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Characterization of Biocompatible Polyelectrolyte Complex Multilayer of Hyaluronic Acid and Poly-L-Lysine

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Abstract A biocompatible polyelectrolyte complex multilayer (PECML) film consisting of poly-L-lysine (PLL) as a polycation and hyaluronic acid (HA) as a polyanion was developed to test its use for surface modification to prevent cell attachment and protein drug delivery. The formation of PECML through the electrostatic interaction of HA and PLL was confirmed by contact angle measurement, ESCA analysis, and HA content analysis. HA content increased rapidly up to 8 cycles for HA/PLL deposition and then slightly increased with an increasing number of deposition cycle. *In vitro* release of PLL in the PECML continued up to 4 days and *ca.* 25% of HA remained on the chitosan-coated cover glass after *in vitro* release test for 7 days. From the results, PECML of HA and PLL appeared to be stable for about 4 days. The surface modification of the chitosan-coated cover glass with PECML resulted in drastically reduced peripheral blood mononuclear cell (PBMC) attachment. Concerned with its use for protein drug delivery, we confirmed that bovine serum albumin (BSA) as a model protein could be incorporated into the PECML and its release might be triggered by the degradation of HA with hyaluronidase.

Keywords: polyelectrolyte complex multilayer, hyaluronic acid, poly-L-lysine, surface modification, peripheral blood mononuclear cell

INTRODUCTION

Polyelectrolyte complex multilayers (PECML) are built up by the alternated adsorption of cationic and anionic polyelectrolyte layers. The electrostatic attraction between oppositely charged molecules is a simple and excellent driving force for multilayer buildup [1]. This widely applicable and promising new technique can be used to fabricate ultra-thin films with determined nanometer thickness, composition, and properties. In other words, nanoscale control of material deposition and versatile surface modification can be made possible through the formation of PECML. There have been many reports on the application of PECML for biosensing [2], optical device fabrication [3], and drug carrier systems for macromolecules [4]. Recently, enzymatically-degradable [5] and hydrolytically-degradable [6] polyelectrolytes were used for the formation of a layer-by-layer assembly for bioactive agent delivery. Proteins were reported to interact strongly with the polyelectrolyte layer regardless of the charges of the multilayer and the protein [7]. Antibodies were reported to retain their reactivity with respect to their antigens when incorporated in a polyelectrolyte complex multiplayer [8]. This remarkable property opens up the possibility to construct multilayers incorporating specific ligands that keep their biological activity and promote the adhesion of specific cells [8].

Hyaluronic acid (HA) is a natural linear polysaccharide consisting of alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with β(1→4) interglycosidic linkage [9]. As a negatively charged polyanion, HA has unique and excellent physicochemical properties, such as biodegradability, biocompatibility, and viscoelasticity [9]. This biopolymer with a molecular weight ranging from 1,000 to 10,000,000 Da has distinctive biological functions. HA is abundant in synovial fluid and the extracellular matrix (ECM). HA acts to control tissue hydration and is present as a hydrated network with collagen fibers. It also constitutes the mammalian connective tissues and the structural backbone of the cartilage proteoglycan assembly [9]. Because of these various functions and physical properties, HA and modified HA have been widely used for arthritis treatment [10], ophthalmic surgery [11], drug delivery [12], and tissue engineering [13]. A number of strategies for the modification of HA mainly through carboxyl and hydroxyl groups have been developed including esterification of HA [14] and chemical modification of HA with carbodiimide [15]. HA has also been modified by crosslinking reaction using divinyl sulfone [16], glycidyl ether [17], or dialdehyde after modification of HA with adipic dihydrazide [18]. Recently, HA hydrogels crosslinked in a mild condition have been used for sustained release formulation of protein drugs [19,20] and tissue engineer-

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ing applications [21,22]. Generally speaking, however, chemical modification of HA has been carried out in highly alkaline or acidic solutions and at elevated temperatures, making it difficult to incorporate sensitive molecules or living cells into the polymer network hydrogel. The counter polycation of poly-L-lysine (PLL) was chosen not only because it is biocompatible, but also because it offers the possibility of being easily conjugated with bioactive molecules [23]. In addition, PLL is widely used for promoting cell adhesion to solid substrates [24]. A significant Schwann cell attachment was observed when crosslinked HA strands were coated with PLL [24].

The objective of this study was to develop a biocompatible PECML film consisting of PLL as a polycation and HA as a polyanion for surface modification and protein drug delivery. Originally, Picart *et al.* reported the buildup mechanism of PECML of HA and PLL by optical wave-guide-light mode spectroscopy, streaming potential measurements, and atomic force microscopy [25]. We could also confirm the formation of PECML of HA and PLL by contact angle measurement, ESCA analysis, and HA content analysis by the carbazole method [25]. The stability of PECML in phosphate buffer solution (PBS) with time and its interaction with proteins were investigated. Its use for surface modification to prevent cell attachment and protein drug delivery was also assessed for possible applications.

MATERIALS AND METHODS

PECML Buildup

All the experiments were performed using double distilled pure water (Barnstead Mega-Pure System, MP-12A). For the preparation of substrate, a cover glass was pretreated with 10^{-2} M SDS / 0.1 N HCl at 100°C for 10 min. After complete washing with pure water, it was brought in contact with 0.1% chitosan (high viscosity grade, Vanson) solution in 0.2 N acetic acid for 30 min. Acetic acid remaining in the film was removed in the form of sodium acetate by neutralizing in 0.2 N NaOH for 1 h and extensive washing with water. As a polyanion, hyaluronic acid (HA, MW of 2 million, Genzyme) was dissolved at a concentration of 0.2 wt% in phosphate buffer solution (PBS, pH = 7.4). As a polycation, poly-L-lysines (PLL, Sigma) with five different MW of 3,970, 14,400, 34,300, 57,900, and 99,500 were tested for PECML buildup at a concentration of 0.5 wt% in PBS. Briefly, polyanion was first adsorbed onto chitosan-coated substrate by incubation in HA solution for 2 min and washing in water for 30 sec. Polycation was then adsorbed onto HA surface from a PLL solution. This layer-by-layer adsorption of a polyanion and a polycation was referred as one HA/PLL deposition cycle. In a same way, HA/PLL deposition up to 14 cycles was carried out on the substrate. To study the interaction of protein with PECML, bovine serum albumin (BSA) was mixed with HA for 1 h at a concentration of 0.2 mg protein and 0.2 mg HA in 1 mL PBS, and then embedded in PECML after 2 cycles of HA/PLL deposition. After a certain number of HA/PLL deposition cycle, the sample was washed under running water for 1 min and then dried in a stream of filtered air.

PECML Characterization

Water contact angle was measured with a standard goniometer (Rame-Hart, Model A-100) and ESCA measurements were done on an SSX-100 spectrometer (Surface Science Instruments) using a monochromatic Al K_α X-ray source with a 5 eV floodgun. HA content analysis was carried out by the carbazole method [25].

PLL Release Test

In vitro release test of PLL from PECML of HA and PLL was carried out in PBS using a shaking incubator at 37°C. PLL concentration was determined by Lowry assay. To check the degradation of HA in PECML film by hyaluronidase (Sigma), a PECML-coated cover glass was incubated in PBS for 7 days and then degraded with hyaluronidase. The remaining HA content was quantified by the carbazole method [25].

Cell Adhesion Test

To confirm the effect of surface modification with PECML on cell adhesion, peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers by density centrifugation [26] and incubated on PECML. The PBMC was seeded at a density of 2 million per well and allowed to adhere for 4 h in a medium of RPMI-1640 (Biowhitaker, Walkersville, MD, USA). Medium was then aspirated and changed to RPMI-1640 plus 10% adult bovine serum (HyClone, Logan, UT, USA). After lipopolysaccharide (LPS) activation at a concentration of 10 ng/mL for 24 h, cell numbers adhering on PECML were determined by measuring the optical density (OD) at 405 nm of lactate dehydrogenase (LDH) with a cytotoxicity detection kit (Roche Molecular Biochemicals, Germany). LDH assay was based on the measurement of cytoplasmic enzyme activity released by damaged cells in supernatant after activation with LPS. The amount of enzyme activity detected in the supernatant represents the number of cells adhering to PECML.

RESULTS AND DISCUSSION

A chitosan-coated cover glass was prepared and used as a substrate to build up PECML of HA and PLL. The formation of PECML could be confirmed from the results of contact angle measurement, ESCA analysis, and HA content analysis. HA has one carboxylic acid group per disaccharide unit with pK_a of *ca.* 2.9 whereas PLL has one amino group per monomer repeat unit with pK_a of *ca.* 9 [23]. The electrostatic interaction between HA and PLL made it possible to form PECML. Fig. 1 shows the schematic representation of PECML of HA and PLL.

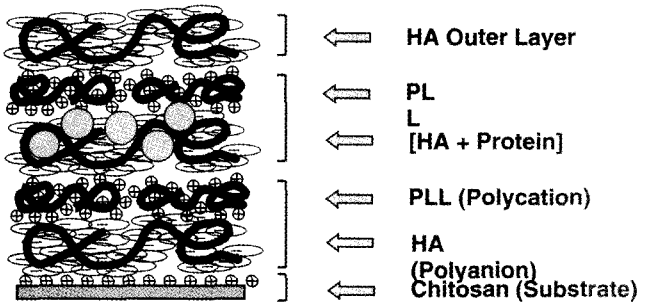

Fig. 1. Schematic representation of polyelectrolyte complex multilayer of hyaluronic acid (HA) and poly(L-lysine) (PLL).

Table 1. Effect of molecular weight (MW) of PLL on its ionic interaction with HA. Also analysis of HA content of coatings after 8 bilayers of HA and PLL are alternately deposited on chitosan-coated cover glass.

MW of PLL	PLL-HA Interaction[a]	HA Content (%)[b]
3970	No precipitation	24 ± 2.6
14400	No precipitation	36 ± 4.3
34300	Precipitation	79 ± 3.8
57900	Precipitation	100

a. Observed after mixing of 2 mL of PLL (0.5 wt%) with 2 mL of HA solution (0.2 wt%).
b. HA content was normalized with that of PLL MW of 57,900.

As specified in Fig. 1, BSA as a model protein could be incorporated in the PECML. Table 1 summarizes the effect of molecular weight of PLL on PECML buildup. At low MW like 3,970 and 14,400, there existed little interaction between HA and PLL. The PECML of HA and PLL could be built up when the MW of PLL was higher than 34,300. However, PLL with MW of 99,500 could not be used because of its poor solubility in PBS.

According to surface characterization, HA/PLL PECML was successfully built up on the chitosan-coated cover glass. Contact angle of cover glass (58 ± 3°) changed to 33.3 ± 1.8° when chitosan was adsorbed on the cover glass. Similarly, contact angle corresponding to the outer layer of HA was 21.3 ± 4.1° and that of PLL was 35 ± 1.6°. To make it clearer, the composition of total polyelectrolyte deposited on the mica was analyzed with ESCA. As is well known, mica consists of Si, Al, and carbon. With increasing number of layers up to 6, Al and Si content on the surface of mica decreased from 11.4% and 14.7% to 1.5% and 1.8%, respectively. To the contrary, nitrogen content increased from 2.5% to 9.2%. The decreases in Si and Al content and the increased nitrogen content represent the addition of PLL and HA. Though accurate quantification was not possible with this measurement technique, the relative changes in chemical composition showed an electrostatic interaction between HA and PLL. More specifically, HA content according to

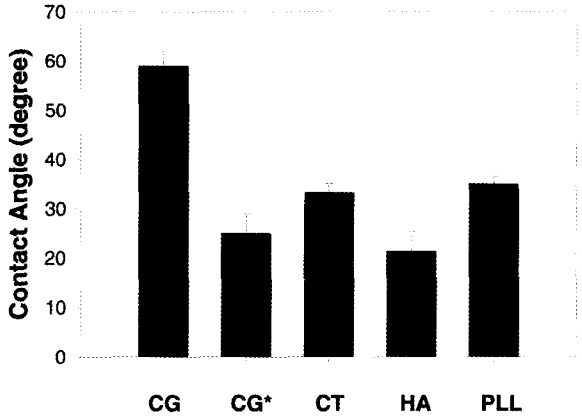

Fig. 2. Advancing contact angle of various samples: [CG] Cover glass, [CG*] SDS/HCl treated cover glass, [CT] Chitosan coated cover glass, [HA] CG/CT/$(HA/PLL)_8$/HA, and [PLL] CG/CT/$(HA/PLL)_8$.

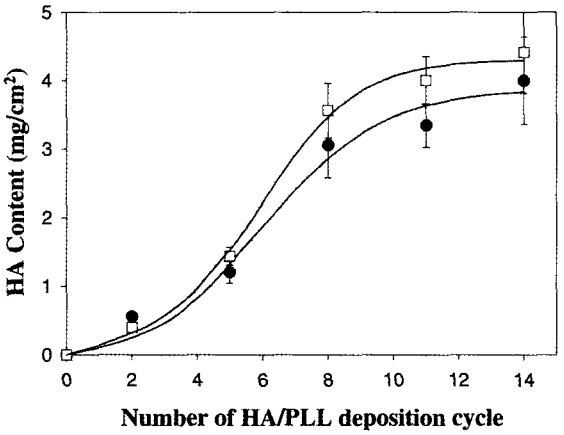

Fig. 3. HA content according to the number of HA/PLL deposition cycle of two different samples. As a substrate, cover glass (CG) was used and coated with chitosan (CT). Protein was embedded after 2 bilayers of HA/PLL had been built up. [□] CG/CT/$(HA/PLL)_2$/ $(HA+BSA/PLL)_n$ and [●] CG/CT/$(HA/PLL)_2/(HA/PLL)_n$.

the number of HA/PLL deposition cycle up to 14 was quantified by the carbazole method [23]. As shown in Fig. 3, HA contents increased rapidly up to 8 cycles for HA/PLL deposition, and then slightly increased with an increasing number of deposition cycle. This might indicate that the adsorption of each polyelectrolyte after 8 deposition cycles was not effective. Based on the results, PECML of HA and PLL after 8 deposition cycles was used for its stability and cell adhesion tests. In the case of PECML with protein, HA content corresponding to the same number of multilayers was higher than that of HA/PLL multilayers. Considering all these results, it was thought that HA/PLL PECML was successfully built up to 8 bilayers on the chitosan-coated cover glass.

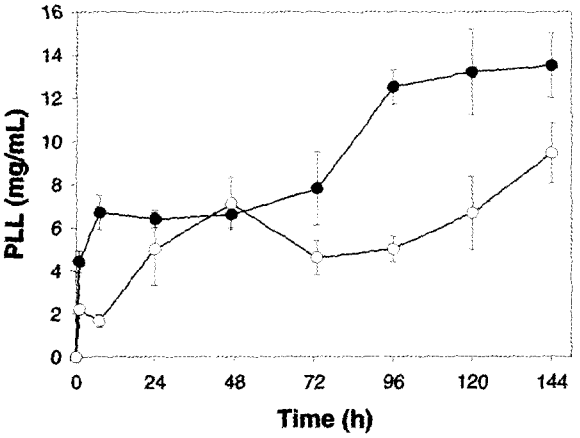

Fig. 4. PLL concentration change of two different samples with time. PLL concentration was determined by Lowry protein assay. [○] CG/CT/(HA/PLL)$_8$/HA and [●] CG/CT/(HA/PLL)$_8$.

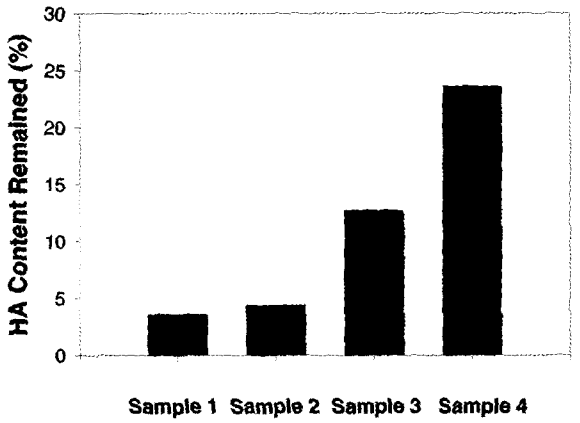

Fig. 5. HA contents remaining on cover glasses after in vitro release test for 7 days for four different samples: [1] CG/CT/(HA/PLL)$_8$ with hyaluronidase treatment, [2] CG/CT/(HA/PLL)$_8$/HA with hyaluronidase treatment, [3] CG/CT/(HA/PLL)$_8$, [4] CG/CT/(HA/PLL)$_8$/HA.

After confirmation of successful formation of PECML, its stability was tested through *in vitro* release of PLL. Fig. 4 shows PLL concentration change with increasing time. PLL appeared to be released up to 4~7 days depending on the outer layer of HA or PLL. PLL concentration was maintained at relatively low levels when the outer layer was HA. In other words, PECML appeared to be more stable when the outer layer was coated with HA. From these results, PECML of HA and PLL appeared to stay stable longer than 4 days. Fig. 5 also shows the stability of PECML of HA and PLL. After *in vitro* release test at 37°C for 7 days, *ca.* 25% of HA remained on the cover glass when the outer layer of PECML was HA. Hyaluronidase treatment reduced the remaining HA content on the cover glass. Less than 5% of HA was detected by the carbazole assay regardless of whether the outer layer was HA or PLL.

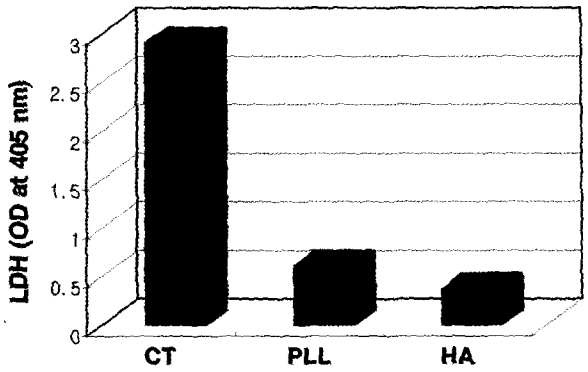

Fig. 6. The optical densities (OD) of lactate dehydrogenase (LDH) representing cell numbers adhering on polyelectrolyte complex multiplayer. Values were obtained by testing with a cytotoxicity detection kit. [CT] Chitosan-coated cover glass, [PLL] CG/CT/(HA/PLL)$_8$, and [HA] CG/CT/(HA/PLL)$_8$/HA.

Finally, its applications for surface modification to prevent cell attachment and protein drug delivery were assessed. In the previous work, we reported PBMC attachment to HA hydrogel was very poor [26]. As shown in Fig. 6, PBMC attachment to the PECML-coated cover glass was drastically reduced. The OD of LDH represents the cell numbers adhering on PECML. Although significant Schwann cell attachment to PLL-coated HA strands was reported [24], PBMC attachment to the outer layer of PLL increased only slightly compared to that of HA (Fig. 6). The result might be ascribed not only to the large hydrodynamic volume of HA in PECML but also to the detachment of small molecular weight PLL on the outer layer surface. As for its use for peptide and protein drug delivery, we confirmed that BSA as a model protein could be incorporated into the PECML and its release seemed to be triggered by the degradation of HA with hyaluronidase. Detailed protein release studies should be performed as a follow-up to these studies.

CONCLUSION

Surface characterization showed that the PECML of HA and PLL was successfully built up on the chitosan-coated cover glass. Contact angle of cover glass (58 ± 3°) changed to 33.3 ± 1.8° when chitosan was adsorbed to the cover glass. Similarly, contact angle corresponding to the outer layer of HA was 21.3 ± 4.1° and that to PLL was 35 ± 1.6°. According to the ESCA analysis of PECML deposited mica, Al and Si content on the surface of mica decreased whereas nitrogen content increased and then reached saturation. More specifically, analysis with the carbazole method showed that HA content increased rapidly up to 8 cycles for HA/PLL deposition and then slightly increased with an increasing number of deposition cycle. PECML of HA and PLL appeared to be stable for about 4 days from the *in vitro* release test results of PLL embedded in the PECML. After *in vitro* release test for 7 days, *ca.* 25% of HA remained on the chi-

tosan-coated cover glass. The surface modification of chitosan-coated cover glass with PECML resulted in drastically reduced peripheral blood mononuclear cell (PBMC) attachment. In addition, we confirmed that BSA as a model protein could be incorporated into PECML and its release could be triggered by the degradation of HA with hyaluronidase.

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