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Characterization of Molecular Transport in Ultrathin Hydrogel Coatings for Cellular Immunoprotection

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Abstract

PEG hydrogels are routinely used in immunoprotection applications to hide foreign cells from a host immune system. Size dependent transport is typically exploited in these systems to prevent access by macromolecular elements of the immune system while allowing the transport of low molecular weight nutrients. This work studies a nanoscale hydrogel coating for improved transport of beneficial low molecular weight materials across thicker hydrogel coatings while completely blocking transport of undesired larger molecular weight materials. Coatings composed of PEG diacrylate of molecular weight 575 Da and 3500 Da were studied by tracking the transport of fluorescently-labeled dextrans across the coatings. The molecular weight of dextran at which the transport is blocked by these coatings are consistent with cutoff values in analogous bulk PEG materials. Additionally, the diffusion constants of 4 kDa dextrans across PEG 575 coatings ($9.5 \times 10^{-10} - 2.0 \times 10^{-9} \text{ cm}^2/\text{s}$) was lower than across PEG 3500 coatings ($5.9 - 9.8 \times 10^{-9} \text{ cm}^2/\text{s}$), and these trends and magnitudes agree with bulk scale models. Overall, these nanoscale thin PEG diacrylate films offer the same size selective transport behavior of bulk PEG diacrylate materials, while the lower thickness translates directly to increased flux of beneficial low molecular weight materials.

Introduction

Synthetic hydrogels have long been studied for the protection of biological species in vivo.^{1,2} Hydrogel architectures have been designed for use in the body for applications ranging from drug delivery particles³ and scaffolds⁴ to large tissue grafts for implantation.⁵ For cell replacement therapies, exogenous cells have been encapsulated in hydrogels to physically prevent the host's antibodies from recognizing the foreign cells while allowing the free transport of water and nutrients throughout the porous mesh.^{6,7} This strikes a careful

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Supporting Information Available

Additional fluorescent and bright field images of PEGDA surface coated Jurkats and fluorescent images of polymer based amplification immunostaining of vimentin and nuclear pore complex of human dermal fibroblasts. Expanded confocal Raman microscopy spectra of polymer and cell samples. Fluorescent image analysis and profilometry data of polymer micro arrays samples. Diffusion model fit at bounds of thicknesses range estimates and associated diffusion constant calculations. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

balance between the transport of the unwanted high molecular weight materials and the beneficial low molecular weight materials. The size selectivity of the hydrogel is a property of the polymer microstructure, where large molecular weight materials do not penetrate appreciably into the hydrogel. Often, these hydrogels are typically formed by bulk polymer methods, where unnecessary thickness imposes additional diffusion lengths for nutrient transport and the release of the desired cellular products.

Some groups have acknowledged the diffusional advantage of thin films and have formed polymer microcapsules around individual cells.⁸ Polymerizing conformal coatings directly around cells has several key advantages for cell transplantation therapies. Thin coatings offer minimal barrier to mass transport of nutrients and small molecules into and out of the cell, increase the surface area for mass flux around each cell, and maximize the effective cell density delivered to the body.^{8,9} For diabetes therapy, insulin producing porcine islets have been encapsulated by soaking the cells in a visible light photoinitiator, and then photopolymerizing poly(ethylene glycol) diacrylate (PEGDA) coatings on the order of tens of microns that allow insulin elution out of each capsule while acting as a barrier to immunorejection.^{10,11}

We seek to encapsulate mammalian cells in suspension by the photopolymerization of ultra-thin hydrogel films to improve the transport of low molecular weight species. Our approach is based on surface mediated polymerization of nanoscale films (Figure 1), where photoinitiator is grafted to the outside of a cell in extremely low concentrations (~ 1 molecule/ μm^2).¹² In these conditions, the photoinitiator content is reaction limiting, and the subsequent growth of polymer is restricted to a thin film at the cell surface.¹³ Upon introduction of a monomer precursor solution containing triethanolamine (coinitiator),^{14,15} the initiator-primed cells are exposed to green light, and a free-radical polymerization process results. We expect this technique will be capable of completely coating cells for size selective control of materials to the cell surface. Prior calculations on 100 μm and 1 mm thick analogues of these materials describe a mesh size of 2–5 nm which prohibits IgG diffusion, yet readily permits diffusion of low MW materials.^{17,18} Critically, the diffusion properties of these ultra-thin films on cells have yet to be measured.

This manuscript directly studies the chemical, physical, and transport properties of nanoscale hydrogel films on a cellular surface. For experimental simplicity, we examined coatings on the surface of Jurkat cells, an immortalized, robust, and easily cultured T lymphoma cell line. We evaluated the viability of these cells after the formation of coatings to support application of these coatings on islets for cell based therapies. The chemistry of the coatings was directly analyzed with Raman microscopy showing shifts consistent with the localization of PEG coatings on the cell's surface. The coatings were imaged by scanning electron microscopy, supporting a surface morphology distinct from native cells and consistent with control samples of surface-initiated polymer thin films.

While structure and chemical characterizations provide valuable insight to the film properties, the most definitive measure of an encapsulation technique for cell transplantation is the capability to exclude large biomolecules from penetrating the coating while allowing the free transport of low molecular weight nutrients. Extrapolation of transport properties of

a bulk hydrogel to thin film geometries is commonplace, yet fundamentally assumes identical and uniform cross-linking in the film.¹⁶ For the surface-mediated, free radical polymerizations employed here, a gradient of double bond conversion arises during the photoreaction yielding a film with non-uniform microstructure.¹⁷ To this end, we measured these transport properties directly on a coated cellular substrate for a measurement of film properties directly on biological supports without any extrapolation of bulk transport properties. Our findings support the current practice of extrapolating bulk hydrogel transport properties to nanoscale coatings, where minimal differences were observed between our diffusion constants and those of the analogous bulk materials. These findings support the potential use of ultra-thin coatings, such as those presented here, for increased transport of low molecular weight species while effectively halting transport of large molecular weight materials above the critical values observed in bulk hydrogel systems.

Experimental Section

Materials

A Jurkat cell line (clone E6.1) was purchased from ATCC. RPMI-1640 with L-glutamine and Dulbecco's Modified Eagle's Medium were both purchased from Corning Cellgro, and supplemented with fetal bovine serum (Gibco) and Streptomycin/Penicillin solution (Thermo Scientific). Deionized and ultra-filtered water was purchased from Fisher Scientific. Eosin-5-isothiocyanate, poly(ethylene glycol) diacrylate ($M_n=575$), 1-vinyl-2-pyrrolidinone, poly-L-lysine (PLL), bovine serum albumin, HEPES-KOH buffer solution, potassium acetate, magnesium acetate, and fluorescein isothiocyanate-dextran (FD) with MW of 4, 10, and 20kDa were purchased from Sigma-Aldrich. Poly(ethylene glycol) diacrylate was passed over a MEHQ removal column twice prior to use. Phosphate buffered saline 10X solution, Nile red carboxyl-terminated 20 nm Fluosphere nanoparticles (NPs), and calcein AM were purchased from Life Technologies. Biotinylated monoclonal mouse IgG anti-human CD45 was from BD Pharmingen, monoclonal mouse IgG anti-human nuclear pore complex was from Covance, biotinylated goat IgG anti-mouse IgG was purchased from Vector Labs. Lyophilized streptavidin, biotinylated bovine serum albumin and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Scientific. Poly(ethylene glycol) diacrylate ($M_n = 3500$) was purchased from Jenkem Technology. Triethanolamine and Triton X-100 were purchased from Acros. Paraformaldehyde (16%, EM grade) was purchased from Electron Microscopy Sciences. Sucrose was obtained from MP Biomedical. Calcein/ethidium homodimer-1 LIVE/DEAD® Cytotoxicity kit was purchased from Life Technologies.

Methods

Preparation of Polymer Encapsulated Cells in Suspension—The T lymphoma Jurkat cell lineage was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37 °C and 5% CO₂. The cell culture was maintained at approximately $0.5\text{--}1 \times 10^6$ cells/ml. A sample of 1.5×10^6 Jurkats was washed once with phosphate buffered saline (PBS) supplemented with 3% FBS (PBS/FBS). Between each rinsing step, the cells were pelleted by centrifugation at $300 \times g$ and 4°C for 5 minutes. The pellet was resuspended in 20μL biotinylated mouse anti-human CD45 in

100 μ L PBS/FBS per 10^6 cells, gently vortexed and incubated at 4°C for 40 minutes. Then, the pellet was resuspended in 25 μ g/mL streptavidin-eosin isothiocyanate (SA-EITC) in PBS/FBS, gently vortexed and incubated for 30 minutes at 4°C. The SA-EITC was synthesized and purified according to a previously reported protocol.¹⁸ After incubation and rinsing with PBS/FBS, the cells were dispersed in 300 μ L of a monomer precursor solution consisting of 420 mM poly(ethylene glycol) diacrylate ($M_n = 575$) (PEGDA 575), 21 mM triethanolamine (TEA), 35 mM 1-vinyl-2-pyrrolidinone (VP) in PBS. In fluorescent imaging studies, 0.05 wt % Nile red 20 nanometer fluorescent NPs were also included in the precursor solution. Just before introduction to cells, the monomer solution was bubbled with ultra-pure N_2 for approximately 5 minutes to remove oxygen (polymerization inhibitor). The monomer/cell solution was gently vortexed and then pipetted onto a standard microscope slide in a Chip-clip chamber well (Whatman). The Chip-clip was then placed in a clear plastic bag and purged with ultra-pure N_2 for 5 minutes. Then, a photopolymerization reaction was initiated with an LED lamp emitting collimated 530 nm light, adjusted to an intensity of 30 mW/cm² for 10 minutes. The Chip-clip was then removed from the purged bag and the cells were removed from the slide with a shearing stream of PBS and washed two times to remove any unreacted monomer solution species and free NPs.

Raman Microscopy Analysis—To probe the surface chemistry of coated cells, first a PEGDA coated Jurkat cell sample was prepared as described, except that Jurkats were fixed in 1% paraformaldehyde for 10 minutes and rinsed three times in PBS, and then incubated in PBS + 3% FBS for 30 minutes before primary antibody incubation. After rinsing the coated cells, the supernatant was removed and the cell pellet was pipetted onto a clean microscope slide and allowed to dry for 2 hr. For a native cell control, 10^6 cells were centrifuged and rinsed once with cold PBS and then fixed in 1% paraformaldehyde for 10 minutes. The cells were then rinsed 3 times in cold PBS. After the final rinse, the supernatant was removed and the pellet was gently pipetted onto a clean microscope slide. A bulk polymer control was prepared by first reacting 25 μ g/mL SA-EITC in PBS on an epoxy-functionalized slide inserted in to a Chip-Clip for 1 hour. After rinsing, 300 μ L of monomer solution was pipetted into the Chip-Clip well. The Chip-Clip slide was purged and irradiated with an LED lamp for 10 minutes, emitting 530 nm light at an intensity of 30 mW/cm². The slide was then washed with deionized water and allowed to dry overnight. The samples were analyzed using a Thermo Scientific DXR Raman microscope. The 532 nm laser and 100X objective were used for all spectral measurements. The pinhole aperture was set to 25 μ m, corresponding to a focal spot of approximately 1 μ m diameter. Spectra were recorded between 300 and 3600 cm⁻¹ at a 5 cm⁻¹ spectral resolution. The system was calibrated to the spectral line of crystalline silicon at 520.7 cm⁻¹. For a spectral measurement, the microscope was focused on the surface of a sample and at least 8 scans at each spot were used to reduce the spectral noise.

Scanning Electron Microscopy—For SEM analyses, a control Jurkat sample was fixed in 1% paraformaldehyde for 10 minutes, rinsed twice in PBS, and placed on a PLL coated microscopy slide for electrostatic attachment. A coated sample was prepared with paraformaldehyde fixed cells, as before. Samples were dried overnight before analysis. The sample slides were then coated in gold/platinum by standard techniques and micrographs

were taken with a Hitachi S-4300 scanning electron microscope (Hitachi, Japan). The working distance was set at 15 mm with an accelerating voltage of 5.0 kV.

Viability Assays—Cellular viability was measured by a calcein assay. Cells were rinsed once with PBS, then the cell pellet was resuspended in cold PBS 1X with 50 nM calcein AM and incubated at room temperature protected from light for 15 minutes. After incubation, the cells were loaded into a BD Accuri C6 flow cytometer (Becton Dickinson), and run was performed at a medium fluidics rate to a total event count of 100,000.

Surface-Initiated Polymer Microarrays—Epoxy-functionalized microscopy slides 25×75mm (CEL-1) were rinsed once with ethanol and dried with ultra-pure N₂. Serial dilutions of biotinylated bovine serum albumin (bBSA) from 0.11 to 1000 µg/mL were prepared in PBS plus one negative control with 0 mg/mL bBSA (12 solutions total). bBSA solutions were then loaded into a 96 well plate and printed on the epoxy slides with an Affymetrix 417 Arrayer and allowed to dry overnight. Two replicate spots were printed for each biotin concentration in an array. A biotin-functionalized slide was loaded into a Whatman Chip-Clip, rinsed once with PBS supplemented with 0.1% BSA (PBSA) for 2 minutes, and then incubated with PBSA for 1 hr. After blocking, a 25 µg/mL SA-EITC solution in PBSA was incubated in the array well for 30 minutes. The slide was then rinsed once with PBSA for 2 minutes, then twice with PBS for 2 minutes each. For this study, three polymerization conditions were investigated that mimicked live cell encapsulation (Condition 1) and conditions used to coat fibroblast nuclei (Conditions 2 & 3). For live cell encapsulation, the monomer solution (1) consisted of: 420 mM PEGDA 575, 21 mM TEA, 35 mM VP, 0.05 wt% Nile red NPs in PBS. For PEGDA-575 thin films around nuclei (2), the monomer solution was: 420 mM PEGDA 575, 210 mM TEA, 35 mM VP, 0.05 wt% Nile red NPs in deionized water. For PEGDA 3500 thin films(3): 25 wt% PEGDA-3500, 210 mM TEA, 35 mM VP, 0.05 wt% Nile red NPs in deionized water. Before polymerization, the solution was bubbled for 5 mins with ultra-pure N₂. After rinsing a slide, 300 µL of the corresponding monomer solution was pipetted into the Chip-Clip array well, and placed in a clear plastic bag and purged with N₂ for 5 minutes. The photoinitiation conditions were as follows: (1) 530 nm, 30 mW/cm², 10 minutes; (2) 530 nm, 10.5 mW/cm², 20 minutes; (3) 530 nm, 10.5 mW/cm², 20 minutes. After photopolymerization, the slides were rinsed twice with deionized water before imaging. After drying overnight, profilometry measurements were performed on each array set with a Dektak 6M profilometer to prevent scratching of the film, scan settings were maintained at 4200 micron for 120 s, with a stylus force of 1mg.

Preparation of Encapsulated Subcellular Species by Polymer-Based

Amplification—Human dermal fibroblasts were cultured to ~80% confluency in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Cells were trypsinized, centrifuged, and seeded onto 35mm cover glass-bottom culture dishes (Mat-tek) in media. For polymer-based amplification of nuclei, a recently published protocol was adapted.¹⁴ Cell dishes were washed twice with cold PBS, and then fixed with 1% paraformaldehyde in PBS for 10 minutes. Cells were then washed 3 more times with cold PBS. The fixed cells were then permeabilized with 0.01% Triton X-100 in PBS for 7 minutes, and washed 3 times with PBS. A blocking solution consisting of 2 mL of PBSA

with 2% FBS was pipetted into the dishes and incubated for 30 minutes at 4°C. Cells were then immersed in a primary antibody solution of 1:1000 monoclonal mouse IgG anti-human NPC in PBSA for 40 minutes at 4°C, followed by a secondary antibody incubation of 1:400 biotinylated goat IgG anti-mouse IgG in PBSA for 40 minutes at 4°C. Cells were rinsed 3 times with PBSA, and then incubated with SA-EITC (25 µg/mL) in PBSA for 30 min at 4°C. The cells were then washed 2 times with PBS and once with deionized water. Two monomer solutions were prepared for polymer films of PEGDA-575 (420 mM PEGDA 575, 210 mM TEA, 35 mM VP, 0.05 wt% Nile red NPs in deionized water) and PEGDA-3500 (25 wt% PEGDA-3500, 210 mM TEA, 35 mM VP, 0.05 wt% Nile red NPs in deionized water). For each monomer molecular weight, 915 µL of monomer solution was pipetted into a petri dish, and placed in a nitrogen-purged bag as before. An LED lamp emitting green light (530 nm, 10.5 mW/cm²) was placed over the dish and the reaction was allowed to proceed for 20 minutes. The dishes were then washed 3 times with deionized water.

Time-Dependent Confocal Microscopy Analysis of Diffusion—To probe macromolecular permeation through films formed by polymer-based amplification, varying sizes of FITC-labeled dextrans (FD) (4kDa, 10kDa, 20kDa) were prepared in transport buffer adapted from Mohr *et al* (2009)¹⁹, consisting of 20mM HEPES-KOH pH 7.4, 120mM potassium acetate, 5mM magnesium acetate, and 250mM sucrose. Solutions of 10kDa and 20kDa FD were prepared at 15µM, however, the 4 kDa FD required a higher concentration of 45µM for quantifiable fluorescence signal. Human dermal fibroblast cell samples were washed twice with transport buffer. Diffusion kinetics were analyzed with a Leica AOBs/TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a HyD photon-counting hybrid detector. A 63x oil objective and an argon laser were used at 30% power. Just before a confocal diffusion study, transport buffer was removed from a glass bottom petri dish and it was loaded onto the microscope stage. The focus was adjusted and centered (z-direction) inside a coated nucleus. An automated timed scan was initiated, with an image taken every 1.5 seconds. After approximately 3–4 scans, 100 µL of FD solution was pipetted directly onto the scanning region and the automated scanning program was allowed to run for at least 160 seconds. For statistical significance analysis Student's t-test was calculated for all samples and in all the cases the p value obtained was lower than 0.0001.

Results and Discussion

Encapsulating Live Cells in Suspension by Surface Protein Recognition

The purpose of this study was to characterize a method of encapsulating live cell species in suspension by binding the type-II photoinitiator eosin to surface marker sites that enables excellent spatial control of polymer formation and ultra-thin coatings for minimal effect on small nutrient transport. Photoinitiated systems are convenient for cell encapsulation, owing to the delivery of uniform initiation energy to the system²⁰, rapid gelation⁶, excellent viability²¹, and the ease of scale-up to large cell populations. For our suspension study we chose the T lymphoma Jurkat cell line because it is a robust immortalized lineage with a high growth rate and abundant expression of CD45, an antigen that is well understood and commonly targeted. The experimentally optimized conditions of 21mM TEA, a 10 minute

reaction time, and an initiation light intensity of 30 mW/cm^2 was used to encapsulate Jurkats with a crosslinked PEGDA film, shown in Figure 1B. To monitor film formation by microscopy and flow cytometry, fluorescent NPs (nile red, 20nm, Fluospheres) were also introduced in the monomer mixture, and have been shown to be physically entangled in the surface-initiated films in a similar system.²² High cell viability was preserved near 90% approximately 1 hour after encapsulation, as measured by a flow cytometric calcein assay (Figure 2). Further, cytometry experiments of coated cells showing low ethidium homodimer-1 fluorescence compared to dead controls also suggest high viability of Jurkats through the coating process. While longer time points are critical to the ultimate application of cell transplantation, this level of initial viability is consistent with that of similar eosin photoinitiated PEGDA encapsulation systems with high long term cell viability.^{9,23,24}

Characterizing Film Surface Chemistry and Morphology

We used Raman microscopy to characterize the surface chemistry of the hydrogel coating. Spectra were collected at the surface of an uncoated Jurkat, the surface of a coated Jurkat, and at the surface of bulk cross-linked PEGDA-575 film (Figure 3). The three spectra revealed the different chemical nature at the surface of each sample. We expect the coated cells Raman spectrum to be the result of the superposition of both, native cells and polymer film spectra. The coated Jurkat spectrum shows a peak at 2850 cm^{-1} associated with the symmetric stretching of CH_2 groups from the lipid-rich cellular organelles²⁵ (lipid bodies) present in native Jurkats, but also displays signals from bulk PEGDA at 2880 cm^{-1} and 2950 cm^{-1} corresponding to CH_2 and CH_3 vibrations, respectively. The presence of peaks associated with both the native cell and the bulk polymer control in the polymer coated cell spectra further supports the presence of a PEG-diacrylate coating observed at the cell surface by fluorescent labeling (Figure 1B).

Scanning electron microscopy was also used to analyze the surface morphology of a polymer coated cell. A scanning electron micrograph of a paraformaldehyde-fixed native Jurkat exhibits a smooth, flattened morphology (Figure 4A), while a PEGDA-encapsulated, fixed Jurkat cell (Figure 4B) possesses a similar size as the uncoated cell with a rough surface. The similarity in size between the coated and uncoated cells supports our hypothesis that thin films on the order of nanometers thick and do not appear to alter the resulting coated cell size. The surface texture of coated cells is also different from that of an uncoated cell and appears to have a rough yet consistent topology. A magnified view of the coated cell surface (Figure 4C) indicates a surface texture similar to that of a surface-initiated PEG-diacrylate film (Figure 4D). While some variation in coating thickness is expected in surface mediated polymerization on the order of tens of nanometers, the surface features in Figure 4C are more consistent with the underlying membrane folds common to Jurkats and other leukocytes.²⁶ Critically, SEM observation clearly supports that the coatings are uniform and complete across the cell's surface, without observable gaps or apparent changes in coating thickness. Further, the covalent PEG crosslinking that forms these films is highly stable and to limit any opportunity for encapsulated cells to shed the films in the transplantation environment.²⁷

Estimation of Film Thickness

Cellular coatings have been routinely proposed to selectively control diffusion in the cell microenvironment after allogenic transplantation to allow the cell to perform a function in host tissue while avoiding immune attack.¹¹ The overall goal of this manuscript is to determine the size-dependent transport properties of nanoscale hydrogels, and to relate them to the established bulk hydrogel transport properties. For accurate scaling of nanoscale barrier films to bulk materials, it is essential to attain an estimate of the coating thickness. The estimation of coating thickness is particularly challenging at thicknesses below direct optical observation. Measurement of coating thicknesses less than ~300 nm is typically accomplished by ellipsometry, atomic force microscopy, profilometry, or electron microscopy. When dealing with a poorly reproducible and non-uniform biological surfaces, it is difficult to obtain accurate baseline data for thickness measurement by ellipsometry, atomic force microscopy, and profilometry. Further, the solvent-dependent nature of hydrogel thickness makes any measurement in a TEM embedding medium impossible to correlate without further understanding the solvent's influence on the hydrogel thickness. As a result, we estimated the thickness based on the fluorescent intensity of the polymer film on a biological substrate by correlation to the fluorescent intensity of an easily measured polymer film grown on a smooth glass substrate.

Biotinylated bovine serum albumin (bBSA) microarrays were prepared with biotin concentrations between 0 and 1000 µg/ml bBSA, where these surfaces were meant to replicate the chemical environment of the biotin-functionalized surface after a cell surface is labeled with biotinylated antibodies. These surfaces were treated with photopolymerization conditions that were identical to cellular coating procedures. As expected, polymer thickness increased with printed bBSA concentration, as more eosin molecules were bound to the array surface which increased radical formation during photoinitiation (Figure 5). From image analysis, it was determined that each a.u. of fluorescence intensity corresponded to 4.0 nm of thickness for the fluorescent nanoparticle loaded PEGDA-575 hydrogel coatings (Table 1). The slope is comparable for the PEGDA-3500 coatings (6.1 nm/a.u.), which suggests that the monomer size does not have a significant impact on fluorescence per polymer volume, and that the PEGDA-3500 films displayed reduced fluorescence simply because of lower polymer thickness. Using this correlation, the fluorescent signal from nanoparticle-loaded coatings on cell structures can be measured to then calculate a film thickness estimate. To obtain a thickness estimate for the coatings used later to study transport kinetics, the fluorescent signal from nanoparticle-loaded PEGDA-575 and PEGDA-3500 films encapsulating dermal fibroblast nuclei was measured by epifluorescent microscopy similarly to the microarray samples described above. To account for the signal contribution from the upper and lower layers of the spherical nuclei, the signal was divided by two to get a representative estimate for the film coating. The thickness of PEGDA-575 and PEGDA-3500 coatings was then estimated to be 158 ± 16 nm and 200 ± 42 , respectively (Table 1). These estimates agree well with previous work with eosin-mediated growth of PEGDA thin films and are used later for mass transfer modeling of the films in this work.^{12,28}

Assessing Permeation Across Hydrogel Thin Films

To assess the transport kinetics of nanoscale hydrogel coatings on cellular substrates, we use time dependent confocal microscopy to measure the transport of fluorescently-labeled macromolecules across hydrogel membranes. There are several advantages for studying diffusion through hydrogel coatings on adherent nuclei instead of an encapsulated whole-cell in suspension. First, using nuclei offer a substrate that is surface-anchored by cellular scaffolding without any further need for attachment factors that could potentially alter cellular geometry or bias the import kinetics. Nuclei are also much more rigidly spherical than other cellular species, including blood cells, that tend to flatten when attached to a surface (Figure 4A), which allows for more accuracy when collecting confocal fluorescence data inside of the nucleus. Finally, the nuclear envelope can be extensively permeabilized (with Triton X-100) without complete loss of structure unlike most cell membranes, providing a scaffold for film coatings to be studied with minimal influence on diffusion kinetics.

Numerous groups have used this time-dependent confocal microscopy technique to study macromolecular permeation kinetics through nuclear pores of permeabilized cells, yet the focus has been mechanistic and on the biological cues to passive or active diffusion of different molecules.^{19,29,30} We modify this established assay by first forming a PEGDA thin film coating around a nucleus of a permeabilized cell with techniques described here and in prior work¹⁴, and measuring passive diffusion of large fluorescent biomolecules through the film by sampling the fluorescence signal (i.e. concentration) inside the nucleus over time.

To establish that permeabilized fibroblast nuclei represented negligible resistance to mass transfer across the nuclear envelope, control samples were fixed and permeabilized with 0.1% Triton X-100. Solutions of fluorescein and various sizes (4, 10, and 20 kDa) of FD were prepared in transport buffer, and the accumulation of fluorescent material in nuclei was monitored by confocal microscopy (Figure 6). To account for differences in absolute fluorescence between permeants, the fluorescent signal was normalized by dividing the signal inside the nucleus by the signal outside the cell. For all of the permeants studied, diffusion was virtually immediate into the permeabilized control nuclei (>90% equilibrium concentration in nucleus at 3s).

In the uncoated permeation control study with 4 kDa FD, there is a clear affinity of the FD for nuclear material. The higher fluorescence of the nucleus versus the cytoplasm and surrounding regions at long timescales acts against the expected concentration gradient at the nuclear membrane and is contrary to the expected diffusive behavior. Further, the dynamics of the increase of fluorescence inside the nucleus after 40 s is linear, rather than the first order decay expected in a diffusive process. Importantly, the rate of this affinity process is less (0.2 change in relative fluorescence over 160 s) than the diffusive behavior (~1.2 change in relative fluorescence over 3 s).

PEGDA (M_n 575 and 3500) coatings incorporating red NPs were grown on the nuclear membrane by immunochemically localizing eosin to sites of nuclear pore complex expression (Figure 6B, 6C), as described previously.^{14,22} Permeant solutions were prepared as in the uncoated control experiments, and Figure 6 shows a representative images at $t=3$ s

and $t=160$ s after introduction of the fluorescent permeant to the slide. For the PEGDA-575 coatings, fluorescein showed an immediate increase to a high F_{in}/F_{out} as the relatively small molecule readily permeated the film. However, the largest molecules investigated of 10kDa and 20kDa FD both showed essentially zero increase in F_{in}/F_{out} during the entire ~ 170 sec experiment, suggesting that both molecules were completely excluded from the diffusing through the film. This exclusion of 10 kDa FD indicates the PEGDA-575 coatings have a polymer mesh size smaller than 3.7 nm (the hydrodynamic diameter of a 10kDa dextran³¹). The trial of 4kDa FD diffusion showed a F_{in}/F_{out} signal that increased, supporting that the mesh size of the PEGDA-575 film is larger than 1.3 nm (the hydrodynamic diameter of a 4kDa dextran³¹). This 1.3 – 3.7 nm mesh size is in agreement with a previous estimate of 2.0 – 5.0 nm for bulk PEGDA-575 with the same mass fraction of monomer in water.²² This prediction also agrees well with the unimpeded diffusion of the fluorescein dye observed in PEGDA-575 coatings, a clear demonstration of molecular weight cutoff behavior in this coating.

In diffusion trials for PEGDA-3500, we hypothesized that because the molecular weight between crosslinks was larger, the film would be more permissive to molecular diffusion. For the largest biomolecules of 10 and 20 kDa, the PEGDA-3500 film showed virtually no increase in F_{in}/F_{out} during the entire trial, demonstrating dextrans of hydrodynamic diameter of > 3.7 nm are excluded by the PEGDA-3500 coatings. The 4kDa dextran (diameter ~ 1.3 nm) does permeate through the coating, indicating the PEGDA 3500 coatings also have a mesh size between 1.3 and 3.7 nm. This estimate agrees closely with the 2.2–2.7 nm mesh size reported for micron thick PEGDA-4000 coatings studied previously by Cruise, Scharp, and Hubbell.¹⁶ To further support the molecular weight cutoff behavior of these interfacial coatings, a trial of just fluorescein showed that the film allowed instantaneous diffusion of the small molecule.

While the absolute cutoff behavior of the PEGDA-575 and PEGDA-3500 yields an identical range of mesh size, the PEGDA-3500 film permitted the diffusion of 4kDa FD at a faster rate than PEGDA-575 (difference in Figure 6 slopes of over the first 10 s are statistically significant with $p = 1.76 \times 10^{-25}$). The faster transport across the PEGDA-3500 coating suggests a larger mesh size when compared to a PEGDA-575 coating. This fundamentally agrees with bulk scale expectations, where a larger diacrylate will yield a larger molecular weight between crosslinks, and larger diffusion than a smaller diacrylate at an identical mass fraction.

It is also observed that the PEGDA-575 or PEGDA-3500 coating did not alter the apparent affinity of the 4kDa FD for nuclear material observed in the uncoated control studies. At long time scales ($t > 90$ s), there is an increase in fluorescence in the nucleus which appears linear and does not follow the expected rate decay of a diffusively driven process.

Estimation of Film Diffusion Coefficients

While these time-resolved images provide quantitative information about the size exclusion properties of the films, the completeness of substrate coating, and the molecular weight cutoff for permeants, we also sought to extend our findings to estimate the diffusion coefficients. Because bulk photopolymerization proceeds through drastically different

initiation conditions than a typical surface-initiated system, the crosslinking profiles generated are inherently different.³² Further, polymer diffusivities are typically measured with a polymer geometries that are several micrometers thick when hydrated, which is a much larger length scale than the thin films generated in this work.^{16,33} Thus, direct measurement of diffusivities of films on cellular substrates would result in a more representative characterization of transport in the cellular microenvironment.

The diffusion constants for 4kDa FD through PEGDA coatings (D_{575} and D_{3500}) were the only analyzed, owing to clear change in nuclear fluorescence over an experimentally-accessible time scale. In addition to permeation data, a coating thickness was estimated (Table 1) using the relationship between film thickness and fluorescence summarized in Figure 5. The coating thickness for the model was further expanded to include an upper and lower bound approximately 50 nm above and below the calculated estimates from our microarray study of film thicknesses to account for potential error in our coating thickness estimate. Thus, a range was defined to be 100 to 200 nm for PEGDA-575 film modeling and 150 to 250nm for PEGDA-3500 film modeling. Coating surface area and nuclear volume were calculated from confocal scans of the nucleus prior to the assay. The nonlinear fluorescence increase during the first 15 s of the polymer coated samples with 4kDa FD appeared to be first order, and the fluorescence change in this region is used to estimate passive diffusion across the polymer coatings. In all 4 kDa FD samples, there was a consistent linear increase in nuclear fluorescence, and this non-diffusive behavior is attributed to an affinity of the dextran for the nuclear material. The contribution from 4kDa FD nuclear affinity was estimated to be 0.03 a.u. per 15 s time span. This contribution is <10% of the overall increase during the initial 15 s analyzed and was not included in the model. This omission potentially introduces a small error in the estimated diffusion constant, and future studies would benefit from identifying a low affinity fluorescent probe in this molecular weight range.

Using these data, Fick's Second law was numerically integrated, and diffusion constants were fit to the data for each condition. Figure 7 shows plots representing a model fit for the estimated film thickness studied for each monomer length. For the lower and upper bounds of thicknesses modeled for PEGDA-575 (100–200nm), the diffusivity of 4kDa FD through the film was calculated as $D_{575} = 9.5 \times 10^{-10} - 2.0 \times 10^{-9} \text{ cm}^2/\text{s}$ (Supplemental Figure 6A and 6B). Similarly, a diffusivity was fit to the $F_{\text{in}}/F_{\text{out}}$ data for the PEGDA-3500 film at 150–250nm, and was calculated to be $D_{3500} = 5.9 - 9.8 \times 10^{-9} \text{ cm}^2/\text{s}$ (Supplemental Figure 6C and 6D). These findings agree with the expected higher diffusivity for the PEGDA-3500 film when compared to the PEGDA-575 film, owing to a higher molecular weight between crosslinks.³⁴

Remarkably, the magnitudes of the diffusion constants for these hydrogel thin films on fibroblast nuclei are consistent with that of bulk materials of similar macromer molecular weights reported in literature. The most direct literature comparison is in Cuchiara *et al.*,³³ where 3 and 10 kDa dextrans diffuse through PEGDA-6000 at a 10% w/v, and the diffusivities were reported to be on the order of 10^{-7} and $10^{-9} \text{ cm}^2/\text{s}$, respectively. Further, our diffusion results also follow the expected trend of increasing diffusivity with increasing PEGDA length. Cruise *et al* (1998)¹⁶ reported an increase in diffusivity as the molecular

weight of the PEGDA monomer increased from 2kDa to 20 kDa for vitamin B-12 (~1.3kDa) permeation across the PEGDA hydrogels. These diffusivities were on the order of 10^{-7} and 10^{-8} cm²/s for the diffusion of a permeant molecule considerably smaller than the 4 kDa FD studied here (Figure 7). This observed agreement in diffusivity between thin film and bulk polymer materials suggest that permeant diffusivity is largely decoupled from substrate encapsulation geometry. As such, we expect the permeability analysis performed here on adherent fibroblast nuclei to extend well to outer cell surfaces with minimal difference in diffusivities for the ultimate application of cell transplantation.

Conclusions

In this work we have demonstrated the encapsulation of both whole cell and sub-cellular species by photopolymerization of PEGDA films. By SEM and Raman microscopy we have shown that complete and conformal films are targeted to cell surfaces, and the coating surface is consistent in morphology to a surface-initiated PEG diacrylate film. This technique is applicable to both adherent and suspended cell configurations. By flow cytometric calcein assays we have shown that encapsulated cells retain viability of ~90%. Time-dependent, photon-counting CSLM analysis demonstrates that large 10- and 20 kDa fluorescein-functionalized dextran molecules are completely excluded by films formed from both PEGDA-575 and PEGDA-3500 monomers, and provides a polymer mesh size estimate of 1.3 – 3.7 nm. Microarray studies suggested these films were 100–200 nm in thickness, which is consistent with previously reported data on glass substrates.²⁸ Diffusion coefficients of 4kDa FD permeation through films calculated from a model of Fick's second law were in good agreement with reported trends and magnitudes in bulk crosslink hydrogel materials of similar precursor monomer units. Notably, our results support that the technique described here can encapsulate cells with biocompatible hydrogel coatings that achieve similar size selective permeability behavior as previously studied immunoisolation strategies, but with drastically lower thicknesses and minimal synthetic material. Thus in the post-transplantation cellular environment, we expect the transport of essential small nutrients across the encapsulated cell membrane is minimally affected while still preventing host immune response. Further, because cells are coated individually in suspension, cell can potentially be seeded by injection into target areas of tissue with much less invasive procedures than for larger bulk crosslinked cell scaffolds, which can potentially improve recovery times and frequency of cell therapy administration. Additionally, we expect the photon-counting CSLM diffusion measurement technique to serve as a convenient assay of coating integrity and performance on other encapsulated cellular materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic of antigen-specific coating process to form nanothin films on the surface of individual cells. (B) Fluorescent image of PEGDA encapsulated Jurkats by recognition of CD45 antibody. Red fluorescence is from Nile red 20 nm fluosphere nanoparticles physically entangled in the polymer film. Scale bar = 20 micron.

Figure 2.

Flow cytometry analysis of coated cell viability by esterase activity. The live control represents calcein stained Jurkat cells from culture and the dead control were polymer coated cells incubated with 70% ethanol for 5 minutes before calcein staining. Image inset shows a representative micrograph of calcein AM (green) and ethidium homodimer-1 (red) staining of coated Jurkats. Student t-test for live control and polymer coated populations yielded $p=0.125$, and for polymer coated and dead control populations yielded $p=6\times 10^{-8}$. Scale bar represents $100\mu\text{m}$.

Figure 3.
Raman spectral comparison of surface of native Jurkat, coated Jurkat, and bulk cross-linked PEG diacrylate (M_n 575).

Figure 4. SEM analysis of uncoated and coated cell surface morphology. (A) Surface morphology of a native and uncoated Jurkat cell. (B) Surface morphology of a PEG diacrylate coated Jurkat. (C) Higher magnification of coating morphology. (D) Surface of an interfacial PEG diacrylate film formed on glass.

Figure 5.
Plot of microarray spot thickness vs. spot fluorescence for different monomer molecular weights.

Figure 6.

Quantification of molecular diffusion through nanoscale coatings. Image summary at $t=3$ sec and $t=160$ sec of passive macromolecular diffusion and time-dependent analysis of fluorescent signal from free fluorescein and FITC-Dextran through (A) permeabilized dermal fibroblast nucleus controls, (B) PEGDA-575 and (C) PEGDA-3500 coated nuclei. F_{in} signal was from the inner domain of nuclei and F_{out} was the signal of the FITC-dextran solution outside the cell. Error bars are shown only on every other time point on plotted data for clarity.

Figure 7.

Analysis of diffusivity of 4kDa FITC-labeled dextrans through nanoscale coatings on cellular substrates. Permeation data was fitted with a time-dependent Fickian diffusion model to calculate diffusion constants for lower and upper bounds of film thicknesses for both PEGDA-575 and PEGDA-3500 films. (A) Model fit and calculated diffusivity for diffusion of 4 kDa FITC-dextran through a PEGDA-575 film of an estimated thickness of 150nm. (B) Model fit and calculated diffusivity of 4 kDa FITC-dextran through a PEGDA-3500 film of an estimated thickness of 200nm. Initial concentration and time parameters were adjusted to a reference point of zero for model curve fitting.

Table 1

Comparison of fluorescent signal for nanoparticle-loaded films encapsulating dermal fibroblast nuclei.

Monomer (M_n)	Fluorescence Intensity From Top and Bottom (a.u.)	Fluorescence Intensity From Top (a.u.)	Intensity to Thickness Conversion (nm/a.u.)	Film Thickness Estimate (nm)
PEGDA (575)	71.9±8.2	35.9±4.1	4.0	158±16
PEGDA (3500)	63.8±13.8	31.9±6.9	6.1	200±42