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Permselectivity Replication of Artificial Glomerular Basement Membranes in Nanoporous Collagen Multilayers

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Abstract

Basement membranes (BMs) play important roles in many biological functions such as tissue regeneration, cancer proliferation, nutrient/drug delivery, breathing, and many others. While there are many theoretical models, adequate experimental analogs of BMs describing basic physicochemical properties of BM, such as diffusion and permselectivity are not available. Taking BMs found in glomerulus of kidneys as an example, adequate reproduction of their permselectivity requires biomimetic membranes with submicron thickness, high uniformity, nanoscale porosity, and size-selective permeability. Artificial kidney BMs were assembled from poly(acrylic acid) and collagen using layer-by-layer (LBL) assembly technology and display multiple structural similarities with glomerular BMs. Diffusional transport through the artificial BMs faithfully replicate cut-off parameters of kidney membranes. Their utilization in understanding of unique diffusion processes in kidneys, *in vitro* studies of blood clearance time of small drugs/nanoscale drug carriers and design of more complex organoids including live cells for cancer proliferation studies is anticipated.

Keywords

extracellular matrix; biological membranes; membrane transport; artificial kidney; layer-by-layer assembly; collagen tissues

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Further advances in tissue engineering, implantable biosensors, nanoscale drug carriers and other biomedical applications traditionally rely on animal models to evaluate biological responses. However animal studies are expensive, very time consuming, and subject to extensive governmental regulations, which tend to become even more complicated. Ethical aspects of animal studies must be considered as well. The need to find adequate *in vitro* methods using artificial replicas of human organs becomes apparent. This challenge is also attractive for fundamental research as an effort to better understand complex biological processes using simpler models. Tremendous activity in the area of three-dimensional (3D) tissue cell cultures with more accurate representation of transport, adhesion, and cell signaling characteristics than the two-dimensional (2D) cell cultures can be one of the best examples of great significance of such efforts.[1]

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One of the unexpected roadblocks in this direction is the fact that even sophisticated 2D or 3D cellular cultures are still insufficient for the replication of some body functions. This is the case because tissues responsible for these functions are acellular. One example of such living acellular structures is basement membranes (BMs) (Figure 1).[2-6]

They are made from sheets comprised from interwoven strands of collagen (Figures 1A,B) and other extracellular matrix proteins. Their thickness is from few hundred nanometers to several microns (Figure 1C-F). In some cases the individual membranes are laminated together into multiple sheets (Figure 1D). BMs can be encountered almost everywhere in the body and typically carry out separation, support, transport, protection, and other functions. BMs play crucial role in tissue regeneration, cancer metastasis, inflammation, nutrient/drug delivery, and many other processes. Artificial BMs are also needed for tissues replicas of higher levels of complexity which may indeed include live cells. The obvious bottleneck in these studies is the availability of biologically appropriate and experimentally convenient replicas of the actual acellular tissues. Two important points need to be made now about biological functions of BMs:

1. BMs are fundamentally different in function and structure than cellular membranes. The latter are much thinner. Made from lipids, cellular membranes have been extensively investigated over many years using a variety of different experimental models. These models are mostly based on mono- and bi-layers of surfactants, and cannot be applied to BMs. In case of BMs, there are many theoretical models of BMs[8-12] but there is a gapping absence of adequate experimental models.
2. The biological function of BMs is determined by fundamental physicochemical processes and their physical and mechanical characteristic to a much large degree than the cellular tissues. Therefore, reproduction of such processes and physical properties of such tissues has direct relevance both to physical chemistry, biology, and drug discovery.

As a demonstration of a fundamental possibility of making artificial BMs, we decided to replicate glomerular membranes found in kidneys. The physicochemical properties of interest here is diffusion and permselectivity, which underlie their biological function. When purifying blood they exhibit quite sophisticated transport behavior[10] which is difficult or impossible to reproduce by current synthetic membranes. Structurally versatile *in vitro* models of kidney membranes would be very useful for modeling different kidney disorders[3, 5, 13] and in drug testing to determine blood clearance time.[14] Also, further development of artificial dialysis devices especially those approaching in size to actual kidney heavily depends on better reproduction of efficient filtration function of kidneys, which also remains a significant technological challenge.[15-17]

In order to closely mimic BM in kidney glomeruli, one will need to make a polymer membrane with structural uniformity, submicron thickness (Figure 1), nanoscale porosity, a certain degree of robustness, and, of course, biocompatibility. Transport properties and, in particular, size selective permeation, are the most fundamental benchmarks for kidney BMs. Their main functionality is to retain higher molecular size particles, cells or proteins (>20-50 kDa) and to let smaller molecules through (< 20 kDa). Theoretical models for the transport permselective transport are very well developed.[8-11] In the same time the experimental models are not: reproduction of permselectivity is still a major challenge in this area.[16, 17] Replicas of glomerular BMs are particularly needed because permeation of many new drug carriers could be quite paradoxical and unpredictable based on theoretical studies. The best example here are carbon nanotubes that were found to have anomalously high permeation through the kidneys.[18] Needless to say permselective properties are also essential in many

other BMs for better understanding of processes in liver, pancreas, blood vessels, and many other organs. Last but not the least, availability of macroscale versions of such membranes suitable for cellular adhesion will make possible 3D organ replicas with greater complexity. [19-21]

Analysis of the challenges for the realization of the idea of artificial glomerular BMs leads to the need of a fine degree of control over the nanometer scale “architecture” of biomaterials. Some of the techniques that were used in the fabrication membranes with biological functionalities related to permeability of small and large molecules include the stereo lithography, wax printers, inkjet printing, the phase-separation technique, particulate leaching, textile technologies, and 3D printing techniques.[22-24] Despite substantial progress in this area, there are still significant challenges in these directions making BM replication a tough project.[25-28] Problems that still need to be resolved include low interconnectivity between the pores and morphologies, inability to reproduce size selectivity of BMs, and the need for support materials that are difficult to remove.[29]

Sequential adsorption of oppositely charged macromolecular species by layer-by-layer (LBL) deposition proved to be a powerful thin film preparation technique that can be applied to the design of many biological materials with well controlled interfacial, mechanical, and biological functions. Alternating adsorption of anionic and cationic polyelectrolytes leads to the formation of regular multilayer assemblies and the films that are remarkably uniform[30-33] and display mechanical characteristics with great similarities to parent biomaterials.[34-36] LBL multilayers from collagen, the dominant component of all BMs, and poly(acrylic acid) (PAA), a common biocompatible LBL partner, produced free-standing macroscale films with distinct structural and functional similarities to BMs. They displayed, thickness, uniformity and size selective permeation for macromlecular species <20 kDa, which is virtually identical to those of the actual BMs from kidneys.

The key insights presented here are related to replication of diffusion conditions, and therefore, permselectivity in glomerular membranes. So far, diffusion in such membranes was not possible to reproduce in any experimental system. In addition to that one can also highlight the fact that diffusion in such glomelular membranes is ultimately related to such physical and physicochemical parameters as thickness and pore size. Realization of porosity with appropriate range of diameters in a membrane with specified nanoscale thickness and specific chemistry is a non-trivial task, which was not possible to accomplish using numerous current methods of membrane making. If you add the need for high hydrophylicity, biocompatibility, and mechanical strength, the task of find such membranes becomes even a tougher challenge representing an interesting nexus between the materials science, fundamental understanding about transport membranes, and biology.

In this work, we prepared LBL assembled free-standing multilayer films with collagen and poly(acrylic acid) (PAA) as poly(cation) and poly(anion), respectively. Earlier work in on LBL-made collagen membranes showed their biocompatibility.[37] Under optimized conditions $(PAA/collagen)_n$ thin films (n is the number of LBL deposition cycles performed) were found to be highly uniform and physically robust for the preparation of free-standing membranes (Figure 2a). LBL assembly required tight control over the pH of both LBL components. The isoelectric point of collagen is 5.5 and, so we chose pH to be below pH 5.5, so that amino groups of collagen were in NH_3^+ form. pKa of PAA is 4.0 which served as a lower limit for initial selection of assembly conditions. It was found that a even a small 0.1 unit change in pH of collagen solution dictated the formation and stability of the $(PAA/collagen)_n$ thin films, which was consistent with previous results on the assembly of weak polyelectrolytes.[38] The best results were obtained for pH of PAA equal to 4.0, while pH of collagen solution was 4.2-4.3. Under these conditions the steady linear LBL growth was

observed (Figure 2b). At lower pH of collagen solution, *e.g*. pH = 4.0, films were very thin and fragile. For collagen solutions with pH > 4.5, much thicker films were formed with large surface roughness, which was probably related to the switch to the hydrogen bonding attachment of PAA and collagen and the exponential growth pattern.[39]

The average ellipsometric thickness for a bilayer in $(PAA/collagen)_n$ multilayers deposited on silicon was found to be around 60 nm (Figure 2b), which was substantially thicker than for traditional polyelectrolytes and indicated the formation of films with substantial nanoscale porosity and volumetric interconnectivity of the chains. AFM images of $(PAA/collagen)_1$ and $(PAA/collagen)_3$ confirmed this notion and displayed complex entanglement of bundles of polymer chains and collagen fibrils with surface features in 10-60 nm range and distinct nanoscale pores (Figure 3a-d). Surface topology in these images was highly reminiscent of those for BMs (Figure 1a,b). SEM images of $(PAA/collagen)_{10}$ confirmed their fibrous morphology (Figure 3e); the analogies the SEM images of BM in Figures 1a-c were also obvious. The thickness of the film was evaluated by cross-sectional TEM images (Figure 3f) and was found to be 600±50 nm for $(PAA/collagen)_{10}$ which coincided really well with the thickness of the glomerular BMs (Fig 1 e, f). Increase in the number of LBL cycles to n=15 and n=20 gave the average thickness of 870±65 and 1280±65 nm, respectively, in agreement with linear mode of LBL growth (Figure 2b).

Permeability Measurements

Permselective properties of $(PAA/collagen)_{10}$ were evaluated in the form of free-standing multilayer membranes (Figure 2a) made on cellulose acetate (CA) under optimized conditions described above. CA was subsequently dissolved in acetone and $(PAA/collagen)_{10}$ was rehydrated in water. No change of the membrane structure/thickness was observed compared to the $(PAA/collagen)_n$ multilayers made on silicon or glass substrates.

The free-standing nature of the membrane unlike the more traditional LBL multilayers on solid supports[40-43] was essential because of the potential integration with well-plates or kidney replicating micromechanical devices.

Permselectivity of $(PAA/collagen)_{10}$ was quantified by measuring the permeability of fluorescein isothiocynate-dextran (FITC-dextrans) molecules with various molecular weights. These experiments were carried out in a specially constructed liquid-liquid diffusion cell, where free-standing $(PAA/collagen)_{10}$ film separated two aqueous solutions with ionic strengths analogous to that in serum (0.15 mol/L). Fluorescence intensity of the solutions was used as a convenient experimental parameter describing the permeation rate of FITC-dextrans from one side of the cell to another (Table 1, SI).

Permeability of FITC-dextrans was found to strongly depend on their molecular size (Figure 4a). Fluorescein in the form of non-conjugated dye and FITC-dextrans with molecular weights (Mw) of 4 kDa, 10 kDa and 20 kDa successfully diffused through $(PAA/collagen)_{10}$ while higher molecular weight FITC-dextrans were retained by the membrane virtually perfectly. This indicates that Mw cutoff for these membranes was 20 kDa.

It is important to have means of fine-tuning the permeability of the membranes. This could be achieved by controlling the thickness of LBL multilayers, which was both experimentally convenient and functionally effective. It could also be potentially attained by chemical treatment of the membranes resulting in potential reduction of, for instance, diameter of pores. Along these lines, permselective experiments were carried out for $(PAA/collagen)_{15}$ and $(PAA/collagen)_{20}$ multilayer films (Figures 4b). As expected the permeability decreased as the number of multilayer layers increased from 10 to 15 and 20 (Figure 4b). The effect

was the strongest for the lowest molecular weight penetrants, which was related to the increase of the tortuosity of the membrane pores (Figure 2a-e).

Permeability through the films could also be affected by cross-linking. The most common cross-linking agent for proteins is glutaraldehyde (GA) reacting with two amino groups in the adjacent chains of collagen. Interestingly, the treatment of $(PAA/collagen)_{10}$ with 8% and 1% GA had a fairly small effect on permittivity both for high and low Mw dextrans compared to non-cross-linked LBL films (Figure 4c). It showed that penetration of dextrans was mainly determined by the diffusion in nanoscale pores in the membrane rather than by the mobility of the macromolecular network. The diameter of the channels in the membrane was apparently unaffected by cross-linking except probably the span of error bars for non-cross-linked version of the film (Figure 4c), which brought the described BM replica even closer to the actual biological membranes.[44] The decrease of error bars after GA treatment is likely to represent the decrease of the number of defects in the film related to improvement of mechanical properties. Interestingly, the tensile strength, σ, of the $(PAA/collagen)_{20}$ after 8% of GA treatment reached 88±7 MPa, which can be compared to that of nylon with $\sigma = 86$ MPa.

In conclusion

experimental reproduction of fundamental physicochemical processes and physical parameters of BM as a typical representative of acellular tissues, which was so far impossible, will lead to replication of their biological properties. The experimental results indicate that $(PAA/collagen)_n$ membranes share components, morphology, thickness, and permselective properties with glomerular BM. In part this was afforded by the versatility of LBL assembly and demonstrated fine level of control over diffusion parameters. One also needs to point out the remarkable mechanical properties of the multilayers which allow them to retain their physical integrity and robustness even for nanometer scale thickness.[45-47]

One can anticipate that beside the Mw cut-off, it is also possible that other permselective properties in respect to biomolecules of different charge and/or different chemical nature can be potentially replicated as well, which must be the subject of the subsequent work.

In perspective, these membranes could be successfully employed for *in vitro* models of BMs in kidney. These studies are difficult to carry out now with natural BM from animals due to high variability of the sources and difficulties of handling micro- and nanoscale scale membranes for reliable measurements. One can also anticipate the possibility to utilize $(PAA/collagen)_n$ in portable/implantable dialysis devices. Similar BMs can be engineered for the drug clearance rate and cancer metastasis studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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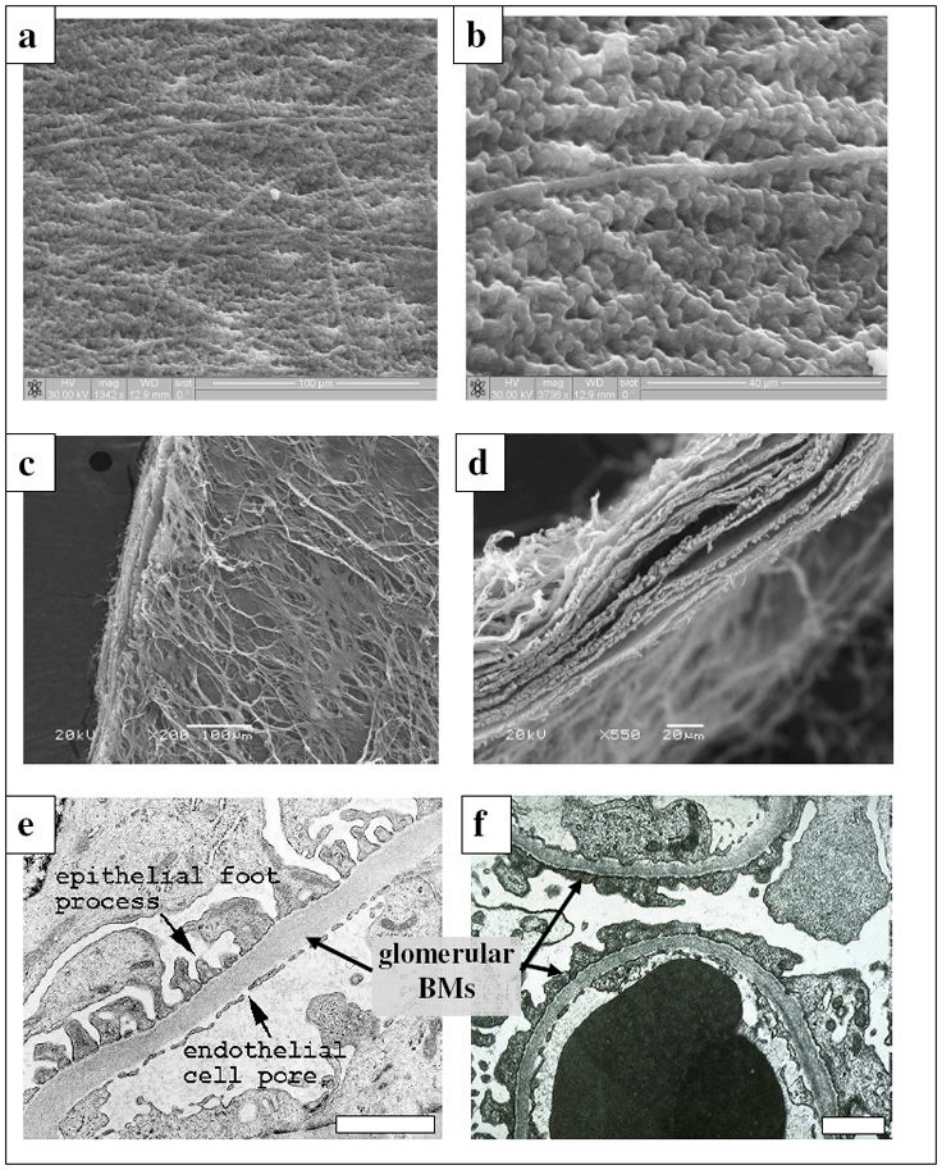

Figure 1.
Microscopy images of different BMs. (a, b) SEM images of egg pouch membrane, (c, d) SEM images of the bovine BM from connecting tissue. (e,f) TEM images of glomerular BM from kidneys.[7] Scales are 1 μm in e,f.

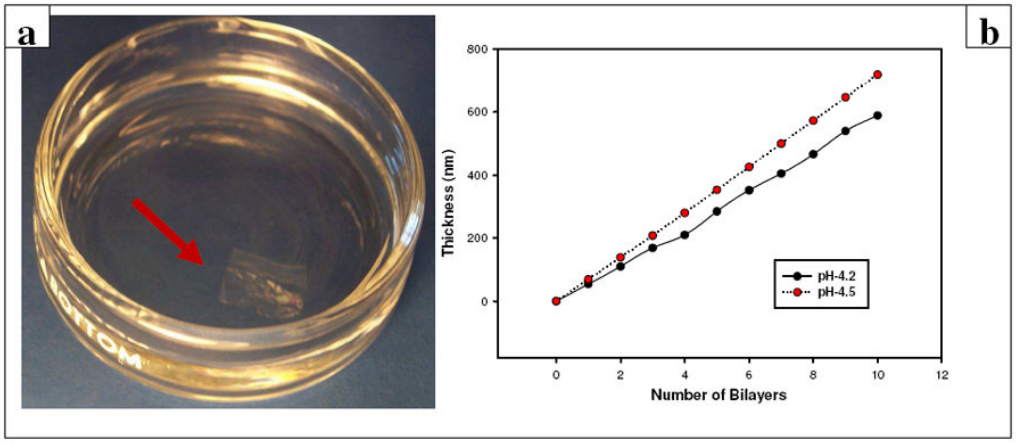

Figure 2.

(a) Image of a $(PAA/collagen)_n$ free standing thin film. (b) Effect of collagen solution pH on the thickness of $(PAA/collagen)_n$.

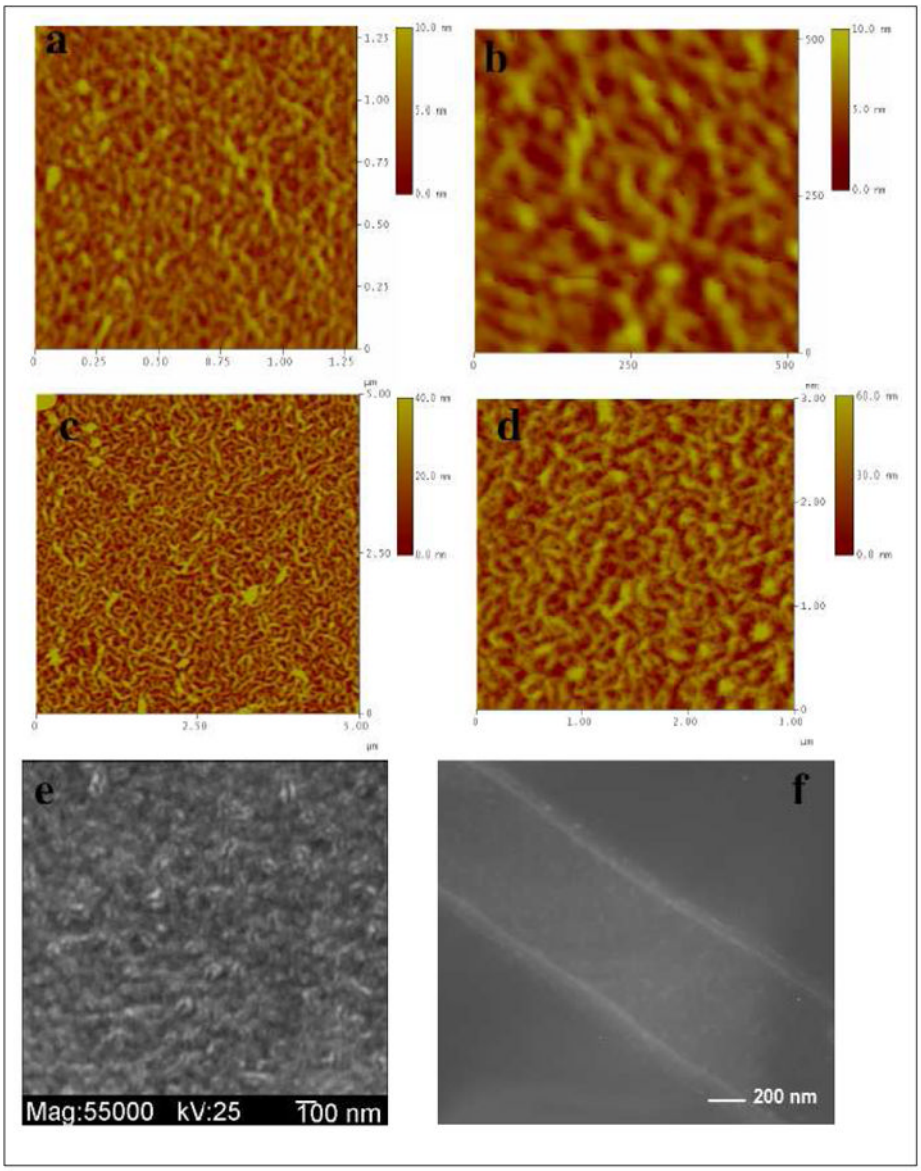

Figure 3.
(a, b) AFM images $(PAA/collagen)_n$ for n=1 and (c, d) n=3. (e, f) SEM images of $(PAA/collagen)_{10}$ films.

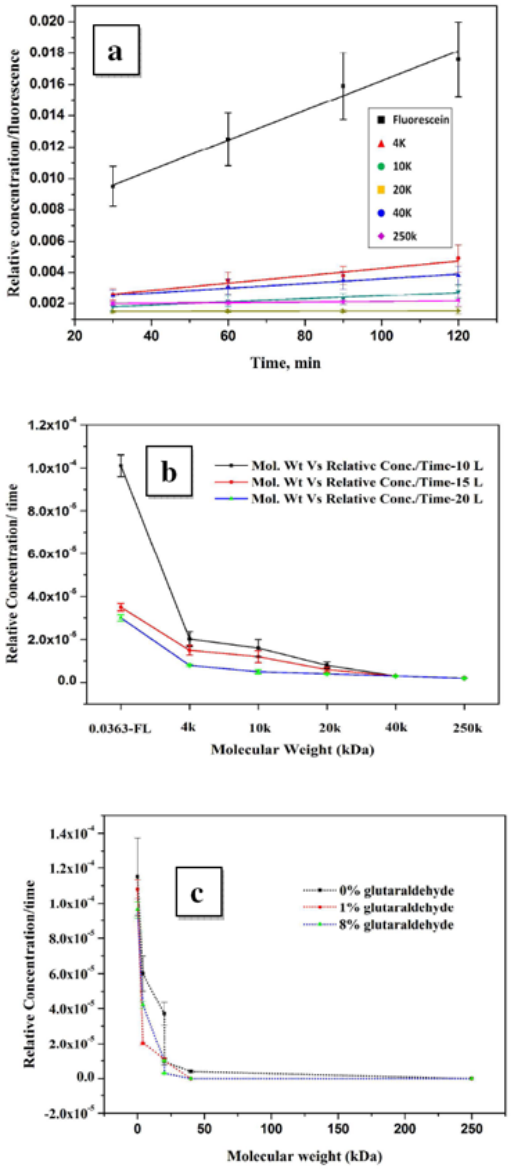

Figure 4.

(a) Temporal dependence of the relative concentration of FITC-dextran and fluorescein on the permeate side of the membrane $(PAA/collagen)_{10}$ film. Color legend is given in the plot. (b) Dependence of permeability on the number of LBL deposition cycles. (c) Effect of cross-linking on permeability for different concentrations of glutaraldehyde for 0%, 1%, and 8% GA cross-linking.

Table 1

Permselective properties of $(PAA/collagen)_{10}$ multilayers. (see Supplementary Information for details of calculations, 5 different batches of collagen were used)

Compound	Molecular Weight (Daltons)	Retention Coefficient (R)
Fluorescein	376	0.22 ± 0.016
FITC-dextran	4,000	0.72 ± 0.054
FITC-dextran	10,000	0.92±0.063
FITC-dextran	20,000	1.0 ± 0.072