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Mini-Review

POLYELECTROLYTE MULTILAYERS FOR ENHANCING CELL ADHESION AND POTENTIAL APPLICATIONS IN TISSUE ENGINEERING

Nicolás E Muzzio[1], Miguel A Pasquale[1], Sergio E Moya[2,*]

[1] *Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), (UNLP, CONICET), Sucursal 4, Casilla de Correo 16, 1900 La Plata, Argentina*

[2] *Soft Matter Nanotechnology group, CIC biomaGUNE, Paseo Miramón 182 C, 20009 San Sebastián, Gipuzkoa, Spain*

* *Autor Corresponsal: smoya@cicbiomagune.es*

Resumen

Las multicapas de polielectrolitos (MPEs) ensambladas mediante la técnica de Capa por Capa (CpC) ofrecen múltiples opciones para la ingeniería de superficies sin hacer uso de la química covalente. Recientemente, las MPEs han recibido atención como un medio de desarrollar matrices e implantes capaces de promover la adhesión celular, la migración y la diferenciación. En esta revisión se presentará el estado del arte en el uso de multicapas de polielectrolitos para mejorar la adhesión celular. En particular, se mostrarán las diferentes estrategias desarrolladas en nuestro grupo combinando polímeros sintéticos y biológicos, y utilizando el recocido térmico para cambiar las propiedades de estos materiales.

Abstract

Polyelectrolyte multilayers (PEMs) assembled by de Layer by Layer (LbL) technique offer multiple possibilities for surface engineering as an alternative to covalent chemistry. PEMs have recently attracted attention as a mean to engineer scaffolds and implants to make their interface with tissue more amenable for cell adhesion, migration and differentiation. In this review we will present the state of the art on the use of polyelectrolyte multilayers for enhancing cell adhesion. In particular, we will show the different approaches followed in our group combining synthetic and bio polymers, and the use of thermal annealing.

Palabras Clave: *Multicapas de polielectrolitos, técnica capa a capa, polielectrolitos biológicos, adhesión celular, recocido térmico.*

Keywords: *Polyelectrolyte multilayers, Layer by Layer technique, biological polyelectrolytes, cell adhesion, thermal annealing.*

1. Introduction.

Cell adhesion is a key process in many physiological and pathological processes such as morphogenesis,[1] wound healing,[2] microorganisms infections,[3] and cancer progression.[4] Controlling cell adhesion is also a fundamental issue in the biological and biomedical fields.[5] Different requisites for cell adhesion are needed for diverse types of applications.[6] For instance,

for biomaterials that have to interact with blood the adhesion of cells and plasma proteins must be prevented to avoid thrombosis and embolism, but materials used in tissue regeneration are required to act as adherent substrates that first promote cell adhesion and then migration, proliferation and eventually differentiation.

Cells can sense a set of complex biological and physicochemical signals and can integrate them to change their dynamic state.[7] Thus, cell adhesion can be largely affected by the physicochemical properties at the surface of the substrates, such as surface charge and energy, wettability, roughness and stiffness.[7,8] Several strategies like plasma etching,[9] Langmuir-Blodgett,[10] or polymer grafting,[11] have been developed to control cell adhesion by modifying the chemistry, topography and mechanical properties of the substrate interacting with cells. These modifications impact on the interaction of the cell with the substrate material while the latter retains its bulk properties.[12]

Introduced in the 90' by Moehwald, Lvov and Decher, the layer-by-layer (LbL) assembly of polyelectrolyte multilayers provides a simple, versatile and cost-effective technique for the noncovalent modification of surfaces and implants and the engineering of scaffolds among other applications.[5,13,14] The LbL assembly is driven by the electrostatic interactions between oppositely charged polyelectrolyte and entropic considerations.[15] By means of the LbL technique, oppositely charged polyelectrolytes (PEs) are sequentially assembled on top of a planar or colloidal charged surface to create a thin polyelectrolyte film, the polyelectrolyte multilayers (PEMs), with thickness and composition controlled in the vertical direction.[16] In addition to synthetic or natural polyelectrolytes,[16,17] nanoparticles,[18] lipid vesicles,[19] biomolecules,[20] and even cells[21] can be used as building blocks provided they interact with previous and subsequent assembled layers electrostatically or by other type of interactions (i.e., hydrogen bonding, hydrophobic or host-guest interactions).[15] Intrinsic properties of the building blocks -such as molecular weight of polymers, charge density or polarity- and assembly conditions -such as temperature, ionic strength or pH- can affect the amount of the polymer assembled and the characteristics of the assembled polyelectrolyte layer, i.e. thickness, density, mechanical properties, etc. These physicochemical characteristics of PEMs have a significant impact on cell adhesion and functions.[12]

PEMs fabricated from bio polyelectrolytes, such as chitosan (Chi), hyaluronic acid (HA), alginate (Alg), poly-L-lysine (PLL), among others are very appealing for the modification of surfaces for biological and biomedical applications due to their expected biocompatibility and biodegradability.[22] These PEMs form a cushion on which proteins, growth factors, peptide sequences and other biomolecules can be assembled, controlling cell functionalities such as cell adhesion, migration and proliferation.[14] Moreover, PEMs offer an appropriate mean to mimic the

extracellular matrix by incorporation of gradients in their physicochemical properties, which would induce a spatial dependence of cell functionalities.[23] Though PEMs composed of bio polyelectrolytes are very promising materials to be used in tissue engineering and regenerative medicine, they present some limitations, such as poor mechanical properties that tend to prevent cell adhesion.[17]

In this review, we will present the state of the art on the use of polyelectrolyte multilayers for enhancing cell adhesion. We will firstly review the advantages and limitations of the use of synthetic and bio polyelectrolytes, and the impact of the PEM composition on cell adhesion. Then, we will comment different approaches for tuning cell adhesion on PEMs. In particular, we will show two strategies followed in our group based on the combination of synthetic and biopolymers and on the use of thermal annealing. Finally, we will present some strategies to spatially control the physicochemical properties of the PEMs to guide cell adhesion.

2. Cell adhesion on biological and synthetic PEMs

The nature of each component in PEMs, i.e., the polycation and the polyanion composition is one of the factors that determines the physicochemical properties of the final multilayer and, in consequence, its behavior towards cell adhesion. Though there is a huge variety of synthetic and biological polyelectrolytes, each group has a certain number of characteristics.[17] Synthetic polyelectrolytes offer a large choice of chemistries, structures and charge densities. They are susceptible to chemical modifications and can be used in large ranges of pH and ionic strength. Usually, synthetic polyelectrolytes are abundant, cheap and available with highly controlled quality. On the other hand, they are generally non-biodegradable or may have harmful degradation products. Biological polyelectrolytes are biocompatible and naturally degraded by different kinds of enzymes.[24] As natural constituents, they have interesting structural and functional properties, such as interaction with specific cell receptors or bioactive molecules. Bio polyelectrolytes have, however, some drawbacks, such as a limited pH and ionic strength working range and chemical modifications can be particularly difficult. PEMs made of bio polyelectrolytes tend to render highly hydrated and soft materials that prevent cell adhesion.[14,25,26]

Cell adhesion on PEMs of different composition was assessed in several works, using purely natural, purely synthetic and combined multilayers. Chondrocyte cells adhesion on purely natural Chi/HA films depended on the number of layers and the surface coverage grade.[27] Mesenchymal stromal cells did not adhere to PLL/HA multilayer films with 3, 6 or 9 bilayers.[28] C2C12 myoblasts adhered poorly to $(PLL/HA)_{12}$ films in comparison to glass.[29] The adhesion of human

chondrosarcoma cells on poly-L-lysine/poly-L-glutamic multilayer films was also poor.[30] $(Heparin/Collagen)_{10}$ multilayer films reduced the initial adhesion of human bone marrow mesenchymal stem cells whilst stimulated cellular differentiation and mineralization.[31] Adhesion experiments have been also performed on PEMs composed of purely synthetic polyelectrolytes. Poly(sodium 4-styren sulfonate)/poly (allylamine hydrochloride) (PSS/PAH) films presented different behaviors towards C2C12 cells adhesion, proliferation and differentiation depending on the composition of the terminal layer, the number of layers or the molecular weight of the PSS.[32] Cell mortality is favored on PAH-terminated multilayers, probably due to the interaction of the positive amine groups with the negative cell membranes. Poly(allylamine hydrochloride)/poly (acrylic acid) $(PAH/PAA)_9$ films terminated in PAH or PAA were tested with normal, noncancerous fibrocystic disease and cancerous human breast epithelial cell lines.[33] Cell spreading area and morphology depended both on the cell line and on the composition of the last layer. Though the spreading area was poor for both $(PAH/PAA)_9$ and $(PAH/PAA)_9PAH$ films, it was a bit larger for the last one. In a study with combined multilayers composed of PLL and PSS it was found that photoreceptor cell viability was less favorable on PSS terminated films than on PLL ones.[34]

All these articles addressed the adhesion or proliferation of eukaryotic cells on PEMs with distinct chemical composition. The different cell lines and assembly conditions of the multilayers such as pH, ionic strength or number of layer makes hard to compare these studies and to draw clear conclusions of the effect of the chemistry on cell behavior. We reported a systematic study about the impact of the chemistry of PEMs on A549 cell adhesion and proliferation.[16] PEMs of different chemical compositions were obtained by assembling natural polycations and polyanions (PLL; HA; Alg; dextran sulfate, Dex) with the synthetic ones (PAH; PSS; PAA; polyethylenimine, PEI; poly(dyallyldimethylammonium chloride), PDAD). All the multilayers had the structure $(Polycation/Polyanion)_7Polycation$ and were assembled at the same pH and ionic strength. Images of A549 cells, the average cell spreading area and the duplication rate on several of the evaluated PEMs are shown in Figure 1. The average cell spreading area was smaller on PEMs assembled with the natural polyanions HA, Alg and Dex than on PEMs assembled with the synthetic PSS, except when the polycation was PDAD (Fig 1b). Indeed, on PDAD composed PEMs cells exhibited scarce adhesion for all polyanions, probably due to its toxicity.[35,36] For PEMs with PSS, the average cell spreading areas were similar to those obtained for cells adhered on glass. The larger spreading area on PSS based PEMs is consistent with the fact that PSS would increase the stiffness of the multilayer,[37] enhancing cell adhesion. Cell adhesion on PEMs with PAA depended on the polycation. While with the natural PLL the average spreading area was larger than that obtained from PEMs assembled with natural

polyanions, with PAH or PDAD the spreading area was lower. PAA retains a large amount of water which makes it a relatively soft material and unsuitable for proper cell adhesion.[38]

In that work, we assessed cell proliferation with the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay 24, 48 and 72 h after seeding on glass and on the different PEMs tested (Fig 1c).

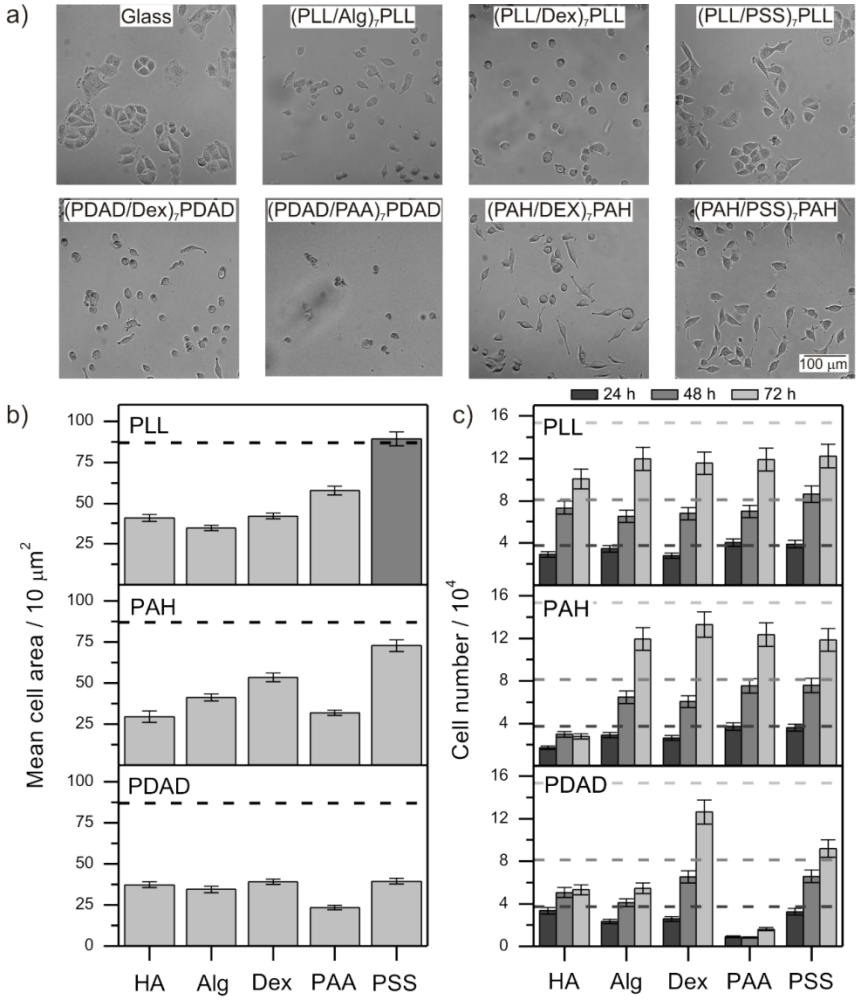

Figure 1. a) Images of A549 cells adhering on glass and purely natural (PLL/Alg, PLL/Dex), purely synthetic (PDAD/PAA, PAH/PSS) and combined (PLL/PSS, PDAD/Dex, PAH/Dex) PEMs. **b)** Mean cell spreading area for A549 cells on PEMs of different composition. For each PEM the average cell adhesion areas were assigned to be smaller (light gray), equal (gray) or larger (dark gray) than on glass employing the ANOVA and Fisher test with 0.05 significance. **c)** Cell proliferation of A549 cells on PEMs coated glass substrates measured by MTT. Cell number was measured after culturing for 24, 48 and 72 h, as indicated in the figure. Dashed lines in b) and c) correspond to the values obtained on glass.

Cell proliferation of cells seeded on all the PEMs was smaller than for cells adhered on glass. Three distinct behaviors for cell proliferation can be distinguished: (i) cell number increased with

an exponentially on glass, PLL/PAA and PEMs assembled with the natural Alg and Dex as polyanions and all tested polycations except PDAD/Alg. (ii) Cell number increased linearly with time on PDAD/Alg multilayers and PEMs with the synthetic PSS as polyanion, except PDAD/PSS. (iii) The rate of cell proliferation diminished with time on PLL/HA, PAH/HA and PEMS assembled with PDAD as polycation, except PDAD/Dex.

To summarize, PEM composition affects cell adhesion and proliferation differently. Cell adhesion on PEMs composed of biological polyelectrolytes was poorer than on glass or on many PEMs with synthetic polyelectrolytes, especially with PSS. On the other hand, cell proliferation was better on natural Alg or Dex based PEMs than on synthetic based PEMs.

3. Strategies to tune cell adhesive properties of PEMs

The development of simple strategies to properly change the physicochemical properties of PEMs made of biological polyelectrolytes without compromising film biocompatibility is fundamental to tune their behavior toward cell adhesion. As diverse cell types preferentially adhere to stiff surfaces and PEMs made from biopolymers usually have low elastic modulus several strategies aim to improve the films mechanical properties have been proposed.

Chemical cross-linking of the multilayers provides stiffness to the PEMs to a degree that correlates with the concentration of cross-linker agent used, leading to an enhancement on cell adhesion. This strategy has been used to enhance the adhesion of placenta and stromal derived mesenchymal stem cells on $(PLL/HA)_9PLL$ films.[28] Preosteoblast and rat skin fibroblast adhesion was also improved on cross-linked Chi/Alg films.[39] The presence of photosensitive groups in one of the polyelectrolytes of the multilayers has been used for photo cross-linking, as an alternative to chemical cross-linking. UV irradiated PEMs composed of PLL and vinylbenzene-grafted hyaluronans showed improved C2C12 cell adhesion in comparison to standard PLL/HA films.[40] Other post-assembly treatments, such as immersion of the PEMs in concentrated salt solution, has been shown to affect film properties and cell adhesion.[41] Another way to improve the mechanical properties of soft PEMs is the addition of nanoparticles. The mechanical reinforcement of HA/PLL multilayers increases the film Young's modulus in an order of magnitude with a concomitant enhancement of mouse fibroblasts adhesion.[18] Cell adhesion has also been improved with the combination of different blocks of PEMs. HT29 cell adhesion gradually increased when soft natural PLL/HA films were capped with PSS or blocks made of one or two bilayers of PSS/PAH.[42] This fact was attributed to an increase in the elastic module due to the penetration of the PSS into the underlying film.[43]

The use of cross-linking agents, nanoparticles or capping with synthetic polyelectrolytes have the disadvantage that they may not be fully biocompatible. We have reported two strategies to

promote cell adhesion while retaining biocompatibility: the use of blocks of polyelectrolytes of different stiffnesses,[16] and the modification of the physicochemical properties of PEMs by thermal annealing.[44–47]

3.1. Di-block PEMs for the enhancement of cell adhesion

With the aim of improving cell adhesion on natural biocompatible films, PEMs were assembled in the form of two blocks with different polyelectrolyte combinations (Fig 2a). The first or inner block was composed of PSS and PLL to reinforce the film mechanical properties, and the outer block was constituted of natural biocompatible polyelectrolytes, like PLL/Alg that displays an exponential like cell proliferation curve (Fig 1c). In this way, in contrast with the capping strategy,[42,43] the top layers of the film facing the cells would be biocompatible.

The assembly of the $(PLL/PSS)_6$ block, $(PLL/Alg)_7PLL$ and $(PLL/Alg)_4PLL$ on top of a $(PLL/PSS)_6$ block was followed by means of the quartz crystal microbalance with dissipation (QCM-D) technique (Figure 2b). Data indicate a rather supralineal growth for the PLL/PSS and the PLL/Alg PEMs assembled on top of glass. The growth of the $(PLL/Alg)_7PLL$ multilayer on top of the PLL/PSS block follows a fairly lineal trend. Thus, the PLL/PSS block underneath affects the growth of the subsequent PLL/Alg block. This fact can also be observed in the atomic force microscopy (AFM) images (Figure 2c). The PLL/PSS PEM is smooth, and this smoothness is retained after the assembly of the PLL/Alg top block. The topography of this PLL/Alg capped film is different from the pure and rough PLL/Alg PEM, highlighting the influence of the inner block of PLL/PSS on the assembly of PLL/Alg layers. Both QCM and AFM data are consistent with the diffusion of the polyelectrolytes throughout the film producing an interdigitated multilayer (Figure 2b).[48,49]

Cell adhesion on $(PLL/Alg)_nPLL$ PEMs (with $1<n<4$) was greatly improved when the polyelectrolytes were assembled on top of a $(PLL/PSS)_6$ block, forming a di-block structure namely $(PLL/PSS)_6(PLL/Alg)_nPLL$ (Figure 3). Fluorescence images (Figure 3a) shows that cells seeded on glass, $(PLL/PSS)_7PLL$ and di-block PEMs exhibit a good cytoskeleton spreading (red) with focal contacts formation (green). In contrast, cells seeded on $(PLL/Alg)_7PLL$ spread poorly and present a diffuse fluorescence image. The average A549 cell spreading area on $(PLL/Alg)_2PLL$ or $(PLL/Alg)_7PLL$ was less than a half of that obtained from cells on glass (Figure 3b). Interestingly, cells seeded on di-block PEMs with n=1 or 2 developed average spreading areas statistically larger than on glass or $(PSS/PLL)_7PLL$ films.

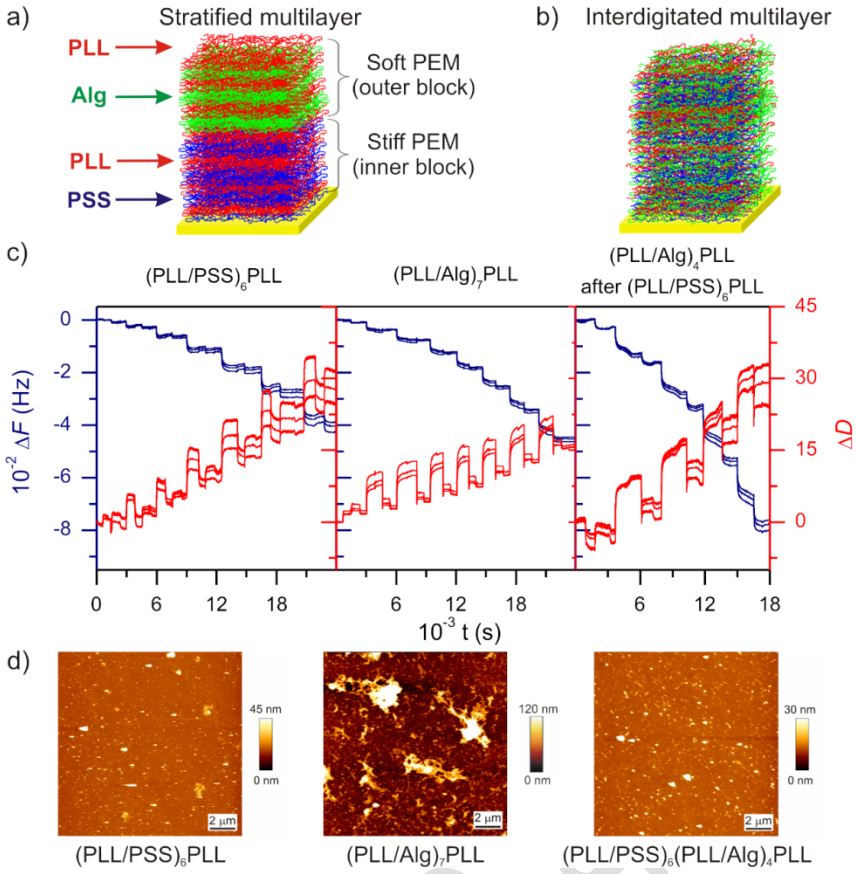

Figure 2. a) Scheme of a di-block polyelectrolyte multilayer. An initial stiff PEM block of $(PLL/PSS)_6$ was first deposited and then a soft PEM block of biocompatible $(PLL/Alg)_nPLL$ was assembled. **b)** Scheme of the possible interdigitation between the blocks. **c)** Frequency and dissipation changes as a function of time for the assembly of $(PLL/PSS)_6PLL$, $(PLL/Alg)_7PLL$ and $(PLL/Alg)_4PLL$ on top of a $(PLL/PSS)_6PLL$ block. **d)** AFM image in dry state of $(PLL/PSS)_6PLL$, $(PLL/Alg)_7PLL$ and $(PLL/PSS)_6(PLL/Alg)_4PLL$.

These results can be interpreted as a consequence of polyelectrolyte interdigitation that generates PEMs with physicochemical properties that are absent in each block separately. We also used the di-block assembly strategy to improve A549 cell adhesion on PLL/Dex biocompatible PEMs and to enhance the adhesion of C2C12 cell line.[16]

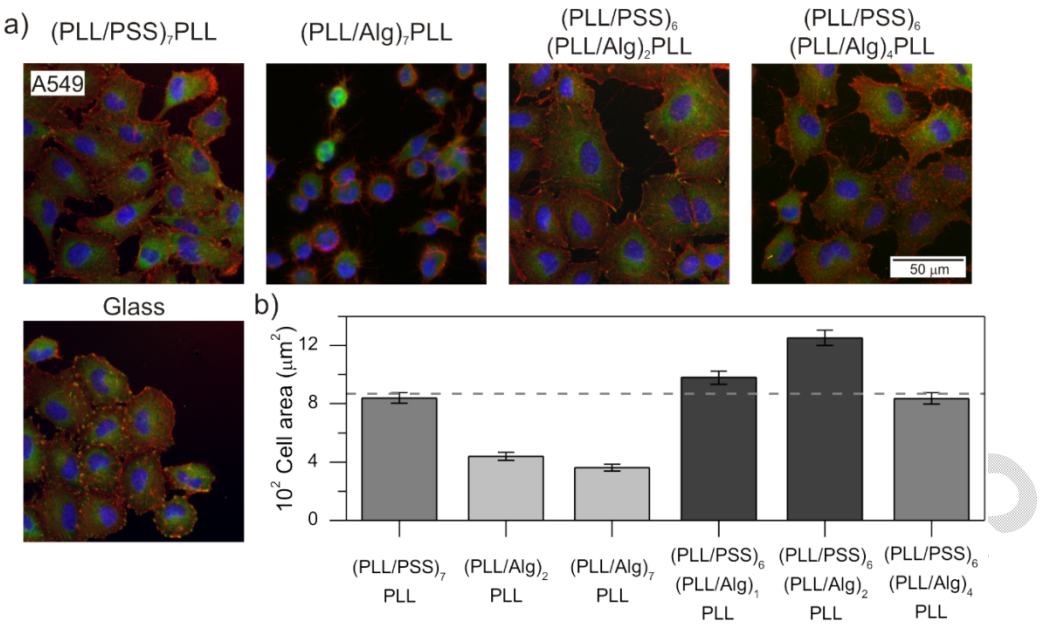

Figure 3. a) Immunostaining of vinculin (green) and staining of actin (red) and cell nucleus (blue) of A549 cells on glass, single and di-block PEMs. b) Mean cell spreading area for A549 cells on single and di-block PEMs. For each PEM the average cell adhesion areas were assigned to be smaller (light gray), equal (gray) or larger (dark gray) than on glass employing the ANOVA-Fisher test with 0.05 significance. Dashed lines correspond to the cell spreading area obtained on glass.

3.2. Cell adhesion improvement by thermal annealing of PEMs

Polyelectrolyte multilayers assembled by the LbL technique are systems outside thermodynamic equilibrium and can be affected by temperature.[50] The post-assembly thermal treatment at 37°C for 3 days generates stable changes in the physicochemical properties of natural polyelectrolyte-based PEMs and induces changes in cell adhesion properties.[44–46] This thermal annealing enhanced the adhesion of A549 and C2C12 cells on biocompatible PLL/Alg and PLL/Dex PEMs.

The changes in the physicochemical properties of $(PLL/Alg)_7PLL$ films upon annealing were assessed with AFM, nanoindentation experiments, water contact angle and zeta potential measurements (Figure 4). AFM images show that after the annealing, the film becomes smoother (Figure 4a) and with height peaks 2 or 3 times shorter than before annealing (Figure 4b). The roughness decreases from a value close to 4.3 nm for the nonannealed film to a value close to 2.3 nm for the annealed one (Figure 4c). The PEM also becomes stiffer, with a Young's modulus one order of magnitude larger after the annealing (Figure 4d). Changes in film wettability were determined by measuring the water contact angle on air dried samples. The contact angle of the PEMs increases from 36±2.8° to 92±4.6° after the annealing, showing an increase of the film hydrophobicity (Figure 4e). The zeta potential of $(PLL/Alg)_7PLL$ coated colloids changes from

about zero on nonannealed films to -14 mV after annealing (Figure 4f), indicating that the density of negatively charged groups on the film surface increased. The above data indicate that the thermal annealing of $(PLL/Alg)_7PLL$ multilayers leads to a reorganization of the film as a consequence of an increase of the polyelectrolyte motility inside the multilayer and a maximization of the polyelectrolyte electrostatic interaction.[51–53]

The reorganization of the $(PLL/Alg)_7PLL$ multilayers induced by the thermal treatment affects its cell adhesive properties (Figure 5). The cytoplasm spreading of A549 and C2C12 cells on nonannealed $(PLL/Alg)_7PLL$ films is poor, as it can be observed in the fluorescence images (Figure 5a). On the other hand, cells seeded on annealed $(PLL/Alg)_7PLL$ PEMs present an extended cytoplasm with well-defined focal contacts and large actin fibers, like the cells seeded on glass. The spreading area of both cell lines on nonannealed $(PLL/Alg)_7PLL$ films is about 2.5 times smaller than the spreading area on glass (Figure 5b). The vinculin area per cell follows the same trend, being 4 times smaller on nonannealed $(PLL/Alg)_7PLL$ multilayers than on glass. Annealing improves the adhesion of A549 and C2C12 cells, and the adhesion parameters reach values statistically equal to the values found on glass.

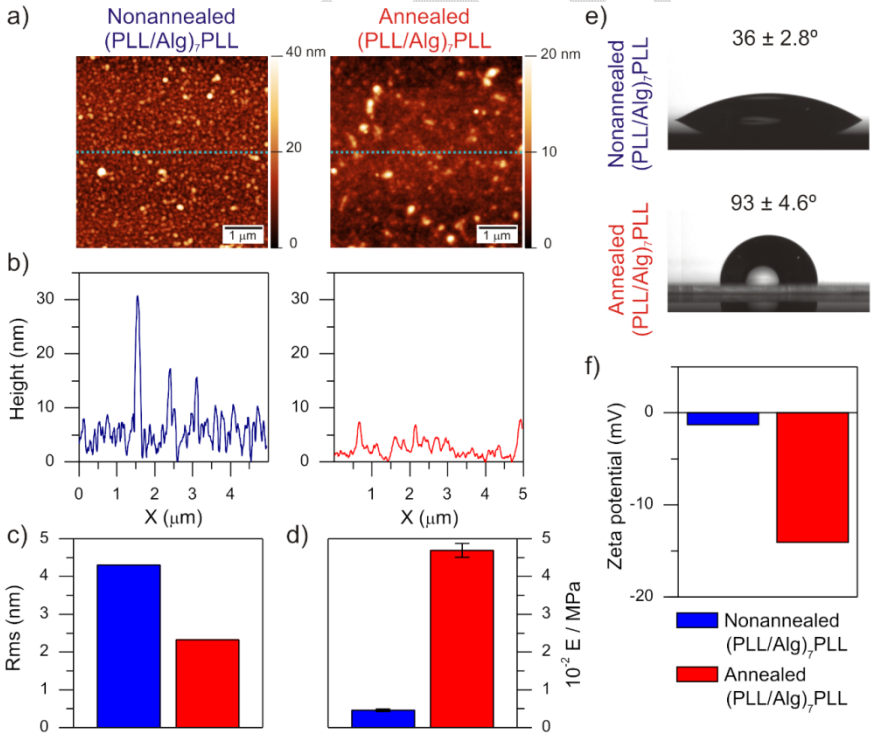

Figure 4. Physicochemical properties of nonannealed and annealed $(PLL/Alg)_7PLL$ multilayers. AFM images **(a)** and roughness profiles **(b)** corresponding to the blue lines in **(a).** Root mean square roughness **(c)** and Young's modulus **(d)** obtained from nanoindentation experiments. Water contact angle measurements **(e)** and surface zeta potential **(f)** of the nonannealed and annealed films.

Thermal annealing represents a simple and cell-friendly strategy to tune the physicochemical and cell adhesive properties of biocompatible multilayers. This strategy was applied to PEMs composed of others pairs of polyelectrolytes, promoting cell adhesion on PLL/Dex films,[47] and inhibiting it in a cell-dependent manner on Chi/HA PEMs.[46]

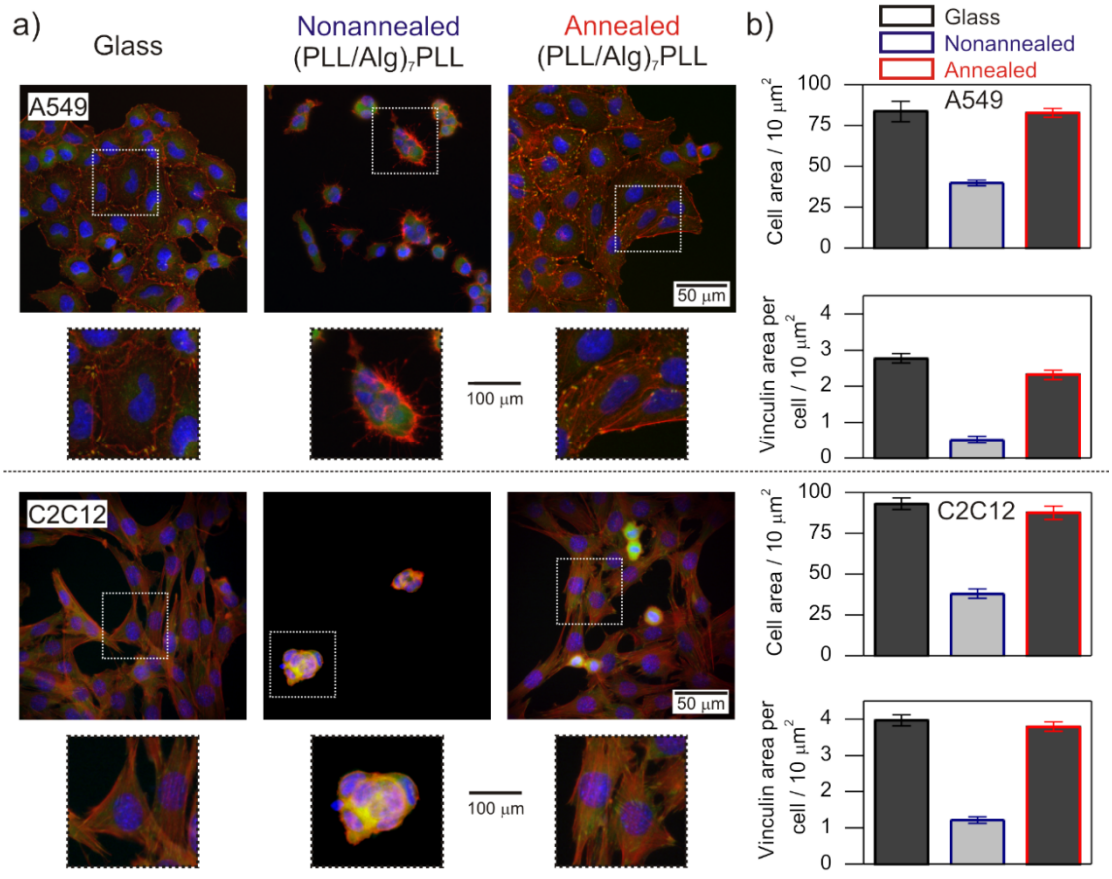

Figure 5. **a)** Immunostaining of vinculin (green) and staining of actin (red) and cell nucleus (blue) of A549 and C2C12 cells on glass, nonannealed and annealed $(PLL/Alg)_7PLL$ multilayers. Enlarged images of the areas indicated by squares are included. **b)** Mean cell spreading area and vinculin area per cell for A549 and C2C12 cells on glass, nonannealed and annealed $(PLL/Alg)_7PLL$ films. For each PEM the average cell adhesion area or vinculin area per cell was assigned to be smaller (light gray), equal (gray) or larger (dark gray) than on glass employing the ANOVA-Fisher test with 0.05 significance.

4. Spatial control of PEMs properties

Surfaces with gradients in their physical properties and with biochemical cues are of great interest for biomedical research and applications as they can better mimic the complexity of the extracellular matrix and guide cell behavior.[54] As PEMs are promising candidates for a biomaterial mimicking the native extracellular matrix, several types of gradients has been developed with these films.[55]

Gradients in pH assembly of PEI/Heparin/Chi multilayers have been made using microfluidic devices. Cells seeded on the resulting multilayers adhered better on the basic region, which is more stiff and hydrophobic than the acid one.[56] Gradients can also be made with post-assembly treatments. A spatially patterned stiffness has been made on PLL/HA multilayers making a cross-linker agent gradient using a microfluidic device.[55] The resulting multilayer presented a spatially dependent cell adhesion. Microfluidic devices have also been used to spatially pattern biomolecules on top of PEMs. Gradients of bone morphogenic proteins on top of PLL/HA multilayers were used to induce a myogenic or osteogenic commitment of C2C12 cells.[20] Photo-cross-linkable PEMs can also be used to create films with gradients of mechanical properties, which were able to guide cell adhesion and migration.[57] These two cellular functions have been tuned on PSS/PDAD films with a spatially controlled swelling ratio generated by a post-assembly treatment in a salt gradient.[41] Moreover, these gradient substrates were used to study the cell-cell interaction dependence of directional cell migration, demonstrating the utility of these materials on basic cell research.

In our group, we have created a spatially controlled arrangement of physicochemical cues on PEMs surfaces with a temperature gradient treatment after the assembly.[47] PLL/Alg films were placed on a silver sheet with one extreme maintained at 10 °C and the other at 50 °C using thermostated chambers (Figure 6a). The resulting temperature gradient was linear with the distance, with a slope close to 0.002 °C m^{-1}. The values of water contact angle at the films extremes held at 10 °C and 50 °C were close to those observed in nonannealed and annealed PEMs, respectively (Figure 4e and 6b). At intermediate temperatures the film presented an intermediate value of water contact angle. Thus, a continuous change in the physicochemical properties of the multilayers appears to set in after the application of a thermal gradient.

These changes in the PEM properties affected the adhesion of C2C12 cells seeded on top (Figure 6c). On the film region maintained at the highest temperature cell adhesion was close to that observed on glass. In contrast, on the region maintained at the lowest temperature cell spreading was poor. The most significant changes in the cell spreading area were observed in the region exposed to temperatures between 22 and 30 °C. This is in accordance with the fact that in the case of uniform thermal annealing of PEMs, a treatment at 37 °C is enough to achieve cell spreading areas close to those found on glass (Figure 5).

The application of a thermal gradient to biological polyelectrolyte composed PEMs enable us to locally modify the physicochemical and cell adhesion properties of films, an issue of interest to better mimic the extracellular matrix properties and for rapid testing of cell functionalities.

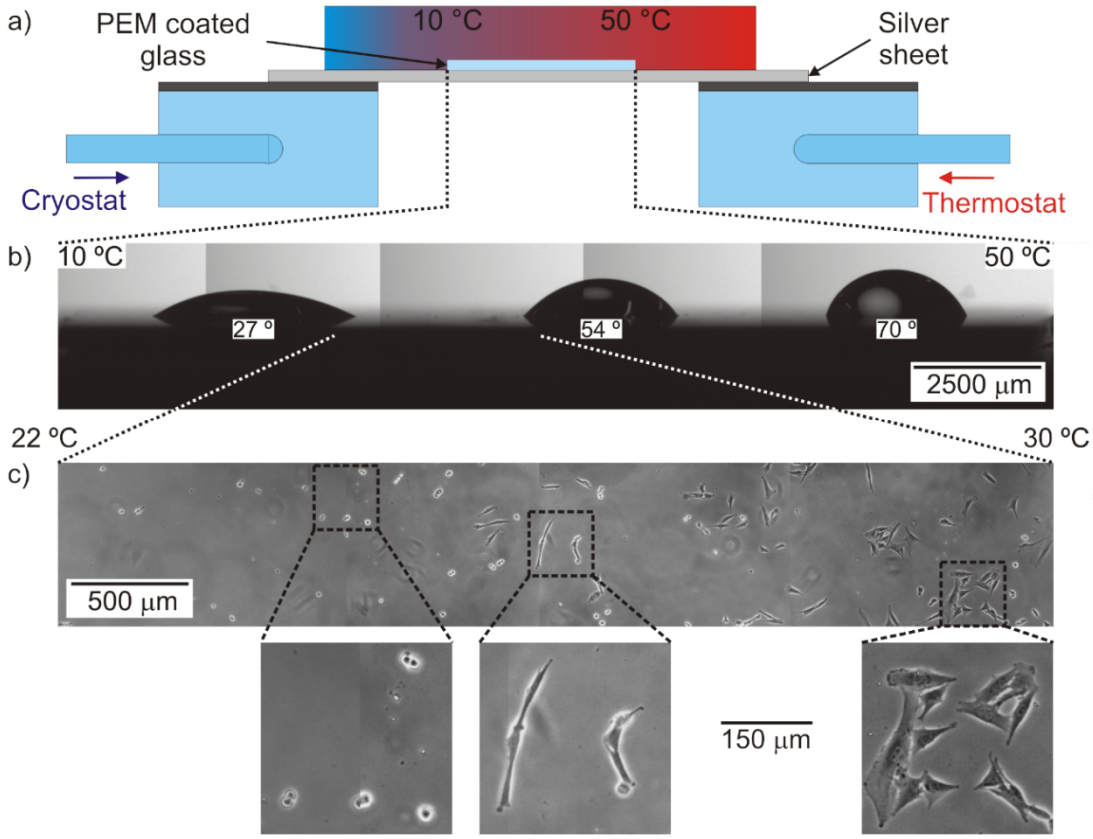

Figure 6. a) Scheme of the experimental setup to produce a temperature gradient on the PEM coated glass. Samples were placed on a silver sheet with one extreme maintained at 50 °C with a chamber connected to a thermostat and the other maintained at 10 °C with chamber connected to a cryostat. Effect of the thermal gradient on the $(PLL/Alg)_7PLL$ surface wettability **(b)** and C2C12 cell adhesion **(c).** The minimum and maximum temperature for all regions are included. Enlarged images of regions indicated by dashed squares in **(c)** are included to appreciate the changes in the cell spreading along the multilayer after the treatment with the temperature gradient.

5. Conclusions

Polyelectrolyte multilayers assembled by the Layer by Layer assembly technique have highly versatile and tunable properties, which make them very attractive materials for various biomedical applications. In this review we have summarized the cell behavior towards purely synthetic, purely natural and combined polyelectrolyte multilayers. As the use of natural polyelectrolytes is highly encouraged because of their biocompatibility and resemblance to the natural extracellular matrix, we have reviewed some of the strategies employed to tune cell adhesion on natural PEMs. We presented two of the approached followed in our group: the combination of blocks of synthetic and bio polyelectrolytes and the use of thermal annealing. Finally, as surfaces with a spatial patterning of physical and biochemical cues are very

interesting for biomedical research and applications, we summarized some of the strategies employed to generate gradients in PEMs properties in order to guide cell behavior.

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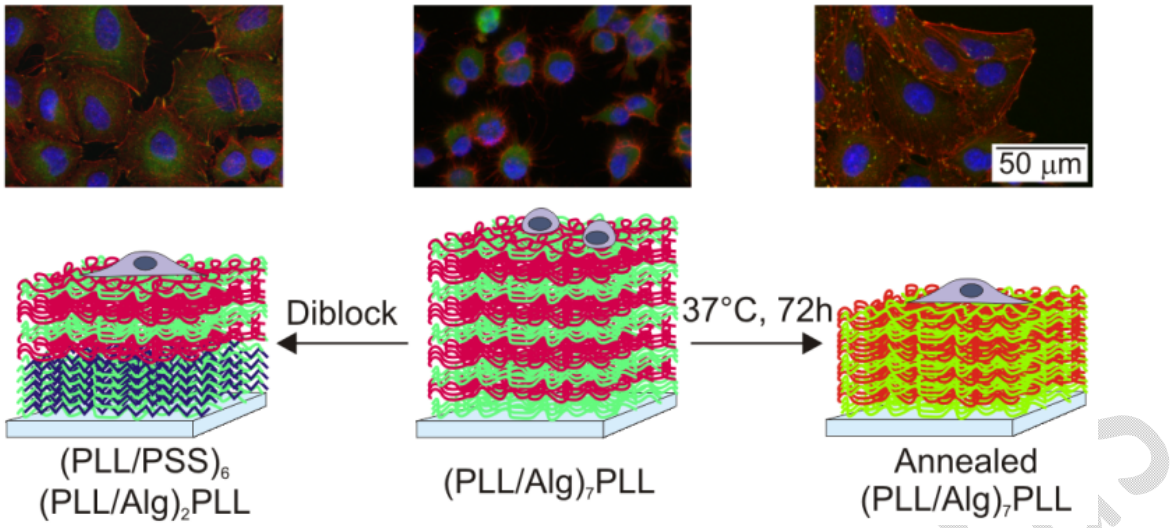
Grafical abstract



Miguel A. Pasquale, Dr., is a researcher at the "Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas" (INIFTA), CONICET, UNLP, Argentina. He is also Professor at "Universidad Nacional de La Plata", where he received his doctoral degree in 2004. His major research interest is the study of complex systems of biological interest, particularly the modulation of individual cell phenotypes and cooperative cell behavior by changing the interactions with different environments.

Sergio Moya did undergraduate studies in Chemistry at the Universidad Nacional del Sur, Argentina. He obtained a PhD in Physical Chemistry from the University of Potsdam, working at the Max Planck Institute of Colloids and Interfaces, Germany. He was post doc at the group of Jean Marie Lehn at the College de France, Paris, and at the Nanoscience Center of the University of Cambridge, UK. After postdoctoral work he moved to Mexico where he worked as Conacyt researcher at the Center of Applied Chemistry in Saltillo. Since 2007 he is group leader at CIC biomaGUNE, San Sebastian, Spain. His research interests cover the use of polyelectrolytes in nanofabrication, the development of hybrid materials, bio non bio interactions, nanomedicine and the biological fate of nanomaterials. He is the author of more than 160 articles. He has coordinated several European and international projects.

Nicolás Eduardo Muzzio joined the research group of Prof. Alejandro Arvia in 2010 as an undergraduate student, focusing on cell motility and the dynamic scaling properties of cell colonies. Upon earning his degree in biochemistry from the Universidad Nacional de La Plata in 2013, he joined the Soft Matter Laboratory and obtained a PhD in chemistry under the direction of Prof. Omar Azzaroni and Dr. Miguel Pasquale direction in 2017. Presently, he is working as post doc at the Soft Matter Nanotechnology group at CIC biomaGUNE, Spain. His current research is primarily on the synthesis of new biomaterials that control cell fate towards tissue engineering and cell studies. He is the author of 9 papers in journals of material science, biopolymers, and physical biology.