Direct Evidence for Vertical Diffusion and Exchange Processes of Polyanions and Polycations in Polyelectrolyte Multilayer Films

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The alternate deposition of polycations and polyanions on solid surfaces leads to the formation of films called "polyelectrolyte multilayers". Described in 1992 by Decher et al.,[1,2] these films have, since their discovery, received considerable attention and present numerous potential applications in fields ranging from electro-optical devices to biomaterials.[3−7] The first investigated polyelectrolyte multilayers grew linearly in both mass and thickness with the number of deposition steps. The poly(styrenesulfonate)/poly(allylamine) system constitutes a typical example of such systems.[8−11] These films present a stratified structure, each polyelectrolyte layer interpenetrating only the neighboring ones, and their growth mechanism involves mainly electrostatic interactions between the polyelectrolytes from the solution and the polyelectrolytes of opposite charge forming the outer layer of the film.[12,13] The motor of the growth mechanism is provided by the charge overcompensation that appears after each deposition step and which shows up by a sign alternating of the zeta potential film during its buildup.[10,14] More recently, Elbert et al.,[15] Picart et al.,[16] DeLongchamp et al.,[17] and Schoeler et al.[18] found new types of polyelectrolyte multilayers which are characterized by an exponential increase of both the mass and the thickness of the film with the number of deposition steps.

The exponential growth mechanism of polyelectrolyte multilayers like [copolymer acrylamide and [3-(2-methylpropionamido)propyl]trimethylammonium chloride]/poly(acrylic acid)[18] or poly(diallyldimethylammonium chloride)/poly(styrenesulfonate)[19] is attributed to the increase of surface roughness with the number of deposited layers. Hyaluronic acid/poly(L-lysine),[20] hyaluronic acid/chitosan,[21] poly(L-glutamic acid)/polyallylamine,[22] and poly(L-glutamic acid)/poly(L-lysine) [23] are several other examples of exponentially growing polyelectrolyte multilayers which rely on the ability of at least one type of the two polyelectrolytes constituting the films to diffuse "in" and "out" of the whole structure during each "bilayer" deposition step. This diffusing mechanism could be due to the such biologically derived nature of the polyelectrolytes. For the (HA/PLL) system, certainly one of the best known systems as far as the growth mechanism is concerned, it was shown that PLL is the diffusing species whereas HA seems not able to diffuse through the polyelectrolyte multilayers.[20] When such a film is brought in contact with the PLL solution, a PLL layer adsorbs on top of the film, and a fraction of PLL chains diffuse into the film in *x*, *y*, and *z* directions. These chains are free to move in the film structure and can be called "free" PLL chains. During the rinsing step by pure buffer, part of these "free" PLL chains diffuse out of the film. After contact with HA the remaining "free" PLL chains in the films also diffuse out of it. As they reach the film/solution interface, they are complexed by incoming HA chains. These complexes are bound to the film top and form the new outer PLL/HA layer. Because the amount of "free" PLL chains that can diffuse out of the film during the contact with the HA solution is proportional to the film thickness, the amount of PLL/HA complexes that forms during this step is proportional to the film thickness itself, and the buildup growth becomes exponential. The diffusion of polyelectrolytes "in" and "out" of the multilayers was first anticipated by analyzing quartz crystal microbalance (QCM) and optical waveguide light mode spectroscopy (OWLS) experiments. In this latter technique one senses the films with an evanescent wave, and when the thickness of the film exceeds the penetration length of the evanescent wave, one remains only sensitive to changes of the refractive index of the film in the vicinity of the waveguide. The "in" and "out" diffusion process of the polyelectrolytes through the films is then characterized by a cyclic evolution of the optical signal with a period corresponding to the buildup step of one bilayer.[16] A direct proof of the "in" and "out" diffusion process of PLL through the PLL/HA films was provided by confocal laser microscopy using fluorescently labeled PLL.[20] This technique also showed the absence of diffusion of HA through the multilayer film. Confocal laser microscopy is useful for the study of these kinds of films because their exponential growth mechanism leads to multilayer films whose thicknesses can be of the order of a few micrometers after deposition of one or two dozen bilayers.

A recent theoretical study showed that the diffusion of a polyelectrolyte "in" and "out" of the film should be strongly related to the concentration of noncompensated charges within the film.[24] These noncompensated charges are at the origin of a Donnan potential which attracts or repels "free" polyelectrolytes into the interior of the film: an excess of noncompensated positive charges should attract "free" polyanions whereas an excess of noncompensated negative charges should attract "free" polycations. It is only when all the charges of the polyelectrolytes are compensated that both polyelectrolytes constituting the multilayer are eventually able to diffuse into the film as "free" polyelectrolytes. Up to now, in most of the reported exponentially growing films only one polyelectrolyte seems to diffuse "in" and "out". OWLS[23] and QCM[25] seem, however, to indicate that poly(L-glutamic acid)/poly(L-lysine) (PGA/PLL) could be

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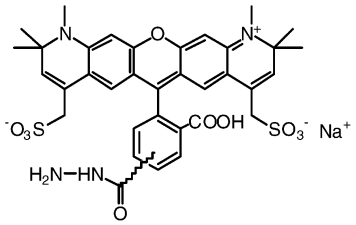
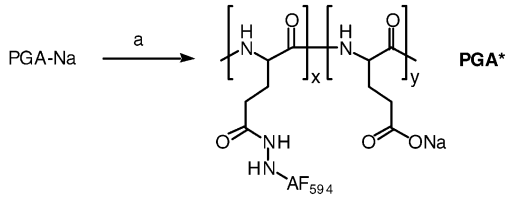
Scheme 1. Structure of Alexa Fluor 594 Hydrazide (AF_{594})



Scheme 2. Synthesis of PGA* (a: AF_{594} Hydrazide, EDCI)



a first example of an exponentially growing system where both polyelectrolytes diffuse "in" and "out" of the multilayer during each bilayer deposition step. Whereas the diffusion of PLL could be shown directly by confocal laser microscopy using PLL-FITC labeled (commercially available),[7] no direct proof of the diffusion of PGA exists up to now. It is the goal of this paper to provide such a proof. This result will be of main importance for a better understanding of the exponential growing buildup process of biologically derived systems where diffusing mechanisms are involved.

The (PLL/PGA) multilayers were built on glass slides by using an automated dipping robot (Riegler & Kirstein GmbH, Germany). The glass slides are first dipped in a PEI solution (poly(ethylenimine), MW = 7.5×10^5 Da, Sigma, France) for 10 min to adsorb a precursor layer. They were then rinsed by simple dipping in 0.15 M NaCl solutions. The polyanion PGA (MW = 7.2×10^4 Da, Sigma, France) and polycation PLL (MW = 3.26×10^4 Da, Sigma, France) were then alternatively adsorbed in the same manner.[26] After deposition of 20 (PGA/PLL) bilayers, we deposited fluorescently labeled PGA (PGA*) or a layer of PGA followed by a layer of fluorescently labeled PLL (PLL*), leading respectively to PEI-$(PGA\text{-}PLL)_{20}$-PGA* and PEI-$(PGA\text{-}PLL)_{20}$-PGA-PLL* films. As PLL*, we used fluorescein isothiocyanate labeled PLL (MW 5.02×10^4 Da, Sigma, France) emitting at 505−530 nm (green). The PGA chains were labeled with the Alexa Fluor 594 probe (Molecular Probe), emitting at 609 nm (red). The synthesis of the labeled polyelectrolyte (Schemes 1 and 2) was achieved by coupling PGA with Alexa Fluor 594 hydrazide (excess EDCI, pH = 4, aqueous solution).[27] After neutralization, the final compound was purified by dialysis against water.

The average grafting rate was measured by UV spectrophotometry and was found to be about 1.1 unit per polymer chain (see Supporting Information). The UV spectra of the free probe and the polymer grafted probe showed no frequency shift (λ_{max} = 588 nm). The same remark applies to the fluorescence emission spectra which exhibit no frequency shift of the emission maximum (λ_{max} = 609 nm) or peak broadening (see Supporting Information). This demonstrates that there is no interaction, such as stacking, between the probe residues. This is mainly due to the choice of a polar probe, along with a low grafting rate. All the polyelectrolyte solutions were used at a concentration of 5 mg/mL.

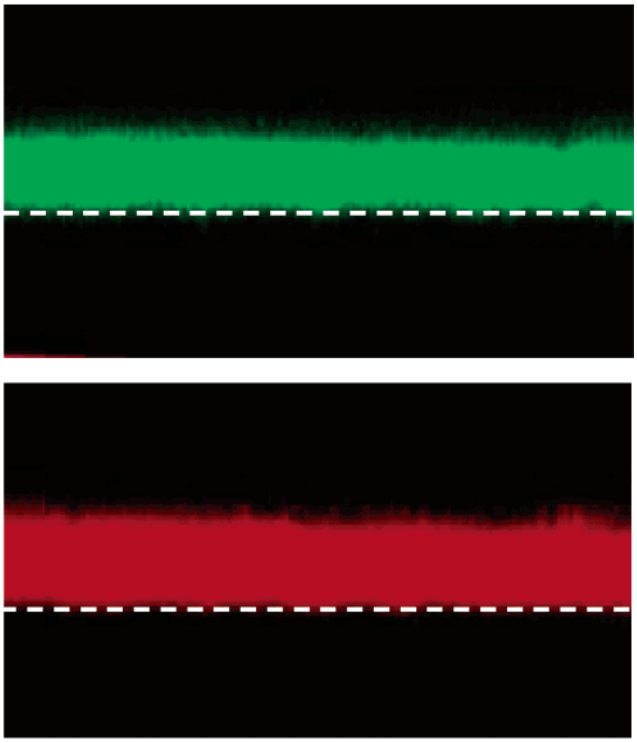


Figure 1. (A, top) Confocal laser microscopy images (x,z) of a virtual section through a polyelectrolyte multilayer film consisting in PEI/$(PGA\text{-}PLL)_{20}$-PGA-PLL* buildup on a glass slide (materialized by a dashed white line). PLL* means PLL containing a green fluorescent probe, FITC (fluorescein isotiocyanate). The image size is 74 × 32 μm^2, and the total thickness of the film corresponding to the green color is about 7.9 μm. **(**B, bottom**)** Confocal laser microscopy images (x,z) of a virtual section through a polyelectrolyte multilayer film consisting in PEI/$(PGA\text{-}PLL)_{20}$-PGA* buildup on a glass slide (materialized by a dashed white line). PGA* means PGA containing a red fluorescent probe, AR (Alexa Red). The image size is 74 × 32 μm^2, and the total thickness of the film corresponding to the red color is about 8.1 μm.

These films were then imaged on a Zeiss LSM 510 confocal laser microscope. FITC fluorescence was detected after excitation at 488 nm, cutoff dichroic mirror 488 nm, and emission long band filter 505−530 nm (green). Alexa Fluor fluorescence was detected after excitation at 543 nm, dichroic mirror 543 nm, and emission long pass filter 585 (red). Figure 1A,B shows vertical sections of these two films taken by confocal laser microscopy.

Before imaging, the films were rinsed with a 0.15 M NaCl solution. Both films appear colored over a similar thickness of around 8 μm. The film containing PGA* appears entirely red, and that containing PLL* appears entirely green. These results prove that both polyelectrolytes diffuse vertically through the entire multilayer during each deposition step. Streaming potential measurements performed on such film indicated an alterning sign of the zeta potential when passing from a multilayer ending with PGA to a multilayer ending with PLL (see Lavalle et al.,[23] Figure 7). This clearly indicates that the vertical diffusion in and out of the film follows the adsorption of the incoming polyelectrolytes on the top of the film, and a real "layer by layer" adsorption occurs.

The study of the HA/PLL film buildup also showed that free PLL* chains can exchange with nonlabeled PLL chains that constitute the film and thus become bound to the film.[28] To show that this exchange process

Table 1. Different Steps of Photobleaching Experiments Described in Figure 2

step 1	$t = 0$	film ending by PLL, bleaching of zone A
	$t = 28$ min	no change in the bleached zone is observed
step 2	$t = 29$ min	film is brought in contact with PGA* solution
	$t = 37$ min	zone B is bleached, the film remains in contact with the PGA* solution
step 3	$t = 38$ min	the film is imaged and appears entirely red
step 4	$t = 41$ min	PGA* solution is replaced by a 0.15 M NaCl solution: partial fluorescence recovery in zone B and almost entire recovery in zone A
	$t = 54$ min	no change in fluorescence recovery when compared to $t = 41$ min

is more general, we performed a series of bleaching experiments aimed to prove that "free" PGA* chains can exchange PGA chains which are part of the PLL/PGA film. We first constructed a PEI-$(PGA/PLL)_2$-PGA*-PLL-$(PGA\text{-}PLL)_{13}$-PGA*-PLL film. No free PGA or PGA* chains are expected to be present in the film since it was in contact with PLL in the last buildup step. The thickness of the film, determined by confocal laser microscopy, was of the order of 3 μm. We focused manually the focal plane at the midheight plane of the multilayer. During the whole experiment the settings of the microscope (position of the confocal plane, laser intensity, and detector sensitivity) were kept constant. At time $t = 0$, we illuminated a 150 $\times$ 20 μm^2 rectangular zone of the film over its whole height and took a 2D image of the zone with a red filter.[28] This zone will be called zone A (Figure 2, step 1). When zone A of the film was illuminated, the PGA* chains present in this zone were bleached. After 28 min no change in red intensity was observed. This proves that the PGA* chains constituting the film cannot diffuse significantly during this period of time. The film then was brought in contact with a 5 mg/mL PGA* solution, and an image was immediately taken (Figure 2, step 2). As expected, zone A of the film appears now red as the remaining of the film. This is due to the diffusion of PGA* chains from the solution into the film and in particular to the focal plane inside the film. These chains are thus "free" PGA* chains. While in contact with the PGA* solution, zone B of the film was bleached at time 37 min (zone B is a 150 $\times$ 20 μm^2 rectangular zone, near zone A), and the film was imaged at time 38 min (Figure 2, step 3). The whole film appears red, proving that nonbleached PGA* chains were able to diffuse rapidly into the bleached zone. These chains can only be the "free" chains since we proved that PGA* chains introduced previously during the film buildup are unable to diffuse over such a short time step. Finally, the PGA* solution was removed and replaced by a 0.15 M NaCl solution at time $t = 41$ min (Figure 2, step 4). A new image of the film was taken. The red fluorescence intensity in zone A is almost entirely recovered whereas only partial recovery is observed in zone B. Finally, at time $t = 54$ min, one observes again the film, but no changes are observed: the image is similar to that seen in step 4 (data not shown). Thus, no observable evolution took place after image corresponding to step 4. The various steps of the experiment are summarized in Table 1. This series of experiments demonstrates that "free" PGA*, which has diffused into the film when it was brought in contact with the PGA* solution, exchanges significantly with the PGA chains constituting the film during the 12 min of contact (between times $t = 29$ min and $t = 41$ min). Indeed, if no exchange process would take place, the red intensity in zones A and B would appear similarly (intensity around zero) after the final rinsing procedure. Moreover, the fact that zone B is only very slightly red after this rinsing step shows that the exchange process needs more than 4 min (between times $t = 37$ min and $t = 41$ min) to be very effective. The partial fluorescence recovery must be due to the early steps of the exchange process (and, to a minor extent, to the "free" PGA*

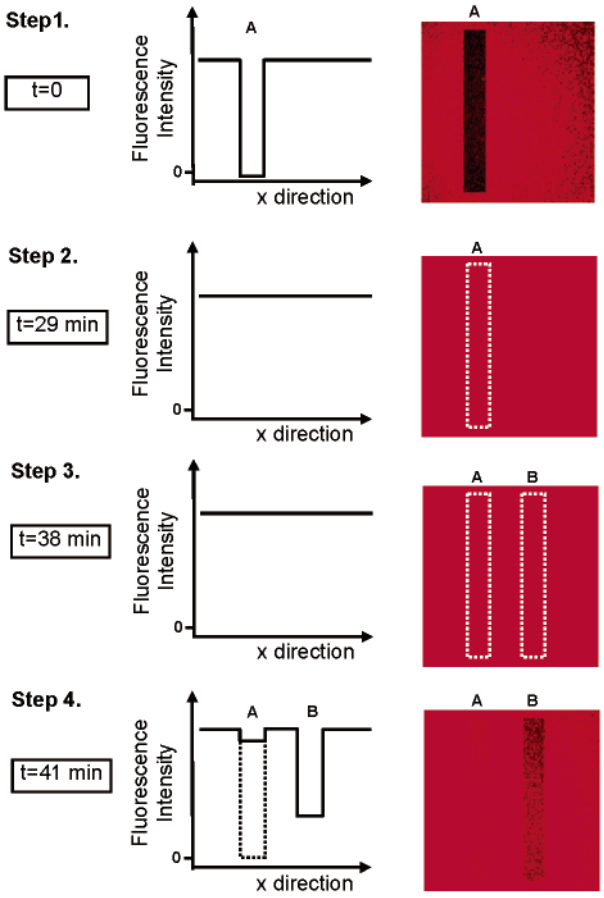


Figure 2. Confocal laser microscopy images (x,y) of a plane locate at the midheight plane of the multilayer consisting in PEI-$(PGA/PLL)_2$-PGA*-PLL-$(PGA\text{-}PLL)_{13}$-PGA*-PLL (PGA* means PGA containing a red fluorescent probe). During the whole experiment the settings of the microscope (position of the confocal plane, laser intensity, and detector sensitivity) were kept constant. Image size is 156 μm $\times$ 169 μm. At time $t = 0$, the film is in contact with a 0.15 M NaCl solution. We bleached a 150 $\times$ 20 μm^2 rectangular zone of the film (zone A), and then we took an image with a red filter (step 1). After 29 min no change in red intensity is observed. Then the film is brought in contact with a PGA* solution and an image is immediately taken (step 2). A new rectangular zone of the film is then bleached in the presence of the PGA* solution, and after 1 min, an image is acquired: the whole film appears red (step 3, zone B). The detector must be saturated by the strong fluorescence intensity, and for this purpose, zones A and B have been delimited by drawing a white dashed line. Three minutes later (at time $t = 41$ min) the PGA* solution is replaced by 0.15 M NaCl solution, and a new image is taken (step 4). No observable evolution took place after image corresponding to step 4. The red fluorescence intensity along the x direction of the image is also represented for each step in the left column (scheme of qualitative data). The dashed line in the graph of step 4 corresponds to what will be expected if no exchange process occurs at all in zone A.

chains that remain in the film after the rinsing step).

Finally, this exchange process is of primary importance in applications where peptides covalently bound to polyelectrolytes like PLL or PGA are inserted in PLL/PGA multilayers as it is described in the work of Chluba et al.[6] Some PGA chains constituting the film can exchange free PGA chains linked to the peptide and deposited on the top of the film. This can lead to a film entirely functionnalized with the peptide. However, the exchange process is probably not sufficient to lead to a homogeneous peptide concentration over the entire film thickness.

In conclusion, previous studies have showed that PGA/PLL constitutes an exponentially growing system and that PLL diffuses "in" and "out" of the entire film during each bilayer deposition step. In this note we proved that, similarly to PLL, PGA also diffuses through the whole film. In addition, "free" PGA chains in the film exchange with chains forming the network of the PLL/PGA multilayer, and the exchange process takes place over a typical time which is of the order of 10 min.

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Supporting Information Available: UV spectra of PGA* and AF594 (Figure I) and fluorescence spectra of PGA* and AF594 (Figure II). This material is available free of charge via the Internet at http://pubs.acs.org.

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