Spontaneous Biomacromolecule Absorption and Long-Term Release by Graphene Oxide

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ABSTRACT: Biomacromolecule loading is the popular research field in the biomedical field. To control the loading amount and releasing profile, various materials and fabrication techniques were developed. In this study, layer-by-layer assembly of multilayer films between collagen (Col) and graphene oxide (GO) was used to control the release of the loading molecule. By mixing GO into the system, ovalbumin (OVA) can be spontaneously adsorbed onto the GO sheet (denoted as GO/OVA) via the hydrophobic interaction. Two kinds of multilayer films (Col/GO/OVA and Col/GO/OVA) were fabricated. The thickness growth curve, quantitative of each layer adsorption, film morphology, stability, cell viability, and OVA release from multilayer films were investigated. The result has shown excellent film stability, macromolecule loading, and sustained release because of GO ability.

1. INTRODUCTION

Drug delivery system is one of the most popular topics in biomedical engineering fields. To deliver therapeutic molecules, various techniques and materials were used. Even though there are some methods which use simple materials and facial techniques such as Chitosan hydrogel microneedles and composite ceramic–polymer hydrogels, they require many complex steps and also show a rapid release of the loading molecule. High loading ability and rapid release property can be achieved via a lysozyme-assisted oil/water emulsion technique. In this technique, a hollow silica nanosphere with large through holes plays a key role. Although complex copolymer and liposome multidomain peptide nanofibers show a good result, both of them need to be fabricated via a complicated method. The easy and simple technique normally shows an uninteresting result, but for the highly effective carrier, the complicated process was needed. Layer-by-layer (LbL) assembly is a simple and versatile method for coating the substrate. By using the sequentially adsorbed opposite charge materials, the LbL film can present a nanoscale-controllable film. This technique allows various kinds of material coating onto the different substrates via a large variety of interactions.

In a drug delivery system, many studies used an LbL assembly to fabricate multilayer structures with drug loading. Many materials were used as building blocks such as polyelectrolytes, block copolymer micelles, and silica nanoparticles. There are several therapeutic molecules that can be loaded in these films such as DNA, protein, anti-HIV microbicide (tenofovir), antibacterial, antibiotic, and anticancer drug.

Graphene oxide (GO) belongs to the carbon family. It can be obtained by exfoliation of natural graphite powder by Hummers’ method. GO with one-atom thickness contained a carboxylic group at the edge and phenol, hydroxyl, and epoxyide at the basal plane. With these functional groups together with the high surface area, GO is one of the popular materials used in many fields such as energy storage, gas barrier, optical, and biological applications. In biomedical applications, GO was used for different functions such as antibacterial and drug delivery and as material stabilizers. Together with another material, GO layer acts as the capping or blocking part to prevent the burst releasing a loading molecule. Collagen (Col) is one of the popular materials in drug delivery fields because of its biocompatibility. In this study, we present a simple technique for fabricating a macromolecule loading and a long-term release material (as shown in Figure 1). Ovalbumin (OVA) was used as a model drug in this study. By using the full advantage of GO, OVA 45 kDa globular protein (pI ≈ 4.6) was adsorbed onto the GO sheet spontaneously. Because of the rich nonpolar amino acid group in OVA, the hydrophobic interaction occurs. Furthermore, GO acts as the capping layer and prevents the
rapid release in our film, resulting in long-term release in this system.

2. MATERIALS AND METHOD

2.1. Materials. The Col type I solution extracted from the rat tail with ≥90% purity was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Phosphate-buffered saline (PBS; 10×) was purchased from Gibco (Grand Island, NY, USA). OVA extracted from egg white was purchased from Bio Basic Canada Inc (Toronto, CANADA). OVA and Texas Red conjugate were purchased from Thermo Fisher Scientific Ltd. Fluorescein isothiocyanate, isomer I, and sodium acetate buffer solution (pH 5.2) were purchased from Sigma-Aldrich. Sodium hydroxide and hydrochloric acid were purchased from Daejung, Korea. Random size GO in this study was prepared from graphite powder (20 μm, Alfa Aesar, MA) via the modified Hummers’ method.

2.2. Film Preparation on the Substrate. In this study, multilayer films were fabricated on a Si wafer or poly(ethylene terephthalate) (PET) film using an LbL assembly dipping technique. The substrate was treated with O2 plasma (Femto Science, Korea) for 2 min to produce the negatively charged surface. The treated substrate was dipped into Col solution (1 mg/mL in acetate buffer solution, pH 5.2) for 10 min, followed by rinsing twice with distilled (DI) water (pH 5.2) for 2 min. Subsequently, the substrate was dipped into GO solution (0.5 mg/mL, pH 6) and washed twice with DI water (pH 6). A multilayer film was obtained by repeating the step described above.

2.3. Col/GO Film Characterization. The thickness growth curve of the Col/GO multilayer film was detected by a profilometer (Dektak 150; Veeco Plainview, USA). The quantity of each Col and GO layer adsorption was measured by using a quartz crystal microbalance (QCM 200; Stanford Research Systems, Inc., USA). The amount of polymer adsorption was calculated from the decreasing frequency (ΔF) by Sauerbrey’s equation

$$\Delta F (\text{Hz}) = -\frac{2F_0^2}{A\sqrt{\mu}} \Delta m$$

where $F_0$ is the fundamental resonance frequency of the crystal, $A$ is the area of the Au-chrome electrode, and $\mu_\lambda$ (2.95 × 10\(^11\) g/(cm·s\(^2\))) and $\rho_\lambda$ (2.65 g·cm\(^{-3}\)) are the shear modulus and density of quartz, respectively. Applying these numerical values, the equation can be simplified as follows:

$$\Delta F (\text{Hz}) = -56.6 \times \Delta m_a$$

where $\Delta m_a$ is the mass change per unit area of the quartz crystal ($\mu g/cm^2$).

The surface morphology of the multilayer film was investigated by atomic force microscopy (AFM) (NX-10; Park Systems, Korea) and field emission scanning electron microscopy (LIBRA 120, Carl Zeiss). The zeta potential of the solution was measured by a nanoparticle analyzer model S7-100 (HORIBA, Japan). The film stability was detected by soaking the film in 1× PBS at 37 °C, and then a profilometer was used to measure the thickness at each time point.

2.4. Cell Culture and In Vitro Cytotoxicity of the Col/GO Film. A human dermal fibroblast (HDF) cell was cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin–streptomycin under 37 °C and 5% CO\(_2\) condition. After that, 80% confluence of cells (1 × 10\(^4\)) were seeded in 12-well plates and cultured overnight. Then, the (Col/GO)\(_{15}\) multilayer film fabricated on the PET film was rolled and put onto the wall of each well. After a certain period (1 and 3 days), the film was removed and cell viability was tested by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 10 μL of the MTT solution was added to each well and incubated for 2 h in the incubator without light. Then, the relative amounts of viable cells were measured by a microplate reader at 540 nm wavelength.

2.5. OVA Loading on the GO Sheet. To load OVA on the GO sheet, OVA (10 μg/mL) in sodium acetate buffer solution (pH 5) was mixed with GO solution (0.5 mg/mL, pH 6) by a 1:1 volume ratio. Then, the mixing solution was stirred overnight to ensure that OVA can be adsorbed onto the GO nanosheet. After centrifuging at 3000 rpm for 10 min, the supernatant was removed, and then DI water (pH 6) was added. The final solution (GO/OVA) was dispersed by using an ultrasonic bath and vortexed.

2.6. OVA Loading on the Col/GO Film. For loading OVA on the Col/GO film, two kinds of multilayer structures (bilayer and triayer structures) were fabricated on the substrate to observe the effect of different interactions for OVA release. For the bilayer structure (Col/GO/OVA) film assembly, the Si wafer was cleaned and dipped into the Col solution, followed by the washing step. Then, the substrate was dipped into the

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Figure 1. Schematic representation of the materials used and the structure of Col/GO/OVA multilayer films (a) and Col/GO/OVA multilayer films (b) fabricated by the LbL assembly method.
GO/ova solution, followed by the same washing step. The multilayer film was obtained by repeating the cycle as described above. The trilayer structure (Col/GO/OVA) film was prepared using the same method. Briefly, the cleaned Si wafer was dipped into the Col, GO, and OVA solution. After each dipping step, DI water was used for washing the unreacted substance, and a multilayer film was obtained by repeating the previous steps.

2.7. OVA Release Test. For measuring OVA release from the multilayer film, OVA-conjugated Texas red (Invitrogen, USA) was used for film fabrication, instead of normal OVA. The bilayer structure (Col/GO/OVA)₁₀ and the trilayer structure (Col/GO/OVA)₁₅ were fabricated on a Si wafer, and the thickness of both films was 150 nm. Then, the multilayer film was cut into 1 × 1 cm² pieces and soaked in 1X PBS solution under 37 °C. The release of OVA–Texas Red was obtained by an FP-8300 spectrofluorometer (JASCO) at the fluorescent emission wavelength of 615 nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of the Col/GO Multilayer Film. The LbL assembly method is used for the fabrication of multilayer films from Col and GO by a dipping technique. The main driving force to assemble this film is electrostatic interaction. During the film fabrication, Col has a slightly positive charge because of its pI ≈ 8.2–8.4 and GO has a negative charge at pH 6. From the result of each bilayer thickness, this multilayer film exhibited a linear growth curve as shown in Figure 2a. The quantitative adsorption of Col and GO in the Col/GO film (Figure 2b) shows a decrease in QCM frequency because of the increasing mass adsorption on the gold electrode. The mass adsorption ratio between Col and GO is 1.7:1. It can be confirmed that the LbL assembly between Col and GO is successful, from the curve of thickness growth and QCM data. To investigate film morphology, scanning electron microscopy (SEM) and AFM were used. The results from the SEM images (Figure 2d,e) illustrated Col/GO film morphology with a wrinkled surface because of the adsorption of GO sheet onto the surface. From the AFM image (Figure 2f), Col fiber was detected. This fiber was distributed in the whole area and covered by the GO sheet. The root mean square of the (Col/GO)₁₀ film was 22.5 nm, which indicates that the film surface is smooth. The film stability was concluded by the decrease of thickness. From Figure 5a, the thickness was decreased by time, and the stability was proportional to the number of bilayers because of the presence of GO. The 15-bilayer film thickness still remained more than 80%, when we measured after 1 week.

3.2. Cytotoxicity of the Col/GO Film on HDF Cells. In order to observe the cell viability, the HDF cell was cultured and seeded in 12-well plates at a seeding density of 1 × 10⁴ cells in each well. Cells were incubated overnight, and then the Col/GO film was placed on the wall of a culture well plate subsequently. At a certain time point (1 and 3 days), the cell viability was measured using the MTT assay. Hu et al. (2011) reported that the physical contact between the cell and the GO sheet can cause damage to the cell membrane. Liao et al. (2011) showed that the toxicity of GO depends on its exposure to the environment whether GO aggregates or not. Furthermore, the interaction with the cell can also be one of the factors to affect the toxicity of GO. Two groups of Col/GO films were prepared to investigate the effect of physical contact with GO sheet on the damaged cell membrane. In the first group, GO was exposed to outside. This group was denoted by (Col/GO)ₙ, where n = 1, 3, and 5 bilayers. In another group, GO was covered by the Col layer to prevent direct contact between GO and the cell membrane, which was...
denoted as (Col/GO)₅. Figure 2 shows the MTT assay of HDF cells treated by multilayer films with Col coverage. There was no significant difference between them (p > 0.05) on day 1 and had a tendency to induce HDF cell proliferation when compared to the control group on day 3, whereas the group with GO exposure shows a decrease in cell viability. From Figure S1 (Supporting Information), HDF cells treated by the GO-exposed film show a significant decrease in cell viability. However, the percentage of cell viability is still higher than 80.

3.3. OVA-Loading Capacity on Multilayer Films. We then observed whether ova protein can be spontaneously bound with GO or released sustainably. Different films were prepared by using the LbL assembly method, and the zeta potential of each material is shown in Table 1. It can be confirmed that the fabrication of (Col/OVA) and (Col/GO/OVA) was successful in OVA loading, which can be proved by the growth curve of film thickness and QCM. The thickness growth curve of the Col/OVA film (Figure 3a) shows a linear relationship with the high standard deviation (SD), which refers to the high roughness of the film surface.

This result was confirmed by the SEM image as shown in Figure 4a. The film surface showed high roughness because of the GO/OVA coalescence and the size within 200 nm to micron size as shown in Figure 4a (inset). The binding of OVA on the GO sheet was due to the hydrophobic interaction of large size GO and 163 nonpolar amino acid group in an OVA structure. The QCM data of Col/OVA show the quantity of layer adsorption, and the adsorption ratio between

| material          | zeta potential (mV) |
|-------------------|---------------------|
| Col pH 5.2        | 9.93 ± 0.9          |
| GO pH 6           | −55.3 ± 2.84        |
| GO/OVA pH 6       | −36.8 ± 1.47        |
| OVA pH 5.2        | slightly positive   |

Figure 3. (a) Thickness growth curve of Col/OVA and Col/OVA multilayer films. (b) QCM data represent the frequency change of Col/OVA and Col/OVA structures. (SD, n = 3).

Figure 4. SEM image of (a) (Col/OVA)₁₀ multilayer films. Inset picture represents the GO-conjugated OVA. (b) (Col/OVA)₁₀ multilayer films.
Col and GO/OVA was 1:5. This ratio indicates that the amount of GO/OVA adsorption was higher than that of Col. The Col/OVA multilayer film shows a linear relationship with lower SD than the Col/OVA film. The SEM image (Figure 4b) shows a more uniform surface film than the surface of the Col/OVA film. The less roughness surface in Col/OVA occurred because the deposition axis of OVA onto the GO sheet was limited. In case of the mixing between GO and OVA in free-standing form, the adsorption of OVA on the GO sheet occurs in all direction. This occurrence causes the complexity between both materials (GO/OVA) and non-uniform deposition, resulting in a rougher surface. The QCM data (Figure 3b) show that the adsorption ratio between Col/OVA was 6:4:1 in a trilayer structure. Comparing the adsorption ratios of Col in the Col/OVA film and the Col/OVA film, we found that the adsorption ratio of Col was similar, but the ratio in the trilayer structure was lower. For fabricating the LbL assembly film, the adsorption onto the substrate was promoted by diffusion-driven kinetics. Because of the deposition of OVA on the GO sheet, the zeta potential of GO in a complexation form was changed from −55 to −36 mV. The reduced zeta potential of GO causes the decrease in the growth rate of the Col layer, as shown in the QCM data. These phenomena can be explained by the previous study of Zou et al., 2014: polyelectrolyte was able to penetrate through the GO interspace and diffuse out by osmotic pressure, resulting in additional deposition. In the Col/OVA system, the presence of OVA onto the GO sheet can obstruct the Col diffusion to GO interlayers and reduce the driven diffusion of Col.

3.4. Release of OVA from Multilayer Structures. To investigate the OVA release from bilayer and trilayer films, OVA–Texas Red was used for fabricating the LbL film, instead of OVA. Figure 5b shows the release profile from (Col/OVA)2 and (Col/OVA)15 films. On the basis of this, the interdiffusion in a film fabrication process, we hypothesize that interdiffusion can be controlled by GO and shows the different release profile from both the films. However, the bilayer and trilayer structure films show the similar release profile. The total amounts of OVA release were 2.19 and 2.07 μg/cm² for bilayer and trilayer films, respectively. It was released bristly at the early stage, followed by a sustained release from the middle period to the end. The time for the release test is over 70 days. These release profiles can be explained by the effective barrier ability of the GO sheet. Many researchers use GO/GO layer as a capping layer to prevent the rapid release of the loading molecule, and the results have shown the success in their work. The upper layer generated a burst release because of the effect of Col diffusion into GO and create a new interface, leading to a large void present between the layer. The sustained release in the middle of the release occurred because the multilayers of GO retard the release of OVA.

4. CONCLUSIONS

From this study, GO acts as a biomacromolecule carrier. GO can bind with a macromolecule such as OVA spontaneously and act as a barrier to prevent the rapid release of the loading molecule. The LbL assembly method was used for fabricating multilayer films between Col and GO. Two different kinds of films were prepared to observe the ability of controlling interdiffusion by GO. Because of the rich nonpolar amino acid group of OVA resulting in the spontaneous binding with the GO sheet, the hydrophobic interaction occurred as the main driving force. The complexation of GO and OVA was successfully prepared without any catalyst or external stimuli. The bilayer and trilayer films with OVA loading show the same release profile, which is different from our hypothesis. Even though GO cannot control the interdiffusion, its capping ability was excellent. Both films can release OVA for more than 70 days with a loading amount of 2.19 and 2.07 μg/cm² for Col/OVA and Col/OVA films, respectively. For future study, the Col/OVA film with OVA loading will be used for treating cells, and various biological tests will be done. Furthermore, to prove more biomacromolecule binding of GO, another kind of macromolecule will be used.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00537.

Toxicity testing of multilayer films with the HDF cell

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Figure 5. (a) Col/GO multilayer film stability. (b) OVA release profile: (●) indicates OVA release from (Col/OVA) multilayer films. (●) indicates OVA release from (Col/OVA) multilayer films. (SD, n = 3).
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