Graphene Multilayers as Gates for Multi-Week Sequential Release of Proteins from Surfaces

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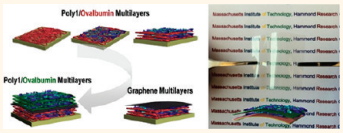
ABSTRACT The ability to control the timing and order of release of different therapeutic drugs will play a pivotal role in improving patient care and simplifying treatment regimes in the clinic. The controlled sequential release of a broad range of small and macromolecules from thin film coatings offers a simple way to provide complex localized dosing *in vivo*. Here we show that it is possible to take advantage of the structure of certain nanomaterials to control release regimes from a scale of hours to months. Graphene oxide (GO) is a two-dimensional charged nanomaterial that can be used to create barrier layers in multilayer thin films, trapping molecules of interest for controlled release. Protein-loaded polyelectrolyte multilayer films were fabricated using layer-by-layer assembly incorporating a hydrolytically degradable cationic poly(β-amino ester) (Poly1) with a model protein antigen, ovalbumin (ova), in a bilayer architecture along with positively and negatively functionalized GO capping layers for the degradable protein films. Ova release without the GO layers takes place in less than 1 h but can be tuned to release from 30 to 90 days by varying the number of bilayers of functionalized GO in the multilayer architecture. We demonstrate that proteins can be released in sequence with multi-day gaps between the release of each species by incorporating GO layers between protein loaded layers. *In vitro* toxicity assays of the individual materials on proliferating hematopoietic stem cells (HSCs) indicated limited cytotoxic effects with HSCs able to survive for the full 10 days of normal culture in the presence of Poly1 and the GO sheets. This approach provides a new route for storage of therapeutics in a solid-state thin film for subsequent delivery in a time-controlled and sequential fashion.



KEYWORDS: layer-by-layer · controlled release · graphene oxide · protein · sequential

Biomedicine faces significant challenges in the areas of drug delivery, *in vitro* diagnostics, *in vivo* imaging, and tissue engineering due to the need for ever more precise temporal and spatial regulation of the dosing of bioactive molecules. There are a number of examples for which it would be desirable to introduce multiple drugs together or in sequence in a manner that is synergistic.[1−9] Particularly, ultrathin films that enable controlled local release for improved therapeutic effects can enhance existing and lead to new biomedical applications.[10−12] The ability to generate ultrathin film coatings on a variety of surfaces to provide both high drug loading and precise control over the release of active biomolecules such as proteins, DNA, growth factors, cytokines, or enzymes from surfaces has the huge potential to broaden the development of new delivery coatings for biomedical technology.[13−16] Because these bioactive materials are often sensitive to processing with solvents and heat, it is highly desirable to incorporate them in aqueous conditions closely mimicking physiological pH and osmolarity.

Layer-by-layer (LbL) assembly, which involves the alternating adsorption of multivalent charged molecular species from buffered aqueous media to build a film,[17−20] provides an alternative approach to traditional polymer-based delivery systems.[21−26] Using this approach, the polymer delivery matrix is built one layer at a time, introducing the drug of choice in alternate layers. The LbL assembly technique is versatile, allowing the incorporation of a broad range of functional polymers, biomacromolecules, and other charged species.[27−32] The drug delivery coating can be constructed one nanoscale layer at a time, ideally achieving release of the compounds of interest in inverse order due to surface erosion with the introduction of degradable polymers.[33] Unfortunately, the interdiffusion and mixing of polyionic species during assembly can limit the ability to control the film architecture for many biomedical applications; thus most such multilayer films release biologic drugs with little or no control of drug sequence.

McEuen and co-workers demonstrated that graphene, a single layer of two-dimensional

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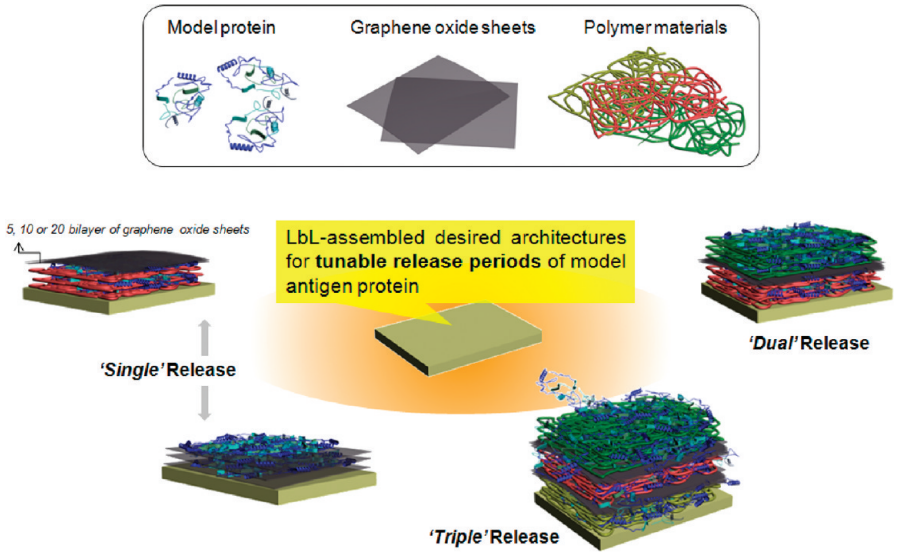

Figure 1. Overall schematic illustrations of architecture toolbox for desired sustained release of model protein.

carbon graphitic network, can serve as the world's thinnest balloon by providing a unique gas separation barrier that is only one atom thick by applying pressure.[34] Graphene, a single layer of two-dimensional carbon lattice, is a promising nanomaterial with outstanding electrical, chemical, and mechanical properties, which can be easily modified by applying the technique used for the chemical oxidation of graphite to graphene oxide (GO). The synthesis was conducted by exfoliation of natural graphite powder with various oxidants followed by the sonication methods developed by Hummers and co-workers.[35]

Here, we present a smart delivery platform with tunable release kinetics of a model antigen protein by capping a degradable polyion architecture with graphene oxide (GO) for systematic release of a model protein. Ovalbumin (ova) is a 45 kDa globular protein (pI $\sim$ 4.6) often used as a model antigen; when simply incorporated into electrostatic multilayers with degradable polyions, it exhibits a rapid burst release of ova over an hour (around 80−90%).[36] Here, we take full advantage of the low permeability of graphene by using GO with charged functional groups as a component in the LbL thin films to create more stable thin films with lowered interdiffusion and sustained and sequential release.

Figure 1 presents the overall schematic representation of the different kinds of LbL architectures examined in this work. We integrate GO multilayer films with desired thickness into films to regulate the release of a single component, as a capping layer, or between sets of multilayers to act as barrier layers that enable sequential release of more than one drug component over time periods out to 100 days.

RESULTS AND DISCUSSION

To assemble a smart film capable of programmable and sequential release, we utilized hydrolytically degradable multilayer films using a poly(β-amino ester) (Poly1) as a cationic LbL component.[37] This particular class of polymers has been extensively studied for gene delivery applications, and the biocompatibility and degradation kinetics of Poly1 have been investigated. Our group has previously employed Poly1 to release a variety of model and therapeutic molecules, including growth factors, antibiotics, oligonucleotides, and enzymes[38−40] from multilayer architectures both *in vitro* and *in vivo* studies. In this study, to demonstrate control of sustained release, we chose to use a model protein, ova, as it is a protein that has exhibited very rapid disassembly with Poly1 in multilayer films in previous work.[36]

We introduce graphene oxide as a means of modulating that release. We first prepared multilayer films for sustained release by alternating adsorption of Poly1 and ova from aqueous buffers onto substrates, utilizing the fluorescence of fluorophore-conjugated ova to confirm the protein incorporation. Ova is relatively small with a low charge density and is able to readily diffuse out of the (Poly1/ova) films upon contact with aqueous buffer solutions at pH 7.4, possibly due to loss of charge along the Poly1 backbone, leading to a charge destabilization of the electrostatically assembled thin film and subsequent rapid dissolution of the multilayer. While the ionic strength of the solutions used for multilayer assembly were similar to the release experiments, it is thought that this rapid release of ova is due primarily to the charge shift in the Poly1 combined with the already low charge density of the protein and its high diffusivity in the LbL film.

To control the release of ova, we utilized an LbL multilayer graphene oxide (GO) film to act as a capping layer on the (Poly1/ova) film. The number of bilayers of (GO-NH_3+/GO-COO-) multilayer films were adjusted to tune the release of ova. Figure 2a depicts the chemical

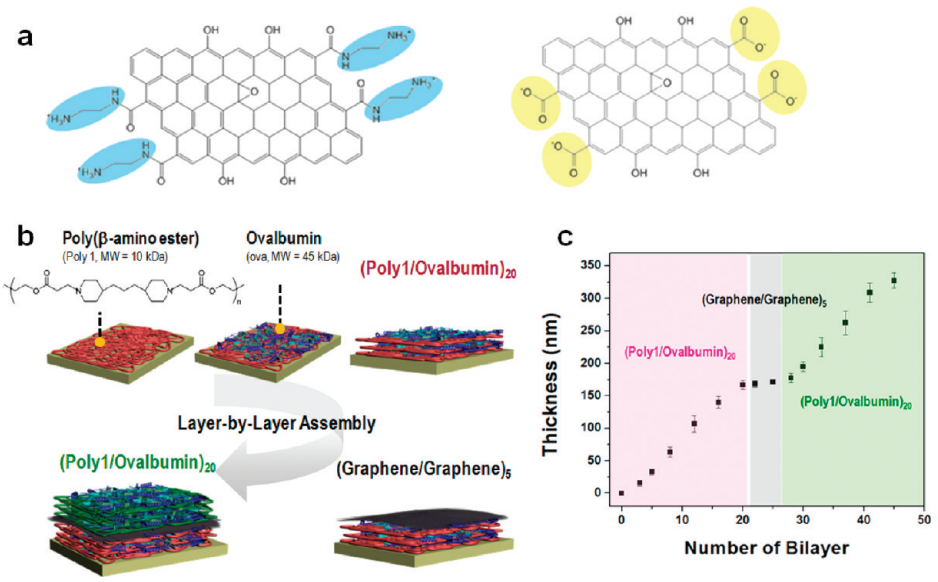

Figure 2. (a) Schematic representation of chemically modified graphene oxide GO-COO- and GO-NH_3+. (b) Schematic illustrations of (Poly1/ova)$_{20}$(GO/GO)$_5$(Poly1/ova)$_{20}$ multilayer films. (c) Growth curve of electrostatically assembled (Poly1/ova)$_{20}$(GO/GO)$_5$(Poly1/ova)$_{20}$ multilayer films as a function of bilayer number.

structures of modified GO sheets. Negatively charged GO was prepared by oxidation with strong acid treatment to create carboxylic acid groups on the GO surface (GO-COO-).[41] Subsequently, positively charged GO sheets were prepared by introducing amine groups (NH_2) on the surface of the negatively charged GO sheets through an *N*-ethyl-*N*′-(3-dimethyl aminopropyl)-carbodiimide methiodide (EDC)-mediated reaction, which resulted in a positively charged GO suspension (GO-NH_3+). The pH for assembly of the GO multilayer films was 6.0, for which both groups should be highly charged, leading to high density multilayer films as previously reported, with a thickness of 4.1 ± 0.8 nm for five bilayers of (GO-NH_3+/GO-COO-). Film growth was linear as a function of the number of bilayers, including hybrid (Poly1/ova), (GO-COO-/GO-NH_3+), and (Poly1/ova), as shown in Figure 2c, with a higher thickness per bilayer pair for the protein/Poly 1 system, consistent with the lower charge density and more globular nature of the protein.

Capping layers of different thicknesses, using (GO-NH_3+/GO-COO-)$_{5,10,\ \text{and}\ 20}$ multilayers, were built atop (Poly1/ova)$_{20}$ films, and the morphology of these GO multilayer decorated films was investigated with scanning electron microscope (SEM) (Figure 3a−c). The graphene oxide sheets formed uniform capping layers onto Poly1/ova film; application of multiple layers leads to defect-free films that give full surface coverage of the underlying LbL multilayers and yields a smooth surface morphology. The root mean square (rms) roughness of a five bilayer GO film measured by AFM was 0.7 ± 0.3 nm. It is very interesting to observe that the two-dimensional GO can conformally coat the relatively rough surface of the Poly1/ova multilayers (rms roughness = 3.7 ± 1.4 nm) without significant undulation. This indicates that the GO multilayers form a stable cap which is able to retain the ova in the film even at five bilayers. The average thickness of the 20 bilayer (Poly1/ova) film was 167.12 nm.

Once we established that proteins could be incorporated into these films, the release characteristics of the films were examined after rehydrating the films in an incubator at 5% CO_2, 37 °C in 1× PBS solution. Release was measured using an enzyme-linked immunosorbent assay (ELISA) of ova. The GO capping layers play a major role in preventing early release of ova, which is remarkably sustained. The delay in release increases with the number of GO multilayers, as shown in Figure 3d. The release profile demonstrates that ova can be released with a linear trend over a period of approximately 90 days, releasing approximately 2.3 μg/cm^2 of incorporated protein from a 20 bilayer film. Sustained release of ova was observed for 5, 10, and 20 bilayer capping (GO-NH_3+/GO-COO-) multilayer films, with total release time of ova ranging from 30 days using a five bilayer GO cap to more than 90 days using a 20 bilayer cap. To verify that the released ova retains its primary and secondary structure, which are directly related with protein activity, we have performed gel electrophoresis and circular dichroism (CD) spectroscopy of collected aliquots of released ova from predetermined time intervals (Supporting Information S1 and S2). Samples from various time points yield a single characteristic band from gel electrophoresis at 45 kDa consistent with intact ovalbumin protein, with no sign of degradation or denaturation products. The CD traces of the released proteins indicate a secondary structure that is similar to native ovalbumin, indicating that the encapsulated and released ova was not denatured during the film preparation and release process.[42]

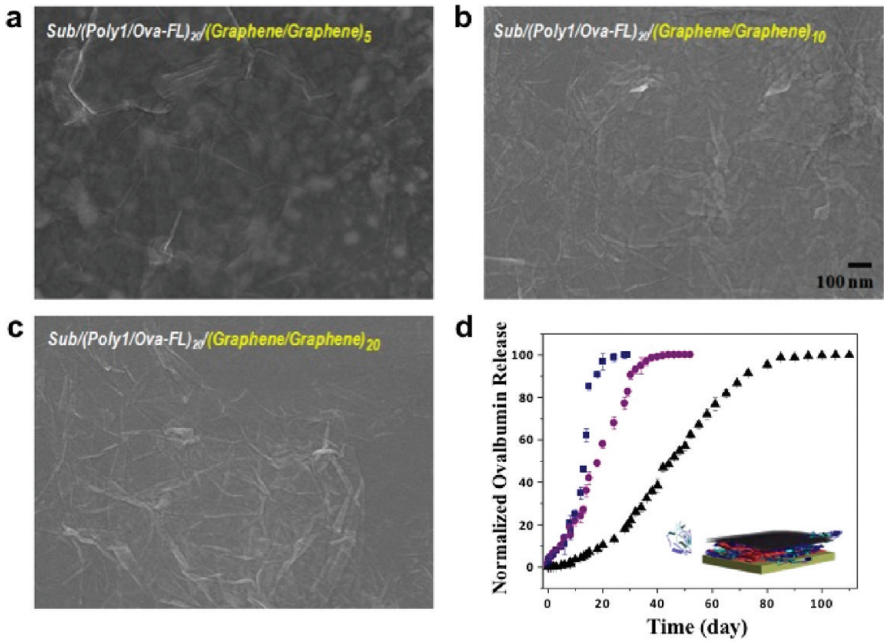

Figure 3. Representative surface morphology of a multilayer films: SEM image of as-assembled (a) $(GO/GO)_5$, (b) $(GO/GO)_{10}$, and (c) $(GO/GO)_{20}$ multilayer on substrate/$(Poly1/ova)_{20}$ multilayer film. (d) Influence of different number of graphene layers in the film architecture based on substrate/$(Poly1/ova)_{20}$ in the multilayer films on the release profile: normalized release profiles of ovalbumin from substrate/$(Poly1/ova)_{20}(GO/GO)_5$ (■, blue line), substrate/$(Poly1/ova)_{20}(GO/GO)_{10}$ (●, purple line), and substrate/$(Poly1/ova)_{20}(GO/GO)_{20}$ (▲, black line) measured by ELISA.

TABLE 1. Overall Release 25, 50, and 75% Times of Ovalbumin Corresponding with Figure 3d

	% of ova release		
number of GO bilayers	25%	50%	75%
5 bi	9 days 21 h	13 days 6 h	32 days 22 h
10 bi	13 days 17 h	19 days 8 h	47 days 2 h
20 bi	16 days 2 h	28 days 11 h	62 days 7 h

The controlled release kinetics of the model protein after topping with different numbers of GO capping layers are summarized in Table 1 by 25, 50, and 75% release time.

It is clear from these data that the GO capping layers can be used as a simple means of tuning release over a very broad range of release times, from zero to at least 80 days, simply by adding a few more GO layers in the cap. Next, we examined the generation of films that use this capping layer concept to release proteins in a predetermined sequence, and release was measured using a fluorescence emission of fluorophore-conjugated ova. More specifically, films were prepared with GO multilayers introduced between two to three modular degradable layers with differently labeled fluorescent ova, depicted in Figure 4. To quantitatively analyze the release behavior, we then examined the release of three different fluorescently labeled ova molecules (labeled with Texas Red, TR; fluorescein, FL; Alexa Fluor 555, AF555). Figure 4a gives the release results from a $(Poly1/ova\text{-}TR)_{20}/(GO\text{-}NH_3{+}/GO\text{-}COO\text{-})_2/(Poly1/ova\text{-}FL)_{20}$ architecture; half of the ova released from the film in less than an hour from the top set of uncapped $(Poly1/ova\text{-}FL)_{20}$ multilayers, yielding a burst release from the top protein layers over the first few hours. In contrast, the underlying ova-TR released over a period of 102 h from the bottom multilayers of the same architecture due to retention from the graphene oxide capped multilayers. This clear separation of protein release is achieved with only two bilayer pairs of GO between the LbL ova containing layers. When five bilayer GO films were used instead, the temporal separation between delivery of the two proteins is dramatically increased, with a clear separation between substantive release of each component; the ova release half-time from the bottom layers extended out to 323 h in the film, with a period of little or no release of the underlying ova-TR for the first 100 or more hours. This example of stepwise release of drugs could prove highly impactful in the staged delivery of drugs, including booster delivery of vaccines, and synergistic temporal release of different growth factors and DNA.

To further demonstrate the ability to differentially tune the release of multiple biomolecules from the same multilayer architecture, we utilized a triple sequential ova releasing film by separating three ova containing $(Poly1/ova\text{-}AF555)_{20}$, $(Poly1/ova\text{-}TR)_{20}$, and $(Poly1/ova\text{-}FL)_{20}$ modules with two capping layers of GO, as depicted in Figure 4c. Ova-AF555 containing multilayers are capped with five GO bilayers, whereas ova-TR containing multilayers are capped with two GO bilayers. As expected, three different release profiles

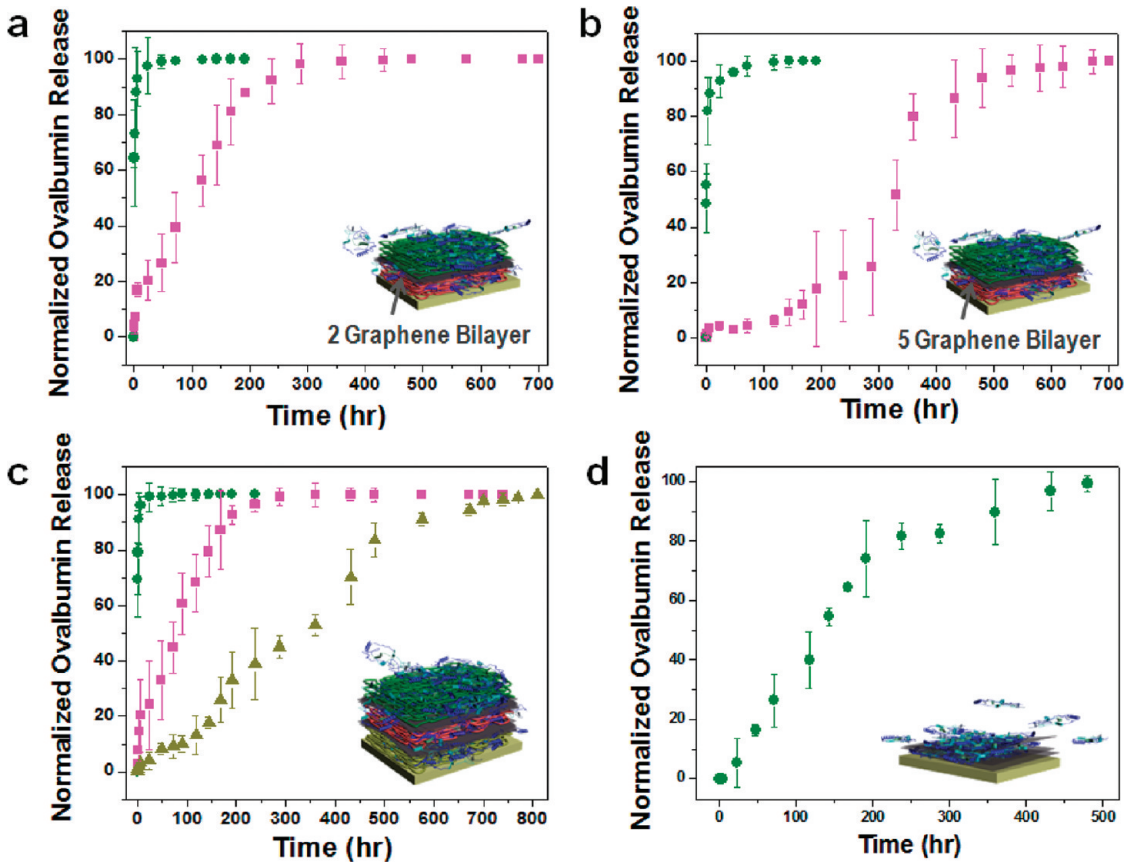

Figure 4. Various kinetics of ova release from dried multilayer films: normalized cumulative release from (a) substrate/$(Poly1/ova\text{-}TR)_{20}(GO/GO)_2(Poly1/ova\text{-}FL)_{20}$, (b) substrate/$(Poly1/ova\text{-}TR)_{20}(GO/GO)_5(Poly1/ova\text{-}FL)_{20}$, (c) substrate/$(Poly1/ova\text{-}AF555)_{20}(GO/GO)_5(Poly1/ova\text{-}TR)_{20}(GO/GO)_2(Poly1/ova\text{-}FL)_{20}$ multilayer films, (d) substrate/$(GO/ova\text{-}FL)_{20}$. The release experiments were conducted in PBS buffer (pH 7.4 at 37 °C, 5% of CO_2): ● (green line), ■ (pink line), and ▲ (yellow line) indicate the ova released from $(Poly1/ova\text{-}FL)_{20}$, $(Poly1/ova\text{-}TR)_{20}$, and $(Poly1/ova\text{-}AF555)_{20}$, respectively.

were exhibited, with the uncapped ova releasing almost immediately, followed in inverse order by the ova-TR and ova-AF555 systems. The half-lives of release of ova-TR and ova-AF555 were 81.4 and 348.7 h, respectively. The ova-TR release is very similar to that observed in Figure 4a, which has the same capping layer thickness; similarly, the five bilayer capping layer also yield time frames similar to the results in Figure 4b, indicating reproducibility of these systems with regard to release behavior with number of bilayers.

When we used GO as one of the charged components of the drug releasing layers rather than as a separate barrier or capping layer, with the film architecture $(GO\text{-}NH_3+/ova\text{-}FL)_{20}$, the half-life of ova release was 137.2 h, as shown in Figure 4d. This suggests that the coverage of a single GO-NH_3+ layer is not enough to provide complete coverage of an entire ova layer; however, the presence of GO still has a strong effect on the release rate of the protein when compared to films with Poly1. The release mechanism for these films is likely based on the slow shift in charge of the amine groups on the GO sheets; we have reported similar effects with nonhydrolyzable polyamines in previous work.[43]

Applying protein multilayers onto flexible, transparent substrates can be advantageous in delivery applications that are coupled with biosensors, implants, and laparoscopic or other optical biomedical devices. Polyethylene terephthalate (PET) has been used as a model substrate because of its excellent mechanical flexibility. Translating our system onto a flexible plastic substrate would provide a basis for developing a therapeutic protein releasing patch. By taking full advantage of LbL deposition, large-scale (6.7 in.) and transparent $(Poly1/ova)_{20}(GO/GO)_{20}(Poly1/ova)_{20}$ films were prepared on 175 μm thick PET substrate. The flexibility conferred by the GO multilayer stems from the presence of a mechanically percolative network structure of graphenes, which provides additional flexibility, mechanical support, and stability that could be important in biomedical applications such as biosensors, actuators, or bioelectronics (Figure 5).

Next, we assessed cytotoxicity by selecting a sensitive population of primary cells which could serve as sensitive *in vitro* sentinals for potential toxicity. For this, we chose human hematopoietic stem cells, cultured with five hematopoietic growth factors in a defined media which we have recently further characterized.[44,45]

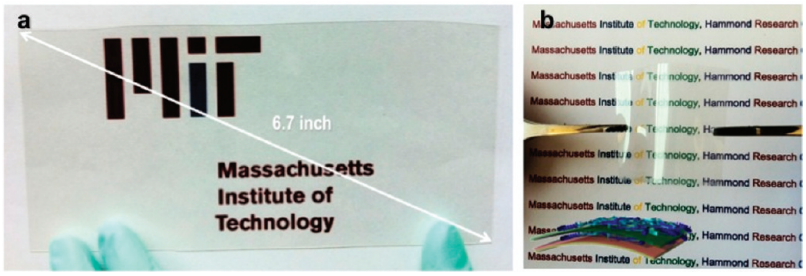

Figure 5. Photo images of thin film of substrate/(Poly1/ova-TR)$_{20}$(GO/GO)$_{20}$(Poly1/ova-FL)$_{20}$ multilayer film: (a) large-area film transferred on a 6.7 in. PET sheet, and (b) assembled multilayer film showing outstanding flexibility.

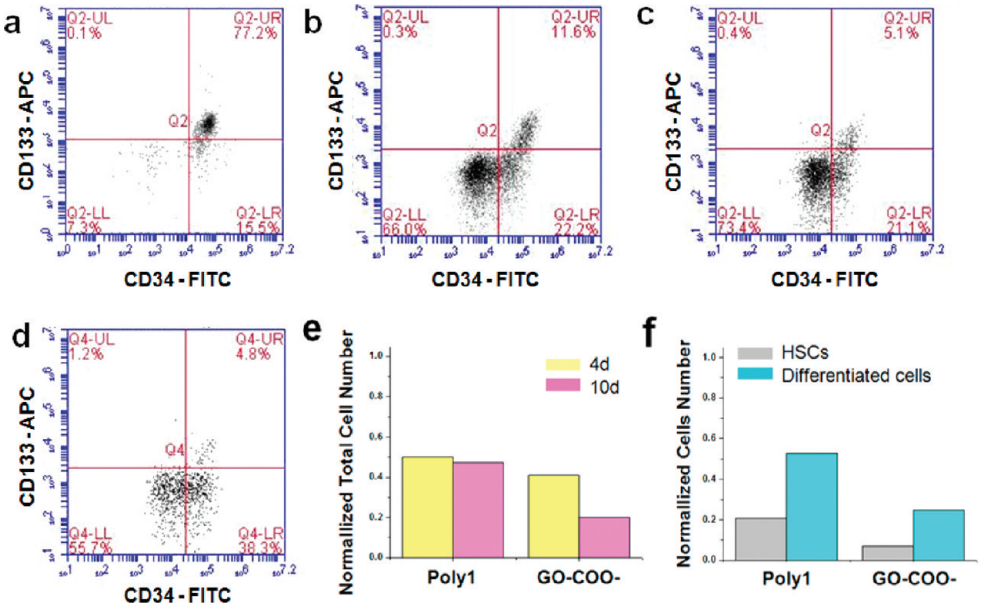

Figure 6. Proliferation was compared between CD133-APC and CD34-FITC subpopulations of hematopoietic stem cell using a cell proliferation from (a) cord blood (1 day), (b) feeder free cytokine culture condition for HSCs expansion and in the presence of (c) Poly1, (d) GO-COO- in control condition for 10 day assay. The expression levels of CD133 and CD34 were examined by FACS analysis. (e,f) Summary of the proportion of cells surviving in culture (e) and assessment of HSC retention in culture (f); data are normalized to a culture without added polymers.

Poly1 has been extensively used for gene transfection and protein delivery applications from LbL architectures. While GO sheets have received significant attention for their potential application in biology and medicine, their physiological effect on sensitive cells, such as hematopoietic stem cells (HSCs), has not been extensively studied. In Figure 6, we show the cell population analyses for 10 day cultures in the presence of high concentrations of Poly1 and GO sheets (modeling close proximity to an implanted film *in vivo*). We assessed toxicity by counting live cells and characterizing them by the expression of HSC markers CD34 and CD133, which we have previously shown correlate with repopulating potential. The CD34 and CD133 expression was assessed in freshly harvested HSCs and in cultured cells by flow cytometry. As depicted in Figure 6a–d, flow cytometry clearly showed the population of HSC containing cells ($CD34^+CD133^+$), progenitors ($CD34^+$), and differentiated cells ($CD34^-CD133^-$). After 10 days in culture in the presence of Poly1 and GO-COO-, the HSC containing population was reduced but still present and progenitor cells could also be observed. The results are summarized in Figure 6e,f, normalized to the control culture without Poly1 or GO-COO- addition; 47 and 20% of cells remained after a 10 day assay in the presence of Poly1 and GO-COO-, respectively, compared with control conditions. This represents modest toxicity when assayed by number and phenotype of stem cells, one of the most sensitive primary cell types to environmental perturbation. Considering the very high concentration of nanomaterials dissolved into the culture media (0.13 mg/mL, each), which is much higher than concentrations that would be used in culture or *in vivo*, this result is a promising first step in demonstrating biocompatibility.

CONCLUSION

In summary, we have established a release platform technology for the release of proteins in preprogrammed

sequences over long time periods of several weeks using GO capping layers as barriers. Using LbL assembly, we can precisely control the architecture of building materials deposited and tune the protein release by manipulating the number of GO deposition bilayers. We believe that the GO multilayers presented in this study for the systematic release of model antigen protein can be used in applications that incorporate numerous therapeutics in cases where control over the temporal release of the molecules is desirable. The films generated are durable, transparent, and ultrathin (<1 μm) with high protein loading and can be used to coat a broad array of substrates, including flexible plastic. The unique chemical and mechanical properties of graphene oxide can be exploited for novel opportunities in biomedical engineering. These observations are an important fundamental advance in biomedical engineering, where carbon-based nanomaterials can play an important role of controlled drug delivery.

METHODS

Materials. Poly(β-amino esters) were synthesized according to previous literature.[37] Alexa Fluor 555, fluorescein, and Texas Red-conjugated ovalbumin were purchased from Invitrogen (Eugene, OR).

Film Preparation. All LbL films were assembled with a modified programmable Carl Zeiss HMS DS50 slide stainer. Typically, films were constructed on a glass/Si wafer slide which was treated in a plasma cleaner (Harrick Scientific Corp.) with O_2 plasma for 5 min prior to use. The substrate was then dipped into Poly1 solution (2.0 mg/mL in 100 mM NaOAc buffer) for 10 min and followed by three sequential rinsing steps with pH-adjusted water for 1 min each. Then the substrate was dipped into ova solution (1 mg/mL in 100 mM NaOAc buffer) for 10 min and exposed to the same rinsing steps as described above.

Preparation of GO Multilayer Coatings. The concentration of the carbon−object solutions used in all of the deposition experiments was fixed to 0.1 wt % without any ionic salts. The GO-NH_3+/GO-COO- multilayer coating was prepared as follows. The substrates were first dipped for 10 min in the cationic GO-NH_3+ (pH 6) solution, washed three times by dipping in water for 1 min, and then dried with a gentle stream of nitrogen. The negatively charged GO-COO- (pH 6) was subsequently deposited onto the GO-NH_3+ (pH 6) coated films using the same adsorption, washing, and drying procedures as described above.

Film Characterization. Release experiments were conducted by immersing a prepared multilayer film into a 20 mL vial containing 3.0 mL of PBS at physiological condition (37 °C, 5% CO_2). At a series of different time points, films were transferred to other vials and fresh PBS solution of the same volume was introduced. Ova release from the film was followed by measuring the fluorescence spectra of released ova-TR, ova-FL, and ova-AF555 in PBS by a plate reader (Infinite 200 PRO, Tecan). Film thickness was measured with a Tencor surface profilometer.

Cell Culture. Human HSCs were isolated and cultured as previously.[43,44] The only alteration was in the formulation of the Stemspan to account of addition of Poly1 and GO-COO-, which were dissolved in PBS and diluted into stemspan (90% stemspan 10% PBS final) with 0.13 mg/mL Poly1 and GO-COO-. The control culture used 90% stemspan/10% PBS without polymers.

Flow Cytometry. Freshly isolated and cultured cells were stained with anti-CD34 conjugated to fluorescein isothiocyanate (FITC) (Biolegend) and anti-CD133 conjugated to allophycocyanin (APC) (Milteyni). Samples were run on an Accuri C6 flow cytometer.

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Supporting Information Available: Additional figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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