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Parylene-Encapsulated Copolymeric Membranes as Localized and Sustained Drug Delivery Platforms

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Abstract—Parylene is a biologically inert material capable of being deposited in conformal nanoscale layers on virtually any surface, making it a viable structural material for the fabrication of drug delivery devices, as well as implant coatings, sensors, and other biomedical technologies. Here we explore its novel drug delivery applications by using parylene to package the polymethyloxazoline-polydimethylsiloxane-polymethyloxazoline (PMOXA-PDMS-PMOXA) block copolymer membrane of a nanoscale thickness (~4 nm/layer) mixed with a therapeutic element, creating an active parylene-encapsulated copolymeric (APC) membrane for slow release drug delivery of dexamethasone (Dex), a potent anti-inflammatory and immunosuppressant synthetic glucocorticoid. Given current needs for localized therapeutic release for conditions such as cancer, post-surgical inflammation, wound healing, regenerative medicine, to name a few, this stand-alone and minimally invasive implantable technology may impact a broad range of medical scenarios. To evaluate the applicability of the APC membrane as a biocompatible drug delivery system, real-time polymerase chain reaction (RT-PCR) was performed to investigate the expression of cytokines that regulate cellular stress and inflammation as a result of *in vitro* RAW264.7 macrophage cell growth on the APC membrane. Significant decreases in relative mRNA levels of IL-6, TNF-α, and iNOS were observed. Dex functionalized APC membranes were further found to effectively slow-elute the drug via confocal microscopy, with a confirmed extended elution capability over a period of several days, undergoing phosphate buffered saline washes between time points. In addition, we examined the membrane surface through atomic force microscopy (AFM) to examine Dex/copolymer deposition, and to characterize the surface of the APC membrane. Furthermore, we evaluated the effects of incubation with the APC membrane in solution on macrophage growth behavior and cellular adhesion, including the physical properties of parylene and the copolymer to elucidate the anti-adhesive responses we observed. The results of this study will provide insight into ultra-thin and flexible devices of parylene-encapsulated copolymer membranes as platform drug delivery technologies capable of localized and precision therapeutic drug elution.

Keywords—Drug delivery, Nanotechnology, Nanomedicine, Medical device, Inflammation, Cancer.

INTRODUCTION

Drug delivery is an essential aspect of medicine as it serves as a mechanism that bridges drug development and patient treatment. During the past decade, the advent of new technologies including tissue scaffolds and drug-eluting stents has improved the ability to determine how, where, and when pharmaceutical agents are delivered.[4,5,8,11,23,28,33,38,45,54,55,57,58,61,62] Furthermore, the rapid development of novel implantable medical devices makes biocompatible/drug-eluting coatings increasingly important toward the prevention of potential patient complications.[19,26,42,48,50,51]

It has been a challenge to create an optimized biocompatible coating capable of therapeutic elution due to several developmental barriers. Such a material would need to be biologically inert and stable, possess anti-inflammatory elution mechanisms, and pliable for use in a wide variety of applications.[45] The drug-eluting stent was created to improve stent longevity and minimize clotting on the stent itself to reduce the risk of neointimal hyperplasia, an excessive immune response to bare metal stent implants that results in narrowing of vessels due to clots, and ultimately medical complications for the patient.[4] By eluting immunosuppressive drugs from the stent, the inflammatory response was lessened near the implant location of the stent, thus inhibiting platelet activation and

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preventing neointimal hyperplasia. The drug-eluting stent has revolutionized cardiology, but conventional systems still rely on non-optimized materials.[5] For example, certain complications may still result from using a drug-eluting stent including severe thrombotic (formation of clot inside a blood vessel) events and restenosis (abnormal narrowing of vessels) which then generate counter-active side effects.[28,38]

Recent studies have been done on natural polymers in the field of tissue engineering with hopes to mimic nature and its evolved benefits.[33] However, these natural polymers alone are not often used to fabricate drug delivery devices because they are often only a few nanometers thick and virtually intangible.[9] For instance, the triblock copolymer used in the active parylene-encapsulated copolymeric (APC) membrane as a nano-scale drug molecule reservoir cannot be handled manually challenging its utility in a clinical setting as an implantable stand-alone device (e.g. patch). Therefore, we used parylene as a tangible complex material that could package the copolymer to maintain its unique nano-scale and drug-sequestering properties, thus creating a backbone for the drug/copolymer layer.[2,47,56] Most importantly, because parylene is coated via vapor deposition of individual molecules at room temperature, the resulting coat is extremely uniform, and conformal to practically any surface shape. This property is critical for many implant devices that require isolation from the body to preserve the function of the device.

Exploring drug delivery as a new field for parylene-based applications fills the need for a biocompatible, functionalized membrane capable of slow-releasing virtually any drug to a localized region for targeted delivery. The result of our studies is exemplified in the APC membrane which introduces a parylene-based functionalized drug delivery membrane with targeted slow-release drug elution capabilities, and the flexibility to be tailored to various therapeutic applications. Currently, chemotherapeutic drugs are capable of killing cancerous cells, but cannot selectively kill only cancerous cells; they exhibit universal cytotoxicity.[57] A similar issue exists in the administration of anti-inflammatory compounds where the indiscriminate introduction of these compounds significantly dilutes drug efficacy and weakens the global immune response, which opens a window for infection. For instance, regarding synthetic corticosteroids, the administration and halting of steroid treatment must be tapered to prevent the occurrence of a patient with abnormally low levels of natural steroids that may lead to a restored inflammatory response.[29] Therefore, it is imperative that drugs are delivered in a targeted fashion. The APC membrane fulfills the need for localized delivery through its flexibility and slow-release mechanism by acting as a reservoir for a spectrum of therapeutic compounds. Because the porous nanoscale layer of the APC membrane acts as a semi-permeable membrane, it is capable of containing the drug and eluting it slowly as the drug comes into contact with surrounding fluids. Previous studies have also examined the application of parylene toward the reduction of bovine serum albumin (BSA) release rates from nanofiber materials, signifying the applicability of parylene as a packaging material capable of facilitating sustained therapeutic elution.[30,60] As such, the effective dose of the drug can be applied in a very controlled and precise fashion that localizes the drug concentration near the surface of the device. This outcome is ideal for the patient because the drug effect is localized and the effective dose is utilized in its entirety. Conversely, without a porous layer, drug delivery would not be localized and have diminished long-term value for the patient due to immediate exposure and dilution of the drug through the patient's physiological systems. As described before, such a result would lead to potential medical complications.[29,48,50,51,57] The importance of localized drug-delivery is unparalleled in overcoming numerous ailments including cancer, infections, and aggressive immune responses. The enhanced level of specificity and biocompatibility that we have effectively engineered into the APC membrane will serve as a flexible, functionalized platform capable of creating a controlled window where neither infection nor implant rejection occurs while localizing drug activity to enhance treatment potency.

Current biological applications of parylene are primarily focused on improving the biocompatibility of stents, PCR chips, neurocages, microfludic probes, neural probes, sensors, and other micro-devices via simple parylene deposition.[7,16,17,24,30,36,41,49,52,55,60,63] In our work we have demonstrated that a parylene-packaged, nanoscale block copolymer-based drug elution device successfully delivers Dex, resulting in a significant reduction in inflammation around the coated substrate. With respect to biocompatibility, localized delivery, and longevity, we have engineered the APC membrane to address these common challenges of biomaterial fabrication as parylene possesses an extended shelf-life lasting many years due to its enhanced biological stability.[21,22,40] In addition, we have engineered slow-elution capabilities into the membrane using a porous parylene layer coupled with the underlying block copolymer network to optimize the dosage duration. Most importantly, we have combined these properties into one single entity to serve as a flexible platform for drug delivery capable of stand-alone pharmaceutical agent transport.

MATERIALS AND METHODS

Preparation of Substrates

Modification of glass slides was achieved via deposition of parylene C (Specialty Coating Systems (SCS), Indianapolis, IN) and deposition of Langmuir-Blodgett thin films. Using a PDS 2010 Labcoater (SCS), 5 g of parylene C was deposited on glass slides sterilized with ethanol and chloroform under vacuum at room temperature. The PMOXA-PDMS-PMOXA copolymer with acrylate endgroups (Polymer Source, QC, Canada) was dissolved in chloroform to a concentration of 10 mg/mL. Solutions were applied to the surface of the nanopure water subphase (18.2 MΩ cm, Barnstead International, Dubuque, IA) used in the Langmuir-Blodgett trough (KSV 2000 LB System, Helsinki, Finland) to a surface pressure of 18 mN/m and allowed to evaporate over a period of 30 min. Dex (Sigma-Aldrich, St. Louis, MO) was solubilized in ethanol to a concentration of 5 mg/mL then applied drop wise (600 μL) evenly over the surface and allowed to integrate for 5 min. Following Dex/copolymer tethering, the functionalized membrane was compressed to a surface pressure of 25 mN/m at a rate of 1 mm/min and five layers were deposited at a rate of 5 mm/min on the desired substrate. After deposition of the hybrid membranes, 0.03 g of parylene C was deposited on the substrates to complete the APC membrane. Unmodified glass slides and slides coated with 5 g of parylene C were prepared simultaneously and stored in foil-wrapped petri dishes under identical conditions as the APC membrane at 4 °C. UV–vis measurements of APC architecture were performed by applying the glass substrate fabrication protocols toward UV–vis compatible cuvettes with subsequent measurements being taken at the wavelengths specified in the absorbance plots.

Atomic Force Microscopy

Atomic force microscopy (AFM) (Asylum MFP3D, Santa Barbara, CA) was performed on four substrates prepared by the methods described previously. The image dimensions were 10 × 10 μm. Measured substrates varied in the functionalization of the copolymer membrane and thickness of the porous parylene layer.

Fluorescent Confocal Microscopy

Glass slides coated with 5 g of parylene C was functionalized via LB deposition of PMOXA-PDMS-PMOXA copolymer and dexamethasone fluorescein (Invitrogen, Carlsbad, CA) as described previously. In total, 0, 0.03, and 5 g of parylene C were deposited on top of substrates A, B, and C, respectively. The substrates were soaked in 15 mL of PBS (pH 7.4) for 0, 2, 4, 16, and 50 h. Dex fluorescence was captured with a confocal microscope for all time points. The fluorescent substrates were transferred to new sterilized petri dishes with 15 mL of fresh PBS between time points.

Cell Culture

RAW 264.7 (ATCC, Manassas, VA) murine macrophage cells were cultured in 1× DMEM (Cellgro, Herndon, VA) containing 10% FBS (ATCC) and 1% penicillin/streptomycin (Cambrex, East Rutherford, NJ) at 37 °C. After the cell culture reached adequate density, the cells were plated into petri dishes containing one of the substrates—glass slide, parylene slide, APC membrane—and incubated for 24 h at 37 °C. Then, 5 ng/mL LPS were added for the last 4 h of incubation, followed by removal of media and cell harvest.

DNA Fragmentation Assay

Cells were lysed in 500 μL lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% Triton X-100). Thirty-minute incubations at 37 °C followed the treatment with RNase A and proteinase K, separately. Following phenol chloroform extraction, nuclear DNA was isolated in isopropyl alcohol and stored at −80 °C overnight. After a 70% ethanol wash, the samples were resuspended in DEPC water and electrophoresed on 0.8% agarose gel, and stained with ethidium bromide (Shelton Scientific, Shelton, CT).

Quantitative RT-PCR

RNA was purified via cell lysis with 1 mL TRIzol® (invitrogen) followed by extraction with 200 μL chloroform. After centrifuging for 15 min at 14,000 rpm and 4 °C, the supernatant was transferred into isopropyl alcohol for isolation and overnight storage at −80 °C. After washing the RNA samples with 70% ethanol, cDNA was synthesized using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with reverse transcriptase according to the manufacturer's instructions and details described previously. RT-PCR was run with the iCycler thermocycler (Bio-Rad) on 25 μL samples comprised of: DEPC water, SYBR Green Supermix (Bio-Rad), the respective forward and reverse primers, and the cDNA sample dissolved in DEPC water. Amplification conditions were: 95 °C (3 min), 45 cycles of 95 °C (20 s), 55 °C (30 s). The β-actin, mTNF-α, mIL-6, and iNOS primer sets as previously described[39] were purchased from Integrated DNA Technologies (Coralville, IA).

RESULTS AND DISCUSSION

Characterization and Configuration of Parylene-Encapsulated Hybrid Nanofilms

Our initial studies pursued the delivery of an anti-inflammatory agent and thus, the APC membranes were functionalized with Dex. Figure 1 provides a cross-sectional view of the APC membrane that outlines the various layers that are incorporated into the structure. Five grams of parylene C constituted the base layer which was a tangible, pinhole free layer of parylene C coated via vapor deposition with a Specialty Coating Systems labcoater (model PDS 2010). The purpose of a substantial base deposition was to provide support for the hybrid copolymeric nanofilm as a standalone drug delivery device. Parylene C was our selected material given that it is biocompatible and a FDA approved implant device coating.

The next component illustrated in Fig. 1 is the hybrid copolymeric layer, a nanofilm of PMOXA-PDMS-PMOXA triblock copolymer functionalized with Dex. Self-assembly of triblock copolymer occurred on a nanopure water subphase (18.2 MΩ cm) in a Langmuir-Blodgett (LB) trough. The self-assembling property of the triblock copolymer is inherent in its alternating hydrophilic-hydrophobic-hydrophilic blocks. In addition, although the arrangement of triblock copolymer resembles that of lipid based membranes, the copolymer possesses superior stability, durability, and porosity making it a suitable material for device fabrication purposes.[35] The PMOXA-PDMS-PMOXA film was functionalized with solubilized 5 mg/mL Dex via dropwise deposition of Dex into the triblock copolymer monolayer, which secured the Dex molecules in a copolymer network. Dex integration was confirmed by monitoring changes in surface pressure following the

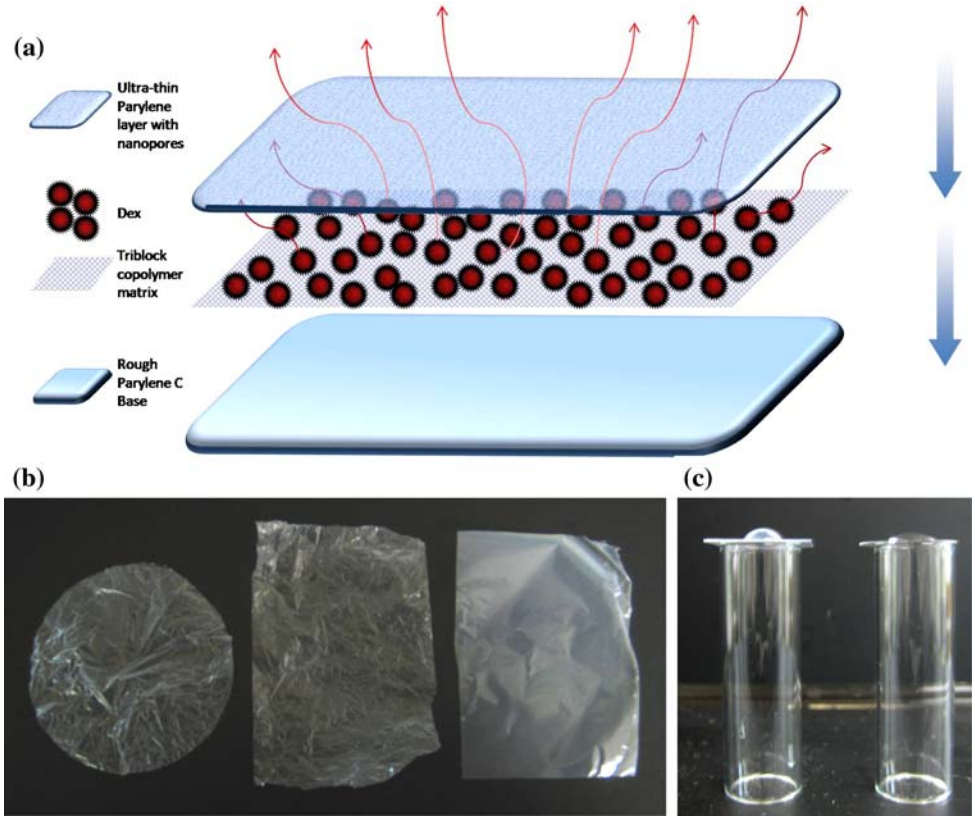

FIGURE 1. Constitutional cross-section view of the APC membrane. (a) The base layer is a layer of parylene that serves as the backbone of the membrane. The top layer is a layer of ultra-thin porous parylene that acts as a semi-permeable membrane. Sandwiched between the parylene layers is a copolymer matrix that can be rapidly functionalized with molecules. The drug used in our studies was dexamethasone (Dex), which was incorporated into the copolymer matrix and deposited onto the parylene base layer with a Langmuir-Blodgett trough. The figure shows drug eluting through the porous layer by diffusing across the membrane. Multiple layers of the Dex/copolymer hybrid membrane may be applied at the cost of only a few nanometers to extend the vvbio-longevity of the membrane. (b) Parylene-based drug eluting APC devices are shown here. Device dimensions/shapes can be easily controlled for versatile functionality. (c) The deposition of the hydrophobic parylene material is shown by a clear increase in the surface contact angle of water.

addition of Dex. Following a 30 min period of chloroform evaporation after the addition of triblock copolymer, the surface pressure was stabilized at ~18 mN/m. The drop-wise addition of 600 μL of Dex increased the surface pressure to ~20 mN/m, which later dropped and stabilized to ~19.5 mN/m after 5 min. Therefore, Dex conjugation to the copolymer established a surface pressure increase of ~1.5 mN/m. The triblock copolymer is a vital component of the APC membrane due to its ability to form a porous network which serves as both a drug reservoir/controlled release mechanism as well as a methodology for controlled drug functionalization of the device. In the absence of copolymer during LB deposition of Dex, isotherms have revealed that the drug molecules are completely assimilated into the subphase, and ultimately failed to be deposited on the substrate.[10] Five layers of the copolymer-Dex film were deposited for the *in vitro* elution studies.

The final element of the APC membrane was the deposition of 0.03 g of parylene C producing an ultra-thin porous layer. Studies on the vapor deposition/polymerization process of parylene indicate that individual monomer molecules are deposited on a substrate where reactions occur to form extended polymer chains.[6,59] Furthermore, the chains may interlink forming a web or mesh network akin to the copolymer effectively trapping the hybrid copolymer-Dex nanofilm between two layers of parylene C. A key distinction is that the porous thin parylene matrix enables the diffusion of Dex across the parylene membrane. Furthermore, the porous layer of parylene shields the copolymer from proteins that may be adsorbed into the copolymer which would be particularly useful for in vivo applications as protein adsorption could lead to cell recruitment and eventual biofouling of the device.[13] In concert with the functionalized copolymeric network, the porous parylene nanoscale layer allowed the Dex to be slowly eluted from the membrane, which was confirmed via confocal microscopy (described in proceeding sections). Figure 1b represents an image of a parylene-based APC device. Device dimensions could be easily controlled for a versatile range of applications and specific configurations. Parylene deposition during APC fabrication was further demonstrated through increases in the contact angle of water due to the hydrophobicity of parylene.

Examination of APC Fabrication via UV–vis and AFM

Figure 2a represents UV–vis spectrophotometry studies to confirm the deposition and presence of an additional layer (0.03 g) on top of the Parylene-C (PC) base layer (5 g) as shown by the increased optical density readings from cuvettes that contained deposited PC. Conditions included a layer of only 0.03 g PC, only 5 g PC, and the APC-based 5 g PC + 0.03 g PC. As such, the demonstration of an increased APC optical density reading over those of the base layer (5 g) and porous layer (0.03 g) indicated the presence of a multilayer PC architecture to serve as the APC device. Additionally, AFM images of four substrates were taken to investigate inherent surface characteristics of parylene and the effects of complex layering on surface properties. The four substrates used were varied in the grams of parylene deposited as the final layer (0.03 or 0.05 g) and the presence of a hybrid copolymer-Dex nanofilm. Figure 2 also compares the AFM images of the substrates and reveals the rough surface of parylene.[6] The vapor deposition/polymerization of parylene was shown to be a dynamic process that involves the constant adsorption-desorption of monomer molecules to achieve equilibrium between the molecules in the gas phase and those deposited on the substrate surface.[59] This unique deposition mechanism may be responsible for the general roughness of the polymer topography.[35] In addition, research has shown that fluctuations of a few mTorr during vapor deposition of parylene can induce formation of nanoscale aggregates of parylene resulting in overall roughness across the surface of the membrane.[20] Furthermore, comparing the AFM images and section analyses of 0.03 g parylene with Dex to 0.03 g parylene without Dex, a clear difference in the topography can be seen, suggesting the presence of the copolymer-Dex membrane; the same observation may be noted from the comparison between the 0.05 g parylene with and without Dex (Figs. 2b and 2c). The AFM images imply that Dex/Copolymer hybrid was successfully integrated onto the parylene surface via LB deposition, but further confirmation was achieved through confocal studies. Most importantly, though section analyses of Fig. 2 images indicate the nanoscale aggregates that range from 50 to 500 nm in height that produced a jagged landscape, the presence of copolymer-Dex under the thin parylene film reduced the frequency and elevation of observed serrations. Furthermore, direct imagery of pore formation, in accordance with previous studies, was challenged by the pervasive presence of nanoscale surface architectures (e.g. indentations, etc.) and as such, the porosity of the top parylene packaging layer was assessed using confocal microscopy-based imagery of drug transport through the parylene as a function of layer thickness (Figs. 2b and 2c).[6] In addition, the application of the APC-defined thickness of parylene as the porous layer on top of the Dex-copolymer hybrid showed a significant reduction in drug elution rate compared with the unpackaged/covered Dex-copolymer hybrids alone, signifying both the presence of a parylene layer as well as its ability to facilitate drug transport. A comparison

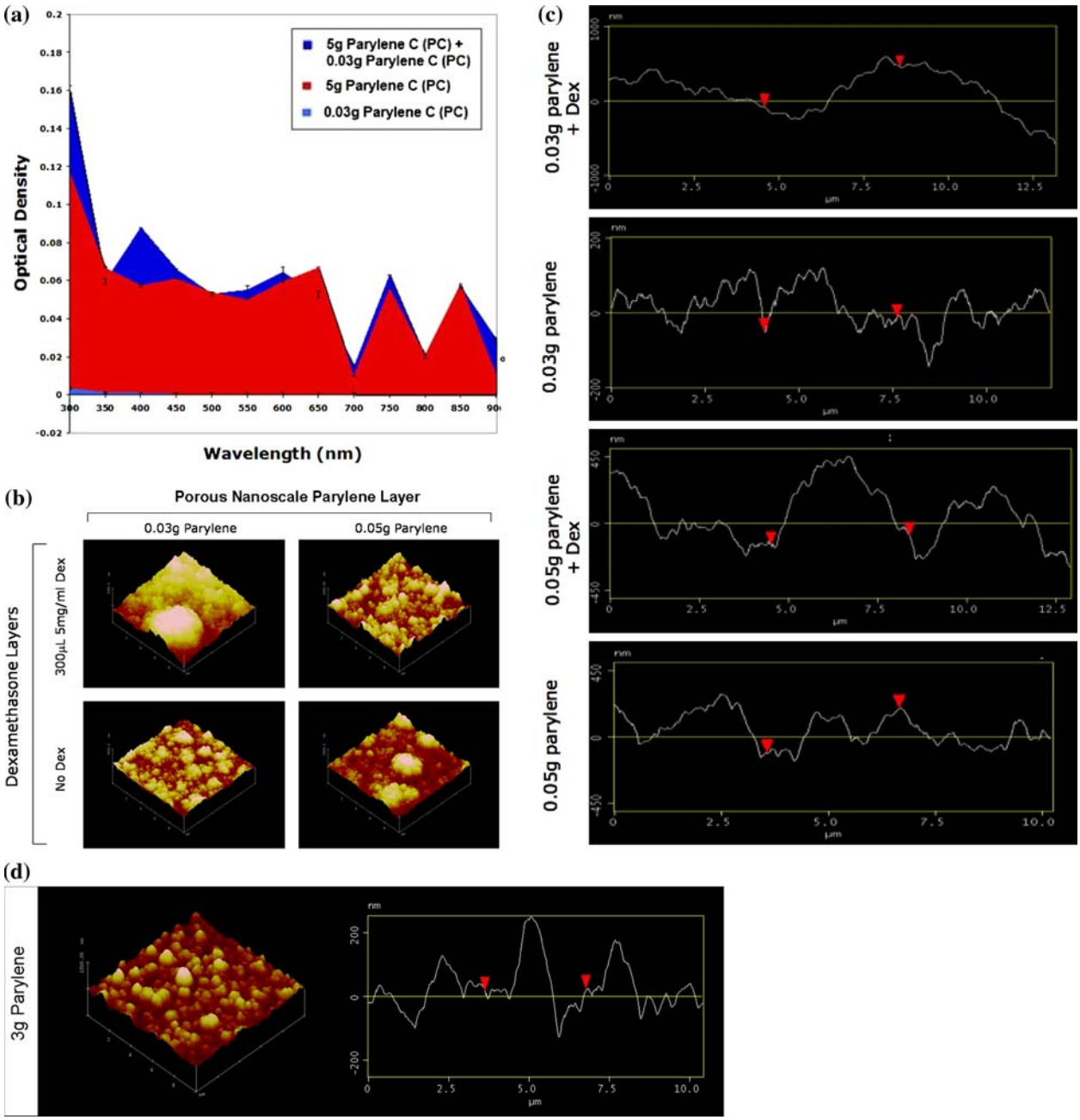

FIGURE 2. UV–vis analysis of APC architecture and AFM images of APC membrane surfaces (top) and parylene only surfaces (bottom) at two different porous layer thicknesses. (a) UV–vis spectrophotometry revealed the deposition and presence of an additional layer (0.03 g) on top of the Parylene-C (PC) base layer (5 g) as shown by the increased optical density readings from cuvettes that contained deposited PC. Conditions included a layer of only 0.03 g PC, only 5 g PC, and the APC-based 5 g PC + 0.03 g PC. (b) Depressions are shown on the surface of the membranes as darker areas suggesting possible pore formation. Differences in surface terrain suggest influence on terrain texture by the triblock/Dex layer, which is absent in the bottom row of images. The geography of parylene exhibits rough terrain. (c) Comparative section analyses of the substrate surfaces reveal diminished terrain roughness on substrates possessing a copolymer-Dex nanofilm. (d) Section analysis of 3 g of parylene without copolymer-Dex and porous parylene.

of section analyses of Figs. 2b–d illustrates the effect that the integrated copolymer-Dex layer has on the topography of the substrate surfaces (Fig. 2d). Because the AFM measurements were taken from arbitrary points on the substrate surfaces, the absolute thicknesses exhibited variation between samples. However, across all samples, the AFM measurements demonstrated a decrease in topographical variance following the

incorporation of the copolymer-Dex nanofilm, which may result in a more biomimetic material based on its less abrasive physical surface structure in addition to its drug-eluting capabilities.

Confocal Studies of Dex Slow-Elution

Fluorescent imaging via confocal microscopy was performed to provide distinct evidence of Dex slow-elution from the APC membrane (Fig. 3). The substrates were prepared to confirm that elution properties differed based on the thickness of the top layer of parylene that was deposited over 5 layers of copolymer/Dex-fluorescein and a 5 g base parylene layer for all three substrates. Substrates A, B, and C possessed 0 g (none), 0.03 g (APC), and 5 g (thick) of parylene deposited as the top layer, respectively. All substrates were soaked in 15 mL of PBS (pH 7.4) for the respective durations of time. In addition, all substrates were transferred to new petri dishes with new PBS between time points to promote elution by preventing the solution from reaching equilibrium. The images confirm the elution of Dex from substrates A and B, but not C. The lack of elution from substrate C was due to the 5 g pinhole-free top layer of parylene that produced a substrate completely encapsulated in parylene. However, at very low quantities of deposition, parylene forms pinholes as a result of interlinking between chains of parylene, of which substrate B also serves as evidence.[59] Substrate B had 0.03 g of parylene deposited as a top layer to mimic the properties of a semi-permeable membrane. At 2 h, we saw the elution of Dex from substrate B starting to occur (Fig. 3). After transferring the APC membrane into new PBS

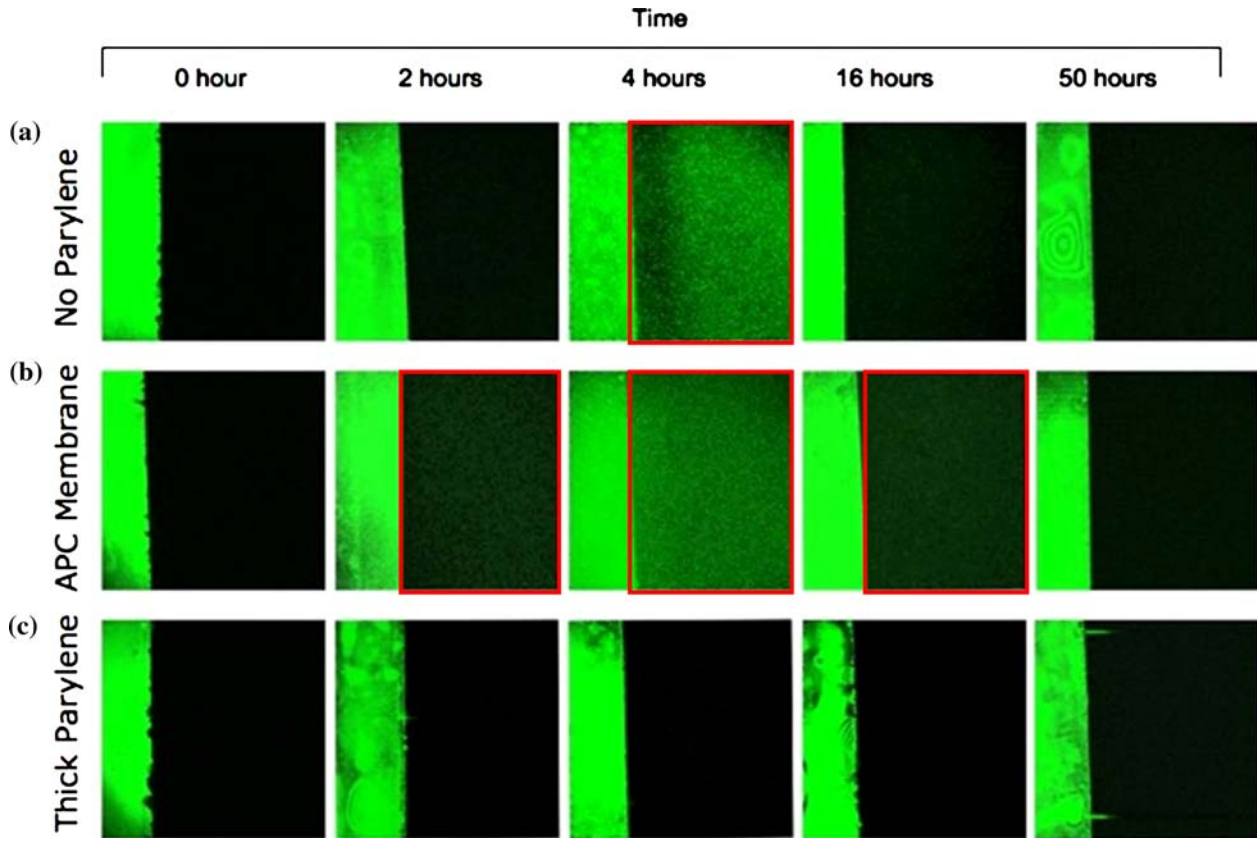

FIGURE 3. Confocal images of fluorescent Dex confirms slow-elution capabilities. The substrates prepared to confirm elution of Dex differed in the thickness of the top layer of parylene that was deposited over 5 layers of triblock/Dex and a base parylene layer for all three substrates. Substrates A, B, and C have 0, 0.03, and 5 g of parylene deposited as the top layer, respectively. All substrates were soaked in 15 mL of PBS (pH 7.4) for the respective durations of time; all substrates were transferred to new petri dishes with new PBS between time points to promote elution by preventing the solution from reaching equilibrium. The images confirm the elution of Dex from substrates A and B, but not C, due to the thick parylene films being pinhole free. However, at very low quantities of deposition, parylene has been shown to become semi-porous, of which substrate B serves as evidence. These pinholes act as a semi-permeable membrane that gives the APC membrane its capability of slow-elution. Compared to substrate A, substrate B eluted less Dex after two PBS washes at the 4 h time point. This observation suggests that the thin porous parylene layer allowed for longer preservation of Dex in substrate B than substrate A. In addition to the capability to slow-elute Dex, substrate B also exhibits automatic dosage tapering. All samples were taken with identical imaging conditions to preserve consistent comparison parameters. Red squares denote observed fluorescent Dex elution.

and 4 h of soaking, the image displayed more elution most likely due to the prolonged soaking time. The same phenomenon was observed at 4 h for the uncoated substrate A. After another transfer of substrates A, B, and C followed by soaking for 16 h, images taken at 16 h indicated no elution from substrate A suggesting that a major portion of the Dex had been washed off from the 4 h soak; substrate B still exhibited significant Dex elution. Since the layers of copolymer-Dex nanofilm were constant for all samples, the images strongly infer that substrate B demonstrated the sustained elution of Dex. The effect may be attributed to the only differential between substrate A and B which was the porous parylene nanoscale layer, an effect that was previously observed with parylene-coated nanofibers.[60] Confocal studies of Dex release (Fig. 3) suggest that the porous parylene nanoscale layer allowed for longer preservation of Dex in substrate B than substrate A, generating the capability to slow-elute Dex, and thus, automatically taper drug dosage.

Interaction of RAW 264.7 Macrophages with the APC Membrane

Tissue culture (Fig. 4) provides qualitative input on the effects that growth on glass, parylene, and APC membrane substrates have on cell development and morphology. Macrophages were plated to approximately 25% confluency on substrates with etchings to

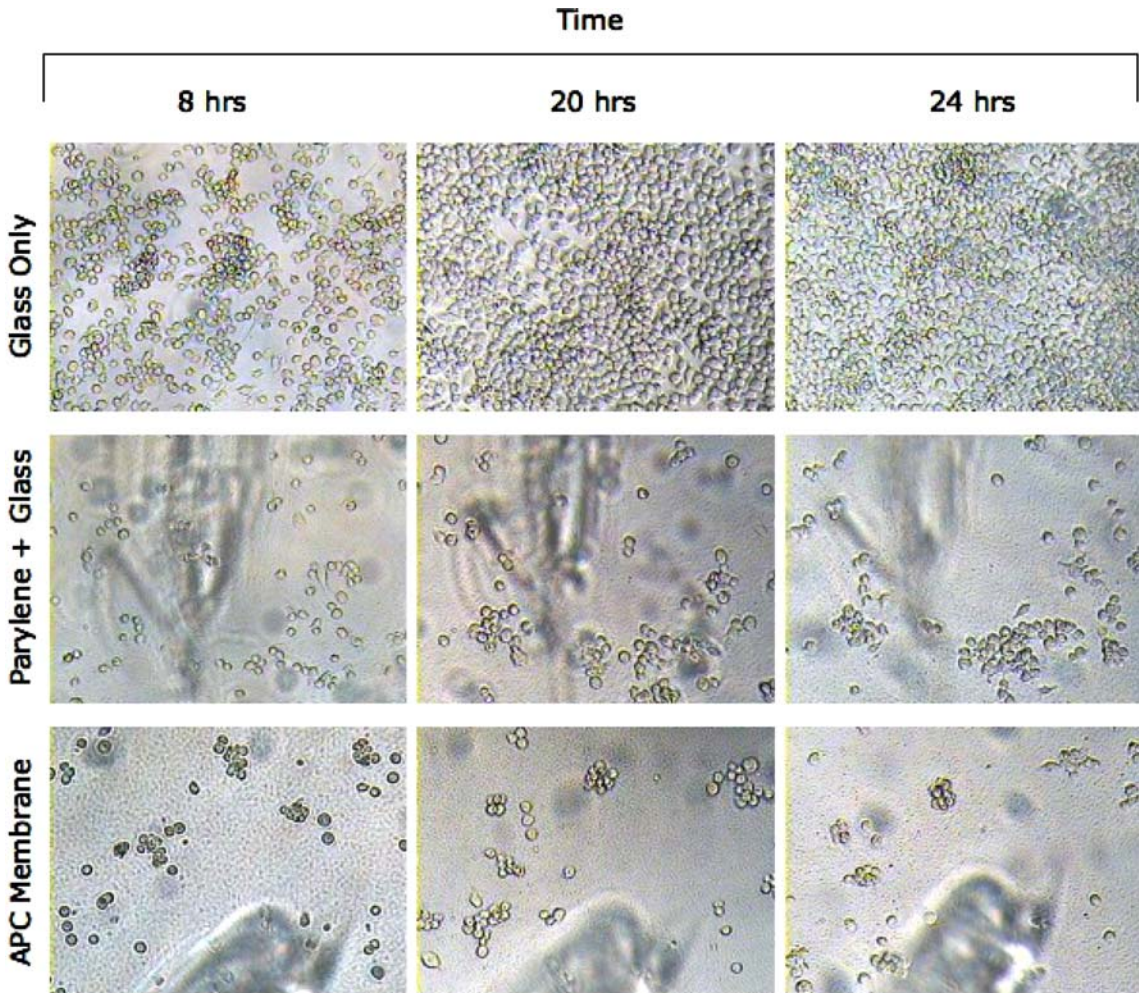

FIGURE 4. Incubation of RAW 264.7 macrophages on glass, parylene, and APC membrane surfaces display differences in growth patterns. LPS stimulation occurred for 4 h beginning after 20 h of growth. Images were taken at 8, 20, and 24 h. Qualitatively, cell growth was less confluent on the parylene and APC surfaces compared to glass. This observation suggests that growth and activity of macrophages are inhibited on the parylene and APC surfaces. Closer inspection reveals that growth was almost completely halted on the APC membrane, while macrophages continued to grow on the parylene surface. Figure 3 shows that parylene and the APC membrane have comparable cell adhesion properties; RT-PCR results reveal contrasting levels of inflammatory cytokines expressed. Comparative expression of cytokines and biomaterial properties as well as DNA fragmentation analysis infers that gene expression was not the result of apoptotic events.

provide accurate representations of change in growth over time. The etchings provided a consistent frame for bright field imaging, and were engraved on the opposing face of the slide to prevent effects on macrophage growth patterns. The images of RAW 264.7 murine macrophage growth on glass, parylene, and APC membrane were taken over three time points. The macrophages were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin for 24 h on their respective surfaces to reach the desired cell confluence on the various substrates; the plain glass slide served as the control. At the 8 h time point (Fig. 4), macrophage growth on the plain glass was about 60–70% confluent with minimal visible clumping. Contrasting growth on glass with growth on parylene and the APC membrane, we observed stunted cell development on the latter two substrates. This observation shows that cells do not readily adhere to parylene and the APC membrane. This could be a favorable property for an implantable drug delivery device because diminished protein adsorption and cell adhesion are less likely to foul the device and interfere with the release process. Images were taken again at 20 h immediately after a 5 ng/mL lipopolysaccharide solution (LPS) was added to each sample to induce inflammation in the macrophages. At 20 h, despite LPS induced inflammation, we saw that cell growth was still virtually halted on the APC membrane and parylene surfaces. Macrophages continued to multiply on plain glass reaching 90–95% confluency by 20 h of growth. LPS stimulation occurred for 4 h and images were taken at the third time point (24 h). Macrophages grown on plain glass were 100% confluent. Growth on parylene increased slightly over these 4 h with LPS stimulation, and there was no visible change in macrophage development on the APC membrane. As previously mentioned, a key distinction of the APC membrane is that it prevents protein adsorption, cell adhesion, and a further benefit of this property is that it is not achieved through material-dependent apoptosis.[3,13] Additionally, LPS stimulation has been shown to increase macrophage adhesion remarkably, but on the APC membrane and parylene this was not the case.[31] Furthermore, comparative analysis of RT-PCR results also showed that apoptosis was not being caused by the parylene surface in that despite less confluent cell growth on parylene, the macrophages cultured on parylene coated slides released a comparable level of cytokines than those grown on plain glass (Figs. 5a–c). Moreover, cytokine expression of cells cultured on the APC membrane often decreased to a fraction of the cytokines released by macrophages on parylene alone. The differences we observed may be attributed to the only differentiating factor between the substrates which was the hybrid copolymer/Dex film, and apoptotic events would not account for these patterns of adhesion and inflammation that are apparent in Figs. 4 and 5. A DNA fragmentation assay also demonstrated that no signs of apoptotic behavior resulted from macrophage growth on parylene or APC when compared to glass (Fig. 6). Therefore, from these data we may infer that the inhibitory effect on macrophage adhesion by parylene C is characteristic of the properties inherent in the biomaterial, rather than apoptosis.

In Vitro Assessment of APC Biofunctionality via Gene Expression

Figure 5 illustrates data from RT-PCR gene expression studies done on the macrophages cultured on plain glass, parylene, and APC membrane. The macrophages were incubated on the substrates for 24 h. After 20 h of incubation, stimulation with 5 ng/mL LPS occurred for 4 h and macrophages were harvested after stimulation. RNA isolation was achieved through cell lysis with TRIzol reagent washes followed by chloroform extraction, centrifugation, and isopropyl alcohol precipitation. cDNA was synthesized with 5× buffer, Oligo DT primer, reverse transcriptase, and the purified RNA samples (iScript cDNA Synthesis Kit). After a water bath at 37 °C for 90 min, the cDNA samples were mixed with SYBR Green Supermix, DEPC water, and the respective primers for RT-PCR analysis. Three cytokines that control inflammation were investigated based on their role in implant degradation[19,34] and activation of nuclear factor (NF)-κB, a key regulator in the immune response of macrophages: TNF-α, IL-6 and iNOS.[25] β-Actin was used to normalize the relative quantities of genes expressed. To attenuate an inflammatory response, we integrated a synthetic glucocorticoid (Dex) into the APC membrane; glucocorticoids are the most potent agents for regulating the immune response and inflammation via binding to glucocorticoid receptors (GR) that mediate expression of genes controlling the immune response.[12,14,53] Specifically, Dex has been shown to reduce expression of IL-6,[1] TNF-α,[32] and iNOS.[15,27,44] Relative IL-6 and TNF-α expression decreased from macrophages grown on the unsoaked APC membrane indicating that an initial release of Dex was occurring (Fig. 5a). The relative iNOS expression showed a modest decrease compared to the parylene-only substrate in the unsoaked devices. The IL-6 expression levels in Fig. 5a clearly showed that the APC membrane decreased inflammatory gene expression up to 2.5 times compared to plain glass and parylene surfaces.

Figure 5b illustrates the effects of slow-elution through RT-PCR gene expression data. The samples represented in Figs. 5b and 5c were prepared

differently from the samples in Fig. 5a. To test slow-elution capabilities, the APC membrane was soaked in a petri dish with DMEM culture media (containing 10% FBS and 1% Pen/Strep) for 1 day prior to tissue culture (Fig. 5b). The plain glass and parylene samples were also soaked for 1 day to maintain uniformity in experimentation. After 1 day of soaking in media, macrophages were cultured on the samples. Soaking allowed the Dex to elute from the APC membrane into the culture media. Figure 5b shows that the APC was

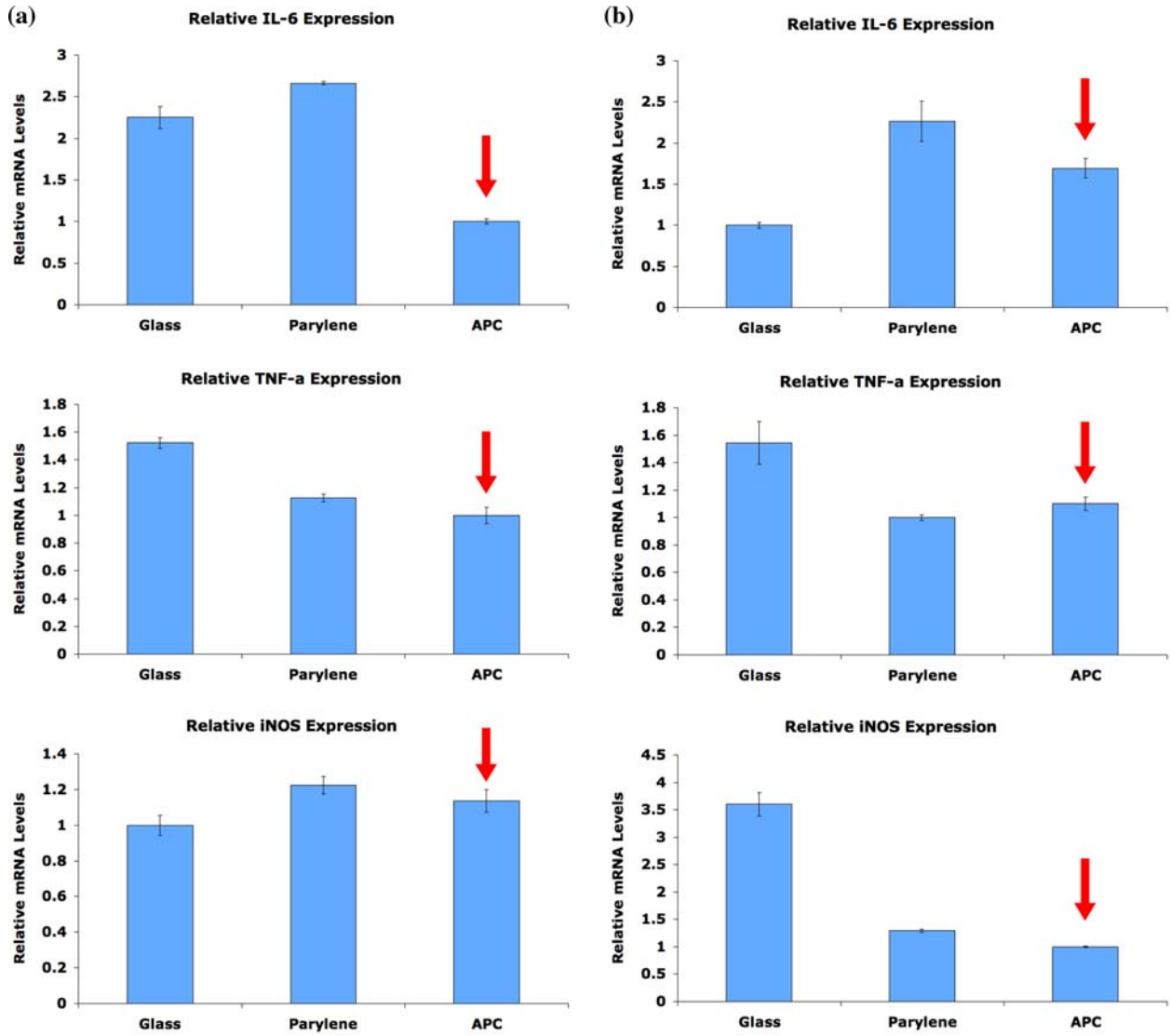

FIGURE 5. Effects of Dex elution from the APC membrane on inhibition of LPS induced inflammatory gene expression in RAW 264.7 macrophages. (a) For culture on unsoaked substrates, a slow initial drug release was observed with attenuated inflammatory gene expression. The expression of IL-6 was *at least* relatively two times higher for the parylene and glass slides compared to an APC membrane. These data show that Dex can successfully survive vapor deposition and soaking, and still be an effective inflammatory cytokine inhibitor. The same pattern of attenuated but apparent suppression was also observed in relative TNF-α expression levels. Suppression of iNOS expression was slightly lower in the APC devices compared to the parylene only substrates, though continued slow elution significantly increased the potency of inflammatory gene expression. (b) Continued ultra-slow slow release was also observed for the 1 day soak parameter. Allowing the substrates and APC membrane to soak for 1 day prior to macrophage culture allowed more of the drug to elute, possibly resulting in a more prominent reduction in relative mRNA levels of iNOS. Slow-release-mediated attenuated suppression was observed for all 3 genes which was a precursor to the highly potent suppression observed with a 3 day soak. (c) For the 3 day soak, multiple conditions were examined. Copolymer-Dex is a functionalized slide with a parylene base and layers of Dex incorporated into a triblock copolymer matrix that lacks the porous parylene top layer. The gene expression of IL-6 is higher for copolymer-Dex compared to the APC membrane that has the porous layer engineered for slow elution capabilities. These data suggest that there is an advantage to slow elution owing to localization of drug elution to near the surface of the membrane. The APC membrane inhibits inflammatory cytokines, up to 18 times more effectively for IL-6 than does plain parylene on glass. One representative experiment of three is shown for all conditions a–c.

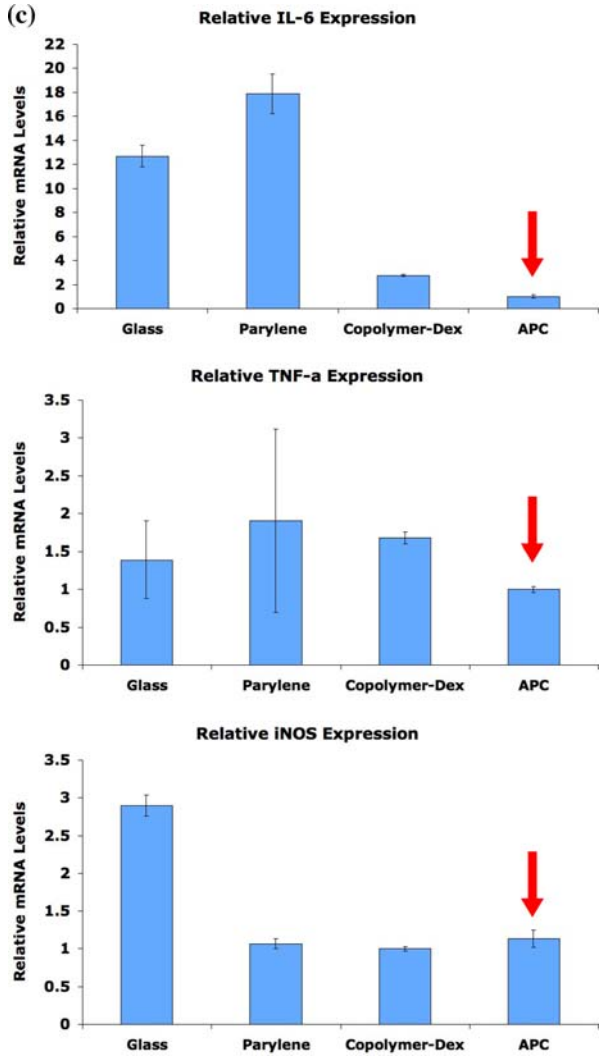

FIGURE 5. Continued.

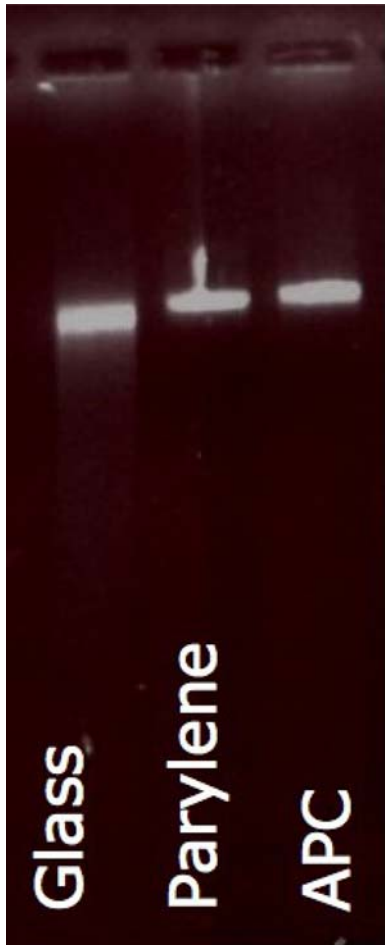

FIGURE 6. DNA fragmentation assay reveals no apoptotic behavior in macrophages cultured on parylene or APC membrane. No significant DNA fragmenting was observed in macrophages cultured on parylene or APC membrane. Decreases in cytokine expression from cells grown on APC membrane can be attributed to drug-elution from the APC membrane and not APC-induced cell death.

capable of generating a tempered release of the therapeutic which still generated significant decreases in relative mRNA levels of iNOS expressed, suggesting that a reduced amount of drug had eluted from the unsoaked membrane to decrease levels of iNOS significantly, therefore demonstrating a sustained release effect (Fig. 5a). In addition, levels of IL-6 increased on the APC and parylene when compared to glass though continued decreases occurred due to slow elution effects though longer term slow release studies generated a more potent response. We saw that drug elution was still in effect, since the IL-6 and TNF-α expression on APC was lower than the relative cytokine expression on parylene and glass, respectively (Fig. 5b). Furthermore, it is possible that the slow release effect induced by the parylene-based sandwich architecture and thus the resultant drug eluted from the 1-day soak was not initially potent enough to either maintain or elicit the continued decrease in IL-6 and TNF-α expression. Moreover, the concentration of Dex functionalized into the membrane may have affected regulation of select cytokines via chronic exposure[37,43,46]; the optimal threshold of transrepression via Dex for each cytokine has yet to be determined. Studies into the standardization and optimization of drug integration will provide solutions and insight into these effects. However, slow release and the observed subsequent gradual increased potency of inflammatory cytokine expression served as an indicator of sustained elution mediated by the parylene-packaged triblock copolymers.

The samples presented in Fig. 5c were prepared to test slow-elution capabilities similar to the substrates soaked for 1 day of Fig. 5b. However, in this case, the APC membrane was soaked for 3 days prior to tissue culture. Again, the plain glass and parylene samples

were also soaked for 3 days to maintain uniformity in experimentation. After 3 days of soaking in media, macrophages were cultured on the samples. An extended 3-day soak was implemented to examine the scope of Dex elution and any potential effects on drug potency due to prolonged elution and exposure to culture media under conditions of incubation. Figure 5c shows that the relative IL-6 expression was *approximately 18 times less* for the macrophages cultured on APC membrane than cells on parylene, *and 13 times less* than macrophages grown on plain glass which demonstrated a significant decrease in inflammatory gene expression and a potent long term drug release functionality. These data suggest that a 3-day soak allowed the Dex to slowly diffuse through the porous top layer, thus eluting more Dex into the media for interaction with macrophages. The samples presented in Fig. 5a were not soaked in media prior to tissue culture. Therefore, the macrophages harvested for Fig. 5a data were exposed to a decreased amount of drug because the Dex had less time to elute into the media due to limited exposure to media for drug diffusion. The consequential difference between soaking and not soaking the membrane may be seen in relative gene expression graphs. The relative IL-6 expression of macrophages cultured on unsoaked membrane was over 2.5 times less than IL-6 expression of macrophages grown on parylene (Fig. 5a), but the soaked membrane resulted in over 18 times less IL-6 expression of macrophages grown on parylene (Fig. 5c). Therefore, through RT-PCR data we saw there was a clear advantage in soaking the membrane in our *in vitro* studies, which translates to effective and prolonged bio-longevity of the biomedical device coated with the porous APC membrane in a patient. In addition, a comparison between Figs. 5b and 5c suggest that allowing for prolonged periods of diffusion is advantageous. In a clinical setting, this effect may result in providing naturally tapered dosages. Figure 5c also compares the APC membrane to a functionalized copolymer membrane with no porous layer engineered for slow elution. These data show that slow-elution is advantageous because it keeps the Dex that is eluted localized near the surface of the membrane where most of the effective dose can interact with macrophages to decrease inflammation. Even in a petri dish, we can see the inefficiency of uncontrolled drug delivery, where localized drug delivery through slow-elution is nearly 3 times more effective in reducing inflammation when compared to the functionalized copolymer membrane that elutes all of the drug at once. This effect could potentially be more pronounced in a clinical environment where the strictly defined boundaries of a petri dish are removed because the drug will have more area to diffuse through, thereby causing the effective dose to be more diluted ultimately resulting in diminished therapeutic value.[18]

Further experiments designed to test drug viability were carried out by performing a 5-day soak. The experimental conditions were identical to the 1-day and 3-day soaks, but soaking was extended to 5 days. In addition, to test the membrane's ability not only to deliver Dex, but also to act as a drug reservoir, the substrates were transferred into new petri dishes containing fresh media after 3 days of soaking. The transfer effectively removed any Dex that was already eluted from the membrane, and any effects on cytokine release to be seen would be due to the release of the drug that was preserved in the membrane. We observed that the suppression of IL-6 expression by macrophages was sustained by nearly 6 times compared to parylene alone, a considerable decrease; additionally, the mRNA levels of iNOS were also abated (data not shown). Despite the substrate transfer that removed all Dex that was eluted from the membrane over 72 h, the APC membrane was able to maintain its activity in decreasing the expression of inflammatory cytokines from the macrophages while DNA fragmentation assays confirmed that the decreased expression was not due to material-induced cell death (Fig. 6). This demonstrates the ability for the membrane to perform drug sequestering and packaging operations. In addition, the transfer scenario may provide strong support for the successful integration of the APC membrane *in vivo* by simulating circumstances analogous to flushing that occurs via natural processes in an animal model. Furthermore, the absence of device-generated cytotoxicity as shown through RT-PCR and DNA fragmentation assays provided supportive biocompatibility evidence toward systemic translation. Furthermore, we believe that drug concentration plays an important role in determining the level of suppression of individual cytokines, and the optimum level of drug integration is a continuing study in multiple fields. These data demonstrate that the APC membrane was able to contain the residual drug preserved in the copolymer matrix, which allowed for slow drug elution via the copolymer-Dex film and porous parylene over varying time points and conditions.

CONCLUDING REMARKS

The parylene-copolymer based localized drug release technology that we have developed addresses the issue of cellular-device interfacing by utilizing the copolymer films to form a network for both drug containment as well as a biologically inert surface for maintaining favorable cell interactions with the device surface.

This was accomplished via the integration of Dex, an anti-inflammatory agent, into a PMOXA-PDMS-PMOXA triblock copolymer monolayer at the air-water interface of a Langmuir-Blodgett trough. The copolymer-Dex hybrid membrane served as a drug containment network encapsulated in parylene C, an FDA approved material for implantation that served both as a backbone and semi-permeable film for the device. Following UV–vis spectrophotometric confirmation of the APC architecture, and after 24 h of growth on the substrate and LPS stimulation during the last 4 h of incubation, RT-PCR was performed to examine the inflammatory response of RAW264.7 macrophages cultured on glass, parylene and the APC membrane. The data showed decreases in inflammation based on relative levels of cytokines released by macrophages including IL-6, TNF-α, and iNOS. The slow-elution capability of the membrane was confirmed via confocal microscopy showing elution of fluorescent Dex from the APC membrane, which was sustained despite several PBS washes. In addition, bright field microscopy revealed the anti-adsorptive properties of parylene and the membrane that are vital to prevention of biofouling of implant devices. These observations may serve as design principles for emerging implant coatings and drug eluting devices that may prolong device functionality by minimizing adverse cellular reactions. The membrane may also serve as a potential standalone drug delivery device for diverse applications including growth factor transport and targeted chemotherapy. Further engineering of the membrane surface will enable optimization of drug integration and standardized rates of elution. Our studies indicate that the APC membrane is a versatile material capable of delivering Dex in a controlled fashion to maximize drug efficacy, making it a suitable platform for chemotherapeutics, steroid treatments, and nanomedicine in the future. Furthermore, the biocompatibility of parylene and copolymer materials we have incorporated into the functionalized nanofilm makes the APC membrane a biomimetic slow-eluting device and therefore, a promising candidate to forge the next generation of localized therapeutic modalities.

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