Supplementary information

Optimization of Amine-Rich Multilayer Thin Films for the Capture and Quantification of Prostate-Specific Antigen

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Effects of Salt and Molecular Weight on PAH/SPS film growth

Figure S1 displays the growth curve profiles for all assembling parameters used in in this work for PAH/SPS films.

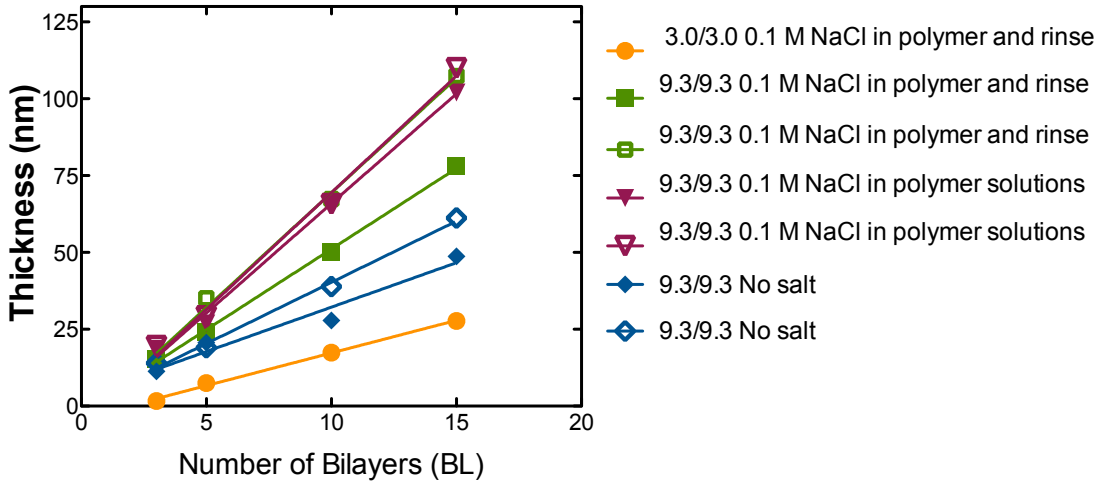

Figure S1. Growth curve of investigated systems for NHS-functionalization. PAH/SPS films were assembled by varying the pH of assembly (pH 3.0/3.3 or 9.3/9.3); molecular weight of PAH of 15 kDa (full symbols) or 56 kDa (open symbols); and by varying the ionic strength of polymer solution or of both polymer and rinse solutions (no salt or 0.1 M NaCl added to solutions).

Compared to films assembled at pH 9.3, when the PAH/SPS system is assembled at pH 3.0 thinner films are observed (Table S1, Figures S1), which is likely due to the high charge density along the polymer backbone due to complete amine protonation at pH 3.0. The electrostatic repulsion along the polymer chain promotes an extended conformation and the numerous ammonium groups causes PAH to strongly pair with the strong polyanion, SPS, during film deposition. Thus, a lower quantity of PAH is needed for charge compensation of SPS in comparison to films assembled at higher pHs, closer to

the isoelectric point of PAH. Previous work has shown that PAH/SPS films assembled at pH 9.3 present a more enriched PAH fraction in the film (70 % mol) due to its poor ionization, thus dramatically increasing the thickness of the films.[1] The lower charge density along the polymer backbone allows PAH to adopt a more flexible conformation and so when deposited, presents more loops and tails that contributes to greater thickness deposited per bilayer. Thicknesses/BL of all films at same ionic strength assembled at pH 9.3 were greater than those deposited at pH 3 (Table S1, Figure S1).

Table S1. Thickness (nm) per bilayer of PAH/SPS films assembled at different parameters. Thicknesses were determined by spectroscopy ellipsometry.

pH Condition:	**Assembly at pH 9.3**			**Assembly at pH 3.0**
Salt Condition:	**No Salt**	**0.1 M NaCl in polymer solutions**	**0.1 M NaCl in polymer and rinse solutions**	**0.1 M NaCl in polymer and rinse solutions**
PAH 15kDa	2.89 ± 0.43	7.14 ± 0.33	5.25 ± 0.13	2.12 ± 0.09
PAH 56kDa	3.98 ± 0.20	7.60 ± 0.45	7.47 ± 0.31	-

In addition to these pH-induced effects, we found that the film thickness of the 9.3/9.3 system increases when salt is present in solely the polymer solution (confirming previous studies)[2] and when present in both the polymer and rinse solutions, but to a lesser degree; thickness per layer incrementally increases from films deposited from no salt in either polymer or rinse solutions (3-4 nm / BL), to salt only in the polymer solution (~ 7 nm / BL), and to salt in both polymer and rinse solutions (5-7 nm / BL) (Table S1). While salted polymer and salted polymer/rinse films likely have similarities during the polymer adsorption, whether salt is present in the rinse appears to have considerable

effect on the resultant film thickness. In the salted polymer/rinse films, it is likely that the polymer deposited maintains a similar conformation before and after the rinse. In contrast, a rinse without salt will likely remove the counterions present in the film that stabilize the polymer conformation by reducing repulsive effects from nearest neighbor charges. As a consequence, it is possible that the polyelectrolytes may undergo conformational rearrangement that exposes additional polymer charges which in the subsequent deposition step requires additional material deposition for charge reversal.

The Mw of the PAH was also found to influence film thickness (Figure S1) - most noticeably for films built with salt in all assembly steps, where assembly with the higher molecular weight PAH became about 29 nm thicker for 15 BL films. When no salt is present, those deposited with higher molecular weight polymer chains (56 kDa) had slightly thicker films than those with lower molecular weight polymer chains (15 kDa) (about 12.5 nm thicker for 15 BL). On the other hand, for films assembled from solely salted polymer solutions, the variation were about 8 nm for the multilayers assembled with the 56 kDa PAH (Figure S1).

Both ionic strength and polymer molecular weight play effects on film deposition that will impact the films functionalization, as will be further discussed in the following sections.

Effects of assembly parameters on amine functionalization

While each of these permutations in film architecture had uniquely interesting effects on the film morphology, we ultimately wanted to evaluate each film system for its ability for functionalization. We probed this by using the NHS-ester of fluorescein, NHS-Fluor,

and used the film's uptake of fluorescein absorbance as an indicator of the relative accessibilities of the primary amines (Figure S2). As controls to determine non-specific interactions, we treated films under the same conditions with fluorescein sodium salt (Fluor) at 5mM for 2 h at room temperature.

Three different parameters were primarily evaluated: the influence of ionic strength of solution during assembly (no salt or 0.1 M NaCl), the Mw of PAH (15 or 56 kDa), and the effect of a post-assembly acid treatment. Figure S2a displays the absorbance values acquired for as deposited (AD) samples, while Figure S2b show the results for those after acid treatment (AT).

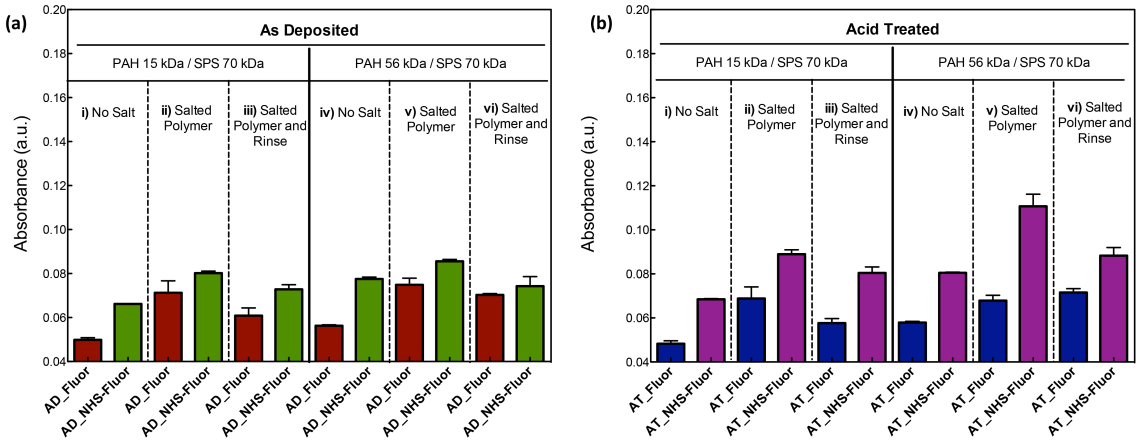

Figure S2. Absorbance at 540 nm acquired by Uv-vis. Effect of Mw, salt and acid treatment on NHS-functionalization performed at pH 8.3 for 2h. **(a)** As deposited samples; **(b)** Acid treated samples (pH 2.5 for 15 min). All tests were performed with 15.5 bilayers of PAH/SPS assembled at pH 9.3 using spin-assisted deposition. As controls for non-specific absorption films were reacted in 5 mM fluorescein sodium salt (Fluor). NHS-Fluor = NHS-fluorescein; AT = acid treated films; AD = as deposited films.

Regarding the addition of salt during assembling, when salt free polyelectrolyte solutions are assembled, the proximity and density of charges within a single polyelectrolyte chain strongly repel each other, resulting in an almost fully extended configuration. When depositing onto a negatively charged surface this typically results in thinner, smoother and more homogenous films, which likely translates into a consistent amine composition present in the films (Figures S2a and S2b, (*i*) and (*iv*)). In the presence of salt, counter ions can screen charges along the polyelectrolyte chain allowing it to fold into a random coil configuration.[3] This is reflected with the addition of 0.1 M of NaCl to the polymer or both polymer and rinse solutions, which generated greater inconsistency in functionalization due to rougher loop-rich structures (Figures S2a and S2b, (*ii, iii, v* and *vi*)). For salted polymer and rinse solutions, the nitrogen will tend to be in the salt form $-NH_3^+ Cl^-$ during film deposition and so less polymer will be deposited compared to films built with rinse without salt, leading to less amine groups compared to salt only. Whereas for the salted polymer samples, the unsalted rinse removes the excess of salt after polymer deposition, which will yield thicker films due to more polymer deposition and, consequently, more amine groups available for reaction. As observed on Figure S2b, the greatest degree of total fluorescein-functionalization in the film came from films assembled with PAH 56 kDa, in 0.1 M NaCl solutions (Figure S2b(*v*)).

By increasing the Mw of PAH from 15 kDa to 56 kDa one can observe that amine functionalization is improved (Figures S2a and S2b). As discussed above, higher molecular weight PAH has an effect on film deposition. Lower molecular weight polyelectrolytes could diffuse through the film. Thus we suggest that high molecular

weight polycation PAH is deposited with more loops and create more pockets when compared to the lower molecular weight PAH.

XPS Analyses

Figure S3 shows a fitting example used on data acquired by XPS analyses. Casa XPS was used for peak fitting and calculation of composition of amine vs. ammonium on films surfaces. Figure S3 demonstrates that ammonium and amine peaks are very distinct.

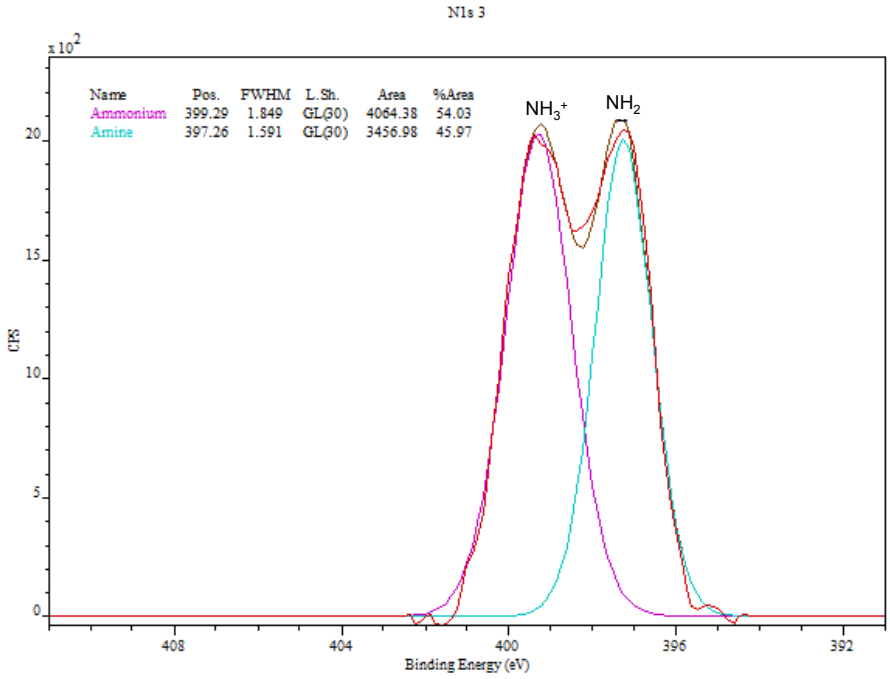

Figure S3. N1s spectra from XPS analyses with peak fitting for ammine and ammonium groups. Spectra of PAH/SPS 9.3/9.3 acid treated films after incubation in pH 10 solution for 2 hours.

Morphology Characterization

Accordingly to previous findings,[4] salted polymer 9.3/9.3 multilayers exhibited higher rose bengal dye uptake when acid treated, if compared to 9.3/9.3 AD and to 3.0/3.0 films. This result demonstrates that the 9.3/9.3 acid treated films presents a higher density of reactive amines (Figure S4).

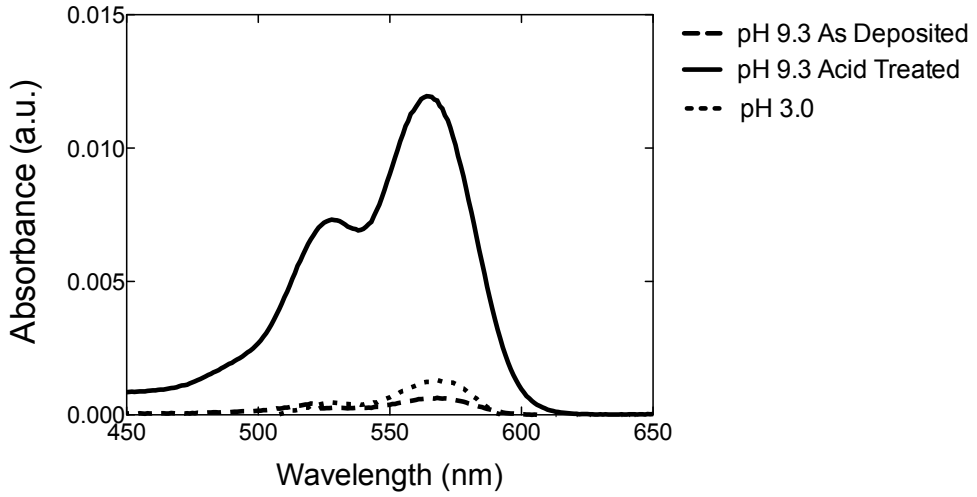

Figure S4. Rose bengal dye uptake absorbance.

AFM fluid was used to characterize surface morphology and roughness of PAH/SPS films in water. As shown in Figure S5, 3.0/3.0 and as deposited 9.3/9.3 multilayers present a very smooth surface. As expected, after acid treatment, 9.3/9.3 films present higher features and an increased roughness, which can be attributed to the opening of hydrophobic pockets and swelling of the system.[1, 4]

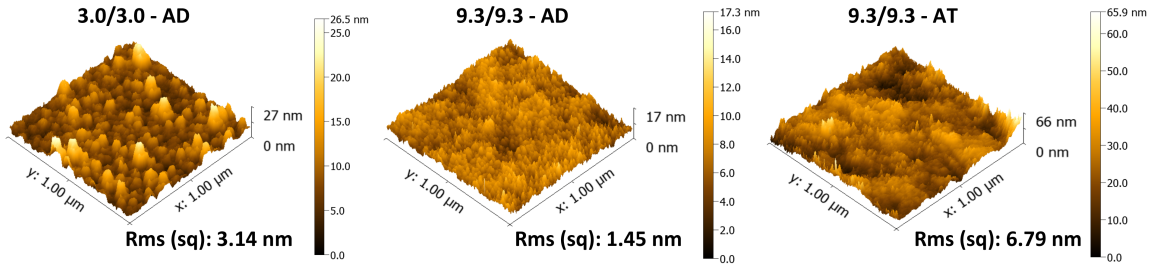

Figure S5. AFM-fluid images of PAH/SPS 3.0/3.0 and 9.3/9.3 multilayers, acquired in deionized water. Effects of pH of assembly (3.0/3.0 or 9.3/9.3), and acid treatment (pH 2.5, 15 min) on film topography and roughness. Films were built with salted polymer solutions (NSR).

Comparison of Post- and Pre- biotinylation on Swelling and capture

We decided to investigate whether the biotinylation of PAH before film deposition (pre-biotinylation) would favor both swelling and capture by those films. PAH was functionalized prior to multilayer assembly by reaction with NHS-biotin in solution (See details in Experimental Section) and then assembled into PAH-biotin/SPS 9.3/9.3 films with salted polymer solutions. These films presented similar thicknesses as those built with non-functionalized PAH with an average of 6 nm/bilayer for the former and 6.2 nm/bilayer for the latter.

Despite these similarities in film growth, their efficacy in capturing Qdots was considerably different. As shown in Figure S6, both acid treated films present higher capture when compared to as deposited films, but post-assembly biotinylation improves capture 2.5-fold over those films assembled with pre-assembly biotinylated, PAH-biotin. The lower capture presented by the latter is likely affected by two factors: 1) the final structure of films after assembly; and 2) the yield of biotinylation achieved on films

surfaces. For the former, the functionalization of the PAH/SPS 9.3/9.3 films will occur on the outermost layer of the films, while on the pre-biotinylated films most of this functionalization will be embedded into the multilayer system. Also, as biotin is a small hydrophobic molecule, on pre-assembled functionalized films it can be possibly hindered inside the hydrophobic clusters presented by those films. In the later case, the Qdot accessibility of biotin conjugated after the film has been assembled is likely greater than the biotin incorporated into the film as PAH-biotin conjugates.

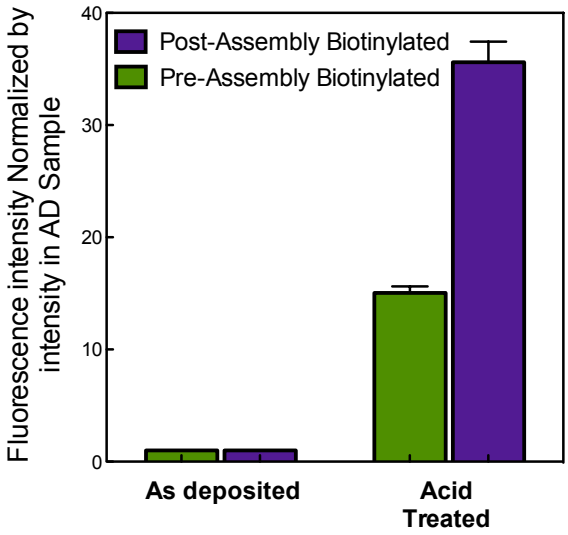

Figure S6. Effect of pre- and post-assembly biotinylation on the capture of streptavidin conjugated Qdtos.

Swelling is also affected on the pre-biotinylated system. Post-biotinylated films presented a 2-fold increase in swelling in water (200 % swelling) compared to pre-biotinylated films (100 % swelling). Similarly to post-biotinylated acid treated films, swelling in PBS buffer was not favored for pre-biotinylated samples – 40 % against 30 % for post-biotinylated films. The lower swelling capability presented by the pre-assembly

biotinylated films is most likely to be correlated with the excess of hydrophobic groups on the film core and surface, when compared to the post biotinylated films.

Swelling of PAH/SPS 9.3/9.3 multilayers

Figure S7 shows the swelling degree (%) for films assembled without salt and salted polymer solutions (no salt on rinse, NSR, or also referred to as "salted polymer solutions") multilayers. Data was acquired by spectroscopy ellipsometry using a fluid chamber[5] in the presence of deionized water or in PBS. For both deposition parameters, no salt and NSR, acid treated films exhibited higher swelling degree, as expected. In this work we found that "No Salt" films swell about 100% in water, once undergo AT (4.5-fold increase in swell when compared to AD films). NSR films swell 3-fold more than "No salt" films upon AT. In contrast, both films present a low swelling behavior in PBS. Even after AT, swelling is not increased. The low swelling degree in PBS buffer can be attributed to film collapse in the presence of salt solutions.

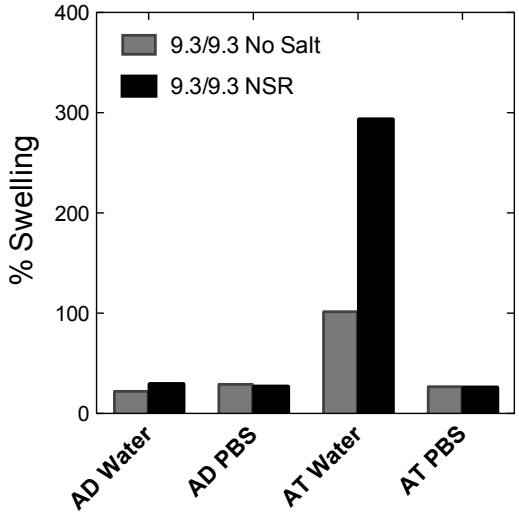

Figure S7. Swelling degree for PAH/SPS 9.3/9.3 films assembled with no salt, and for films assembled with 0.1 M NaCl on polymer solutions (no salt on rinse, NSR). Swelling was measured for as deposited (AD) and acid treated (AT) films in deionized water and in PBS buffer.

PSA detection in PBS

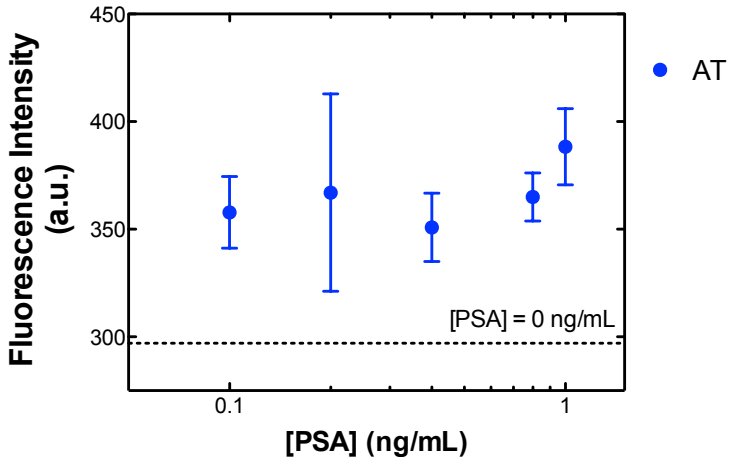

Figure S8. Semi log fit for PSA antigen detection in the range of 1.0 ng/mL to 0.1 ng/mL. Quantification was achieved by using streptavidin conjugated Qdots 655. PSA was diluted in PBS buffer. Fluorescence intensity of Qdots was observed by fluorescence microscopy and then quantified using Image J.

Fluorescent images of PSA capture performed in FBS

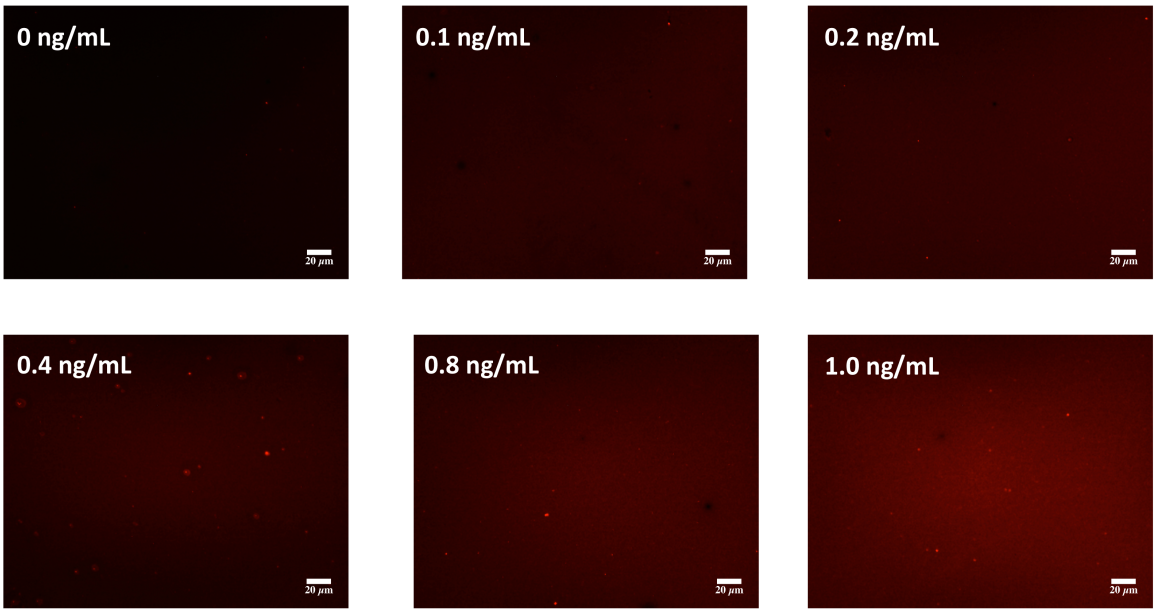

Figure S9. Fluorescent images of PSA capture performed in FBS. PSA antigen was detected in the range of 1.0 ng/mL to 0.1 ng/mL. Quantification was achieved by using streptavidin conjugated Qdots 655. Fluorescence intensity of Qdots was observed by fluorescence microscopy and then quantified using Image J. Scale bars are 20 µm.

Release of particles upon acid treatment

The swelling behavior after the capture protocol is shown in Figure S10. As expected, after biotinylation and capture protocol, the system presents low swelling capability of about 20 %. In this case, the acid treatment also does not recover the high swelling of the system (40 %). Also, upon acid treatment 67 % of the particles are released, most probably due to denaturation of proteins. POC diagnose devices might require further investigated for concentration and release of rare biomarkers.

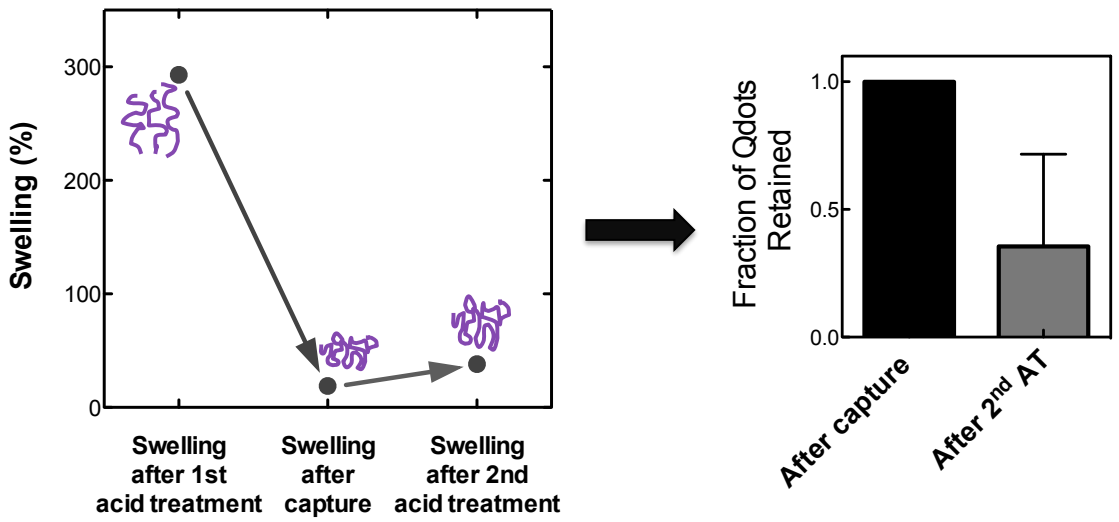

Figure S10. Swelling degree and respective fraction of released particles (Qdots) after acid treatment.

Experimental Section

Rose bengal dye uptake: Rose Bengal dye uptake was used to confirm the presence of free cationic charges in the films. Samples were immersed for 15 min in a 1 mM solution of Rose bengal solubilized in water pH 7. The samples were rinsed twice with deionized water and air-dried. Absorbance was measured with a Varian Cary 5E spectrophotometer.

Atomic force microscopy: Surface morphology of PAH/SPS films was investigated by using a Nanoscope IV/D3100 with glass cantilever mount according to the literature.[6] Processing and analysis of images was carried out using Image J software.

Pre-assembly NHS functionalization: PAH (1 mg / mL) was solubilized in 50 mM MES buffer pH 8.1 was reacted with NHS-Biotin (22 mg / mL), previously solubilized in DMSO. The reaction was carried out for 4h at room temperature. The sample was dialyzed against ultrapure water for 3 times of 2h each by using a dialysis cassette of 10 kDa Mw cut-off. After dialyses 0.1 M NaCl was added to polymer solution and the pH adjusted to 9.3 using 1 M NaOH. PAH-Biotin was assembled with SPS at pH 9.3 with 0.1 M NaCl on polymer solutions.

References

(1) Itano, K.; Choi, J. Y.; Rubner, M. F. Mechanism of the pH-induced discontinuous swelling/deswelling transitions of poly(allylamine hydrochloride)-containing polyelectrolyte multilayer films. *Macromolecules* **2005,** *38* (8), 3450-3460.

(2) Ladam, G.; Schaad, P.; Voegel, J. C.; Schaaf, P.; Decher, G.; Cuisinier, F. In situ determination of the structural properties of initially deposited polyelectrolyte multilayers. *Langmuir* **2000,** *16* (3), 1249-1255.

(3) McAloney, R. A.; Sinyor, M.; Dudnik, V.; Goh, M. C. Atomic force microscopy studies of salt effects on polyelectrolyte multilayer film morphology. *Langmuir* **2001,** *17* (21), 6655-6663.

(4) Lichter, J. A.; Rubner, M. F. Polyelectrolyte Multilayers with Intrinsic Antimicrobial Functionality: The Importance of Mobile Polycations. *Langmuir* **2009,** *25* (13), 7686-7694.

(5) Hiller, J.; Mendelsohn, J. D.; Rubner, M. F. Reversibly erasable nanoporous anti-reflection coatings from polyelectrolyte multilayers. *Nat. Mater.* **2002,** *1* (1), 59-63.

(6) Palmer, A. F.; Wingert, P.; Nickels, J. Atomic force microscopy and light scattering of small unilamellar actin-containing liposomes. *Biophys. J.* **2003,** *85* (2), 1233-1247.