobic polystyrene substrate was used for protein adsorption and for subsequent LBL assembly growth over protein base layer. All materials (polymers/ protein/PBS/BSA) used during assembly growth were filtered through 0.2 μ m syringe filters prior to its use. A monolayer base streptavidin layer is formed (1 mg/ml streptavidin in PBS, 2 minute static incubation) and the excess protein is removed by PBS rinses. BSA blocking is provided to avoid non-specific adsorption during assembly growth (5 wt% in PBS, 100 μ l, 2 minute incubation). Subsequent additions were provided using alternating polymer Biotin-32/PAA 90 polymer (PAA ~ 1mg/ml, 100 μ l) and Streptavidin (2 μ M, 100 μ l) incubation for 2 minutes. After LBL assembly formation, a final rinse is provided using DI water under sink conditions. The films were dried at room temperature prior to AFM imaging.

LBL assembly topography and surface roughness was imaged in a non-contact (tapping) mode AFM (Agilent Technologies, Series 4500). LBLs were imaged in air medium (non-liquid mode) using a Tap 300Al-G cantilever (Budget Sensors®, Bulgaria) with a radius of curvature less than 10 nm (force constant- 40 N/m, resonant frequency-300 kHz). The images were processed using Gwyddion software for visualization and analysis. The only image correction tool employed was plane leveling and line correction.

Affinity based Layer-by-Layer (LBL) studies

Radiolabeled (^{125}I) streptavidin was used for studying LBL self-assembly development. Here, LBL systems of different number of assembly layers (NoL) were developed to study self-assembly development.

In vitro LBL

In vitro LBL studies were carried out on high protein binding radioimmunoassay (RIA) plates (96 well plates, hydrophobic, non-treated poly(vinyl chloride) substrate) at room temperature. Base streptavidin (Layer 1) was formed on the RIA microplates (Streptavidin concentration = 200 nM, 100 μl /well, 2 min incubation). The vacant adsorption sites in base layer were blocked using bovine serum albumin (BSA) (5 wt% solution, 200 μl /well, and 2 minute incubation). The unbound BSA was removed by triplicate rinsing with PBS. To the base streptavidin layer, alternating rinses of biotinylated PAA (PAA 1 mg/ml, 100 μl /well, 2 minute incubation) and streptavidin (2 μM , 100 μl /well, 2 minute incubation) were provided. Two minute incubations were used to simulate maximal oral rinsing times. The excess biotinylated polymer or streptavidin was removed by intermittent PBS rinses. Layers were kept hydrated during all steps. To study the LBL development, self-assemblies with an increasing number of streptavidin layers (NoL = 1, 3, 5, and 7) were developed for various synthesized biotinylated PAA materials.

Chemical stability tests on in vitro LBL assemblies

Chemical stability tests were carried out on earlier developed *in vitro* LBL systems. Either unstimulated whole saliva (UWS) or solutions of proteolytic enzyme were used for studying LBL chemical stability. UWS was collected by the spitting method, with a harvesting lag time of at least 2 hours from meal intake. Other variable salivary factors were kept minimal by abstinence from smoking or drinking.^[22] For proteolytic stability tests, pronase medium (0.01 wt %, 100 μl) was used. LBL systems were incubated in UWS or pronase solution for different times, after which the remaining mass of radiolabeled streptavidin was measured using a gamma counter (Perkin Elmer 2470 Wizard²).

LBL degradation mode

LBL systems with the same NoL in the self-assemblies (N=5) were developed using a modified *in vitro* LBL procedure. For base layer degradation (Layer 1), the innermost protein layer was radiolabeled, and all other layers were developed by addition of cold (non-radioactive) streptavidin. A similar procedure was adopted for studying degradation of the middle (Layer 3) and outermost layers (Layer 5). Protein mass remaining in individual layers was measured for increasing salivary incubation times using a gamma counter.

Ex vivo LBL Formation Studies

Porcine skin patches (5 mm $\times$ 5 mm) were used to evaluate the adhesion barrier function of the assemblies. Epidermal side was placed face down, and LBL assembly was prepared on the exposed subdermal surface using alternating polymer/protein rinses, adopting the same procedure as described earlier for *in vitro* LBL studies. For *ex vivo* LBL studies, biotinylated PAA (MW 50,000 Da) was studied.

Mechanical stability tests on ex vivo LBL assemblies

Mechanical stability was studied on circular *ex vivo* LBL assemblies using a Bose Electroforce® 3300 test system equipped with a 1 kg load cell. Porcine skin patches (16 mm diameter) were wetted with PBS to avoid dryness and emulate oral surfaces. Tissues were mounted to acrylic platens using cyanoacrylate such that the subdermal surfaces faced each other.

In the adhesion tests, a compressive load of 3 N (305.8 g) was applied with a ramp rate of 10 g/sec under load control and was stabilized at the load for 1 minute. Under displacement control, pull-off was conducted at a rate of 0.1 mm/sec until full tissue separation occurred. Adhesion tests were performed on tissues before (tissue trend) and after LBL development (LBL trend). LBL assemblies were developed on tissues fixed to the lower platen using the procedure described earlier in *ex vivo* LBL studies, using cold streptavidin (non-radioactive) for the studies.

In LBL fatigue resistance tests, a minimum of 20 cycles of contact loadings (load pull-offs) was conducted after LBL growth. During these wear tests, tissue dryness was avoided by constant wetting with PBS between the cycles. Tissue trends were examined prior to assembly development to ensure that variations between tissue specimens were not significant; contact loadings were carried out for a minimum of 10 cycles to study this trend.

Statistical analysis

Principal component regression (PCR) analysis was performed on the results of LBL chemical stability tests using statistical software (The Unscrambler®). Response variables ($t_{1/2}$ and mass loss for $t_{1/2}$) derived from chemical stability analysis were used in studying the different LBL factors (MW, conjugation and NoL). In PCR analysis, tests of significance were performed on regression coefficient 'B' (principal component slope) using uncertainty tests. Paired t-tests were performed on trends of the mechanical stability data. For all statistical tests, effects were considered significant only if $P < 0.05$. Main effects of LBL factors were studied using various 3-way ANOVA models (General Linear model (GLM) and 3-way nested ANOVA using a statistical software Minitab. Further post-hoc analysis was performed using Bonferroni, Sidak and Dunnett methods in GLM model. Level of significance used $P = 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Mr. Ravinder K. Garlapalli (Department of Chemical & Materials Engineering, University of Kentucky) for AFM imaging of LBL assemblies and Mr. Sandeep K. Ramineni (Center for Biomedical Engineering, University of Kentucky) for his assistance with Bose Electroforce equipment during adhesion tests. The authors would also like to thank Dr. Nihar Shah for his stimulating discussions and advice on analysis. The authors also give thanks to Cardiac Rhythm Laboratory (Center for Biomedical Engineering, University of Kentucky) for their generous donation of porcine skin samples. This work was funded by National Institutes of Health (Grant R03 DE019496).

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Figure 1. Scheme showing overall proposed application of LBL self- assemblies in oral drug delivery

Figure 2. Development of affinity based LBL assemblies

Self-assembly growth was studied by radiotracing protein (^{125}I -streptavidin) in LBL layers and by analyzing fractional streptavidin mass from base layer (M_L/M_1) with increase in assembly layers. (a) Scheme showing LBL assembly development using biotin-streptavidin affinity linkages by alternate additions of protein streptavidin and synthesized biotinylated PAA. (b) *In vitro* LBL assembly formation using high MW (90,000 Da) PAA of various extent of biotin conjugation. (c) *In vitro* LBL assembly formation using low MW (50,000 Da) PAA of various extent of biotin conjugation. (d) *In vitro* LBL assembly formation using low MW (10,000 Da) PAA of various extent of biotin conjugation. (e) Principal component regression analysis on *in vitro* LBL response variable (M_L/M_1) to study effects of various LBL factors (MW, conjugation and NoL). Bar plot shows principal component regression (PCR) analysis on regression coefficient (B) for various LBL factors effects, while considering response variable (M_L/M_1) during analysis. Test of significance on regression coefficient (B) (obtained from PCR analysis) was performed by uncertainty tests and factor effects were considered insignificant if uncertainty limits crosses the zero axes. Table below shows summarized results of PCR analysis, where interactions (2-way and squared interactions) were found to be significant. All LBL factors like MW, conjugation and NoL

also showed significant main effects for response variable. Level of significance used, $P = 0.05$.

Figure 3. Stability studies on LBL assemblies under protease environment

(pronase), analyzed through extent of streptavidin mass loss (%) from layers where reduced mass loss demonstrates better barrier stability. (a) Effect of extent of biotin conjugation. Plot shows biotin conjugation effect by analyzing self-assemblies developed from polymer PAA of same MW (90,000 Da) with equal number of assembly layers (NoL=7) and providing comparison with different extent of biotin conjugation. Similar analysis was performed for lower MW (10,000 Da) biotinylated PAA materials (results not shown). (b) Effect of number of LBL layers (NoL). Plot shows layer effect from LBL stability tests in self-assemblies developed from high MW (90,000 Da) biotinylated PAA (Biotin-7.1 / PAA 90). Similar analysis was performed for other synthesized biotinylated PAA materials (results not shown). (c) Effect of polymer MW. Plot shows MW effect by analyzing self-assemblies developed of equal number of assembly layers (NoL=7) and providing comparison with different MW (90,000/10,000 Da) PAA materials. Similar analysis can be performed with different number of LBL layers. (d) Loss (%) - streptavidin mass loss (%) at M_{∞} . Plot shows streptavidin mass loss (%) at M_{∞} obtained from protease stability plots of different LBL systems (MW / biotin conjugation / NoL). (e) Summarized results of principal component regression (PCR) analysis on LBL stability response variables (mass loss at $t_{1/2}$

and $t_{1/2}$) to study effects of various LBL factors (MW, conjugation and NoL). Results were obtained by conducting test of significance (uncertainty tests) on regression coefficient (Slope “B”) derived from principal component analysis. Table shows effects of various factors on LBL response variables, where interactions were found to be insignificant. Factors like MW, NoL showed significant main effects for LBL response variables. Also role of conjugation in affecting mass loss from assemblies were found significant. Level of significance used, $P = 0.05$.

Figure 4. Stability studies on LBL assemblies under salivary medium

(unstimulated whole saliva), analyzed through extent of streptavidin mass loss (%) from layers where reduced mass loss demonstrates better barrier stability. (a) Effect of extent of biotin conjugation. Plot shows biotin conjugation effect by analyzing self-assemblies developed from polymer PAA of same MW (90,000 Da) with equal number of assembly layers (NoL=7) and providing comparison with different extent of biotin conjugation. Similar analysis was performed for lower MW (10,000 Da) biotinylated PAA materials (results not shown). (b) Effect of number of LBL layers (NoL). Plot shows layer effect from LBL stability tests in self-assemblies developed from high MW (90,000 Da) biotinylated PAA (Biotin-7.1 / PAA 90). Similar analysis was performed for other synthesized biotinylated PAA materials (results not shown). (c) Effect of polymer MW. Plot shows MW effect by analyzing self-assemblies developed of equal number of assembly layers (NoL=7) and providing comparison with different MW (90,000/10,000 Da) PAA materials. Similar analysis can be performed with different number of LBL layers. (d) Loss (%) - streptavidin mass loss (%) at M_{∞} . Plot shows streptavidin mass loss (%) at M_{∞} obtained from salivary stability plots of different LBL systems (MW / biotin conjugation / NoL). (e) Summarized

results of principal component regression (PCR) analysis on LBL stability response variables (mass loss at $t_{1/2}$ and $t_{1/2}$) to study effects of various LBL factors (MW, conjugation and NoL). Results were obtained by conducting test of significance (uncertainty tests) on regression coefficient (Slope “B”) derived from principal component analysis. Table shows effects of various factors on LBL response variables, where interactions were found to be insignificant. Factors like MW, NoL showed significant main effects for LBL response variables. Level of significance used, $P = 0.05$.

Figure 5. Study on mode of *in vitro* LBL degradation

LBL assemblies with individually radiolabeled assembly layers were developed and subjected to various incubation times in salivary medium (unstimulated whole saliva), thereby measuring protein loss from individual LBL layer. (a) Scheme shows 5-layered LBL systems developed to study degradation rate from innermost, middle and outermost layers by radiolabeling the respective layers in self-assemblies. (b) Results from *in vitro* LBL degradation. Assemblies were developed from high MW (90,000 Da) biotinylated PAA (Biotin- 7.1 / PAA 90) and compared with unconjugated PAA (Biotin-0 / PAA 90) using mass loss (%) from individual radiolabeled layers.

Figure 6. *Ex vivo* LBL Mechanical Stability - Adhesion Tests using Repeated Contact Barrier Fatigue model

(a) *Ex vivo* tissue LBL assembly formation. Plot shows LBL assembly formation from PAA of MW (50,000 Da) with biotin conjugation ($[Biotin^{++}] = 2.75$ and $[Biotin^{+}] = 1.23$). (b) Scheme for *ex vivo* LBL adhesion testing. Developed tissue-LBL assemblies (lower platen) were subjected to adhesion loading through load-pull off cycles. (c) Repeated contact barrier fatigue tests. By providing repeated contacts of loading cycles (load-pull offs), adhesion trends were studied before and after LBL development, thereby studying LBL wear resistance. Plots show significant wear resistance from LBL barriers developed from biotinylated PAA (Biotin- 7.1/PAA 90/14 Layers) with reduced work of separation (WoS) on repeated LBL loading cycles. Inset plot shows load vs. displacement curves collected

during adhesion tests before and after LBL development using biotinylated PAA (Biotin-7.1 / PAA 90 / 14 Layers). Decrease in adhesion was shown by reduced WoS (area under the curve). (d) Summary of results from mechanical testing. 14-layered LBL assemblies developed from various PAA material (unconjugated (Biotin-0/PAA 90), (Biotin 2.75/PAA 50) and (Biotin 7.1/PAA 90)) were subjected to repeated loading cycles. The extent of adhesion on repeated contact was shown by tissue normalized work of separation (WoS / % tissue control). A hypothesis test using paired t-test was carried out on all LBL loadings trend and was found significant with $P < 0.01$.