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Mobility of lysozyme in poly(L-lysine)/hyaluronic acid multilayer films

Natalia Velk[a,b], Katja Uhlig[a], Anna Vikulina[a,c], Claus Duschl[a], Dmitry Volodkin[a,c,*]

[a] Fraunhofer Institute for Cell Therapy and Immunology, Branch Bioanalytics and Bioprocesses (Fraunhofer IZI-BB), Department Cellular Biotechnology & Biochips, Am Mühlenberg 13, 14476 Potsdam-Golm, Germany
[b] University of Potsdam, Institute for Physics and Astronomy, Karl-Liebknecht-Str. 24-25, 14476 Potsdam-Golm, Germany
[c] School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom

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ABSTRACT

The spatial and temporal control over presentation of protein-based biomolecules such as growth factors and hormones is crucial for in vitro applications to mimic the complex in vivo environment. We investigated the interaction of a model protein lysozyme (Lys) with poly(L-lysine)/hyaluronic acid (PLL/HA) multilayer films. We focused on Lys diffusion as well as adsorption and retention within the film as a function of the film deposition conditions and post-treatment. Additionally, an effect of Lys concentration on its mobility was probed. A combination of confocal fluorescence microscopy, fluorescence recovery after photobleaching, and microfluidics was employed for this investigation. Our main finding is that adsorption of PLL and HA after protein loading induces acceleration and reduction of Lys mobility, respectively. These results suggest that a charge balance in the film to a high extent governs the protein–film interaction. We believe that control over protein mobility is a key to reach the full potential of the PLL/HA films as reservoirs for biomolecules depending on the application demand.

1. Introduction

Advanced biomaterials research is a dynamically developing field in modern biotechnology. In particular, materials that form interfaces between biological systems and technical substrates and which can be employed for tuning the biological response are of high interest [1]. A well-known modern technique — layer-by-layer (LbL) assembly [2] — opens a wide range of possibilities for surface functionalization. Oppositely charged polyelectrolytes (PE) can serve as building blocks of LbL films. Among drug delivery systems polyelectrolyte multilayers (PEM) are highly ranked due to a broad spectrum of characteristic advantages, such as preparation conditions similar to the physiological ones, precise control over the geometrical organization, mechanical properties, chemical composition of the films and ability to host biomolecules [3–8].

A variety of bioactive molecules were reported to be immobilized within the PEMs [4,9–13]. Binding specificity of the agents loaded into the PEMs is crucial for practical applications [5–7,10,14]. The macromolecular interactions between the film and loaded compounds can affect a state of the loaded biomolecules and induce a pronounced influence on cell behaviour [10,15]. However, despite an evident applied interest to employ biomolecule-laden PEMs for cellular applications, there is a lack of knowledge concerning the mechanism of interaction between the loaded biomolecules and PEMs.

Analysis of the loading/release of biomolecules into/from polymer-based matrices is a well-established approach for characterizing intermolecular interactions [12,13,16–18]. Fluorescence recovery after photobleaching (FRAP) is a powerful technique for probing the diffusion of fluorescently labelled species at different spatial scales [9,11,16,19–23]. FRAP-approach allows one to evaluate a "mean" diffusion coefficient of compounds [20] and perform multifractional analysis to identify populations of diffusing molecules [24].

Poly(L-lysine) (PLL) and hyaluronic acid (HA) are widely studied biopolymers. The PEMs of these polyelectrolytes are so-called "exponentially" growing films, which are generally much thicker than the "linearly" growing ones [4]. Therefore, the PLL/HA films have a particularly high capacity for loading biomolecules [11]. Noteworthy, the considerable thickness of PLL/HA PEMs makes it possible to analyse the film structure using optical microscopy [25].

In this study we aimed at understanding protein–PEM interactions using the model protein lysozyme (Lys) and PLL/HA PEMs.

* Corresponding author at: School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom.
E-mail address: dmitry.volodkin@ntu.ac.uk (D. Volodkin).

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We employed FRAP and confocal laser scanning microscope (CLSM) to study kinetics of protein loading/release and protein mobility within the film as well as protein distribution as a function of PEM composition and environmental conditions. Variation of the last deposition "layer", temperature, ionic strength and pH are considered for that. Microfluidics is adopted allowing reducing the amount of used compounds and controlling the polyelectrolyte/protein mass transport. Instead of commonly used flat substrates, microfibres were implemented as recently suggested [9]. This approach allowed us to examine at once the whole cross section of the film. This leads to a higher temporal, spatial resolution [9] and degree of control over the sample position. We believe, a combination of high-resolution fluorescence imaging with microfluidics [26] might prove to be a useful platform for probing intermolecular interactions within PEMs.

2. Experimental

2.1. Materials

PLL hydrobromide (~15–30 kDa), fluorescein isothiocyanate (FITC), PLL hydrobromide labelled with FITC (PLL^{FITC}, ~15–30 kDa) and polyethyleneimine (PEI, ~750 kDa) were purchased from Sigma-Aldrich (Germany). HA (~357 kDa) was purchased from Lifecore Biomedicals (USA). Lys was purchased from Calbiochem (Canada). If not additionally specified, Tris buffer containing 10 mM Tris (Sigma, Germany) and 15 mM NaCl (Sigma, Germany) at pH adjusted to 7.2–7.4 with hydrochloric acid (Merck, Germany) was used (standard conditions) as rinsing medium and to dissolve the PEs and Lys. To study the influence of assembly conditions, Tris buffer of different amount of NaCl (150 mM) or pH value (pH 9) was used. PEI, PLL and HA were prepared at a concentration of 0.5 mg/ml. The water was prepared in a three-stage Millipore Milli-Q Plus 185 purification system (resistivity >18.2 MΩ cm).

2.2. Lys labelling

Lys was labelled with FITC (Lys^{FITC}). The concentration of Lys^{FITC} solution in Tris buffer was estimated with the spectrophotometer PEQLab NanoDrop ND-1000 to be 0.88 mg/ml (for details see supporting information).

2.3. Preparation of the films

The films were prepared by the LbL deposition technique on glass fibres (cross section: 100 μm, Hilgenberg, Germany) [9,27] or, if specified, on glass coverslips (diameter: 12 mm, Menzel, Germany) using a dipping robot (DR 3, Riegler & Kirstein GmbH, Berlin, Germany). The PEM coating was performed by alternately dipping the substrates into PLL and HA solutions for 10 min with intermediate washing steps with buffer. PEI was used as an activating layer and adsorbed (in place of PLL) during the first deposition cycle. Film build-up was conducted at 37 °C (for coverslips at 24 °C) for 24 cycles. Further, the designation $(PLL/HA)_i$ will be used, where i defines the number of deposited "bilayers" during assembly and post-treatment of the PEM. The films were stored in Tris buffer at 4 °C.

2.4. Scanning electron microscope (SEM)

The PEMs on the microfibres were lyophilized by using Christ Alpha 2–4 LSC freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). For SEM-imaging, a Gemini LEO 1550 electron microscope (Carl Zeiss, Germany) was used at an accelerating voltage of 3 kV.

2.5. CLSM

Micrographs of the films were taken with a 510 Meta confocal microscope (Zeiss, Germany). Measurements were performed using a 64 × 1.4 oil immersion objective in reflection (λ_{ex} = 488 nm) and transmission (λ_{ex} = 633 nm) mode.

2.6. Microfluidic experiments on Lys loading and retention in the film

A special CLSM–microfluidic setup was developed (Fig. S1). CLSM images were taken every 30 s (for details see supporting information).

2.7. Automated calculation of the fluorescence signal from the film and the thickness of the film

Based on the CLSM images the fluorescence signal and the thickness of the film were calculated using the MATLAB R2014a software package (for details see supporting information, Fig. S2).

2.8. Estimation of Lys concentration in the film

Concentration of Lys^{FITC} in the films was estimated by fluorescence measurements (for details see supporting information, Fig. S3).

2.9. Preparation of samples for FRAP measurements

Prior to FRAP measurements PEMs were incubated in Lys^{FITC} or PLL^{FITC} solutions (for details see supporting information).

2.10. FRAP

The sample was placed in a self-made chamber with glass bottom filled with buffer. FITC molecules were bleached with the maximum laser intensity of CLSM in a region of ~2 μm width. A lower intensity was used to record the fluorescence recovery. For details see supporting information.

2.11. Calculation of Lys and PLL diffusion coefficient in the film by FRAP data

To estimate the "mean" diffusion coefficient of Lys or PLL from the FRAP data we used the procedure described by Seiffert and Oppermann [20]. For some experiments prior to fitting the experimental profiles to the Gaussian function the upper and the lower part of the profiles were averaged, which led to the reduction of the standard deviations. For multifractional analysis a program AFA running in the MATLAB environment developed by G. I. Hauser et al. [24] was used.

2.12. Diffusion of Lys during loading into the film

After refreshing (incubation in the PE solutions in the sequence continuing the assembly process), an HA-terminated film was incubated in Lys^{FITC} solution. After 10 min, 30 min, 1 h and every further hour with a total incubation time in Lys^{FITC} solution of 5 h, FRAP measurements were performed in different regions of the film.

2.13. Diffusion of Lys during post-treatment

During the incubation of the PEM loaded with Lys^{FITC} in the PE solutions the FRAP experiments were performed in different regions of the film (for details see supporting information).

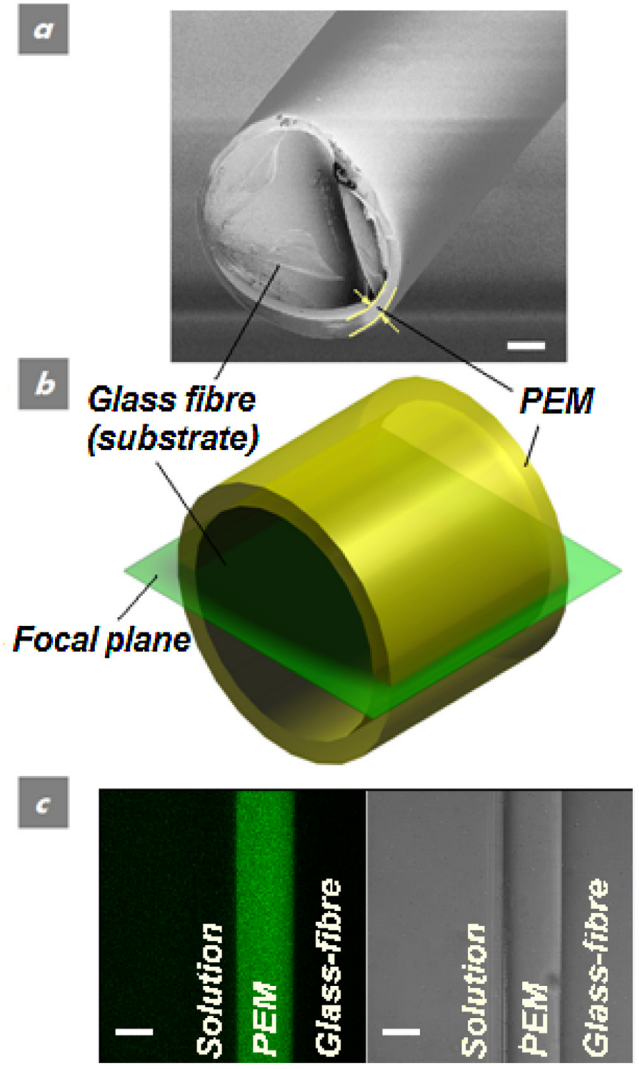


Fig. 1. a) SEM image of the PEM-coated microfibre (the scale bar is 20 μm); b) scheme of the microfibre coated with the PEM and the focal plane; c) CLSM images of the PEM loaded with Lys^{FITC} in reflection (left) and transmission (right) modes (the scale bar is 20 μm).

3. Results and discussion

3.1. Lys loading into the film

PLL/HA films were assembled on cylindrical microfibres (Fig. 1a) [9]. By fluorescence imaging via CLSM (Fig. 1b,c) the loading process of Lys^{FITC} into the PEMs was recorded. Implementation of the microfibres allowed us to examine the cross section of the film in the horizontal plane of the CLSM (Fig. 1b,c). We found that from the beginning of Lys^{FITC} loading the fluorescence signal was homogeneously distributed throughout the whole cross section of the film, and it increased with time (Fig. S4). The absence of a gradient in the concentration of protein points towards a fast diffusion of Lys in the PEM.

Importantly, Lys^{FITC} was penetrating in both – HA- and PLL-terminated films. The time course of the fluorescence signal from the film during Lys^{FITC} loading for these two cases is presented in Fig. 2a, b (Part 1: Loading of Lys^{FITC}). Measurements were performed twice for either PLL- or HA-terminated film (thus, four in total). In Fig. 2 one exemplary measurement is presented for each case. The experimental data were fitted to the function

$$I = I_{max} - A \cdot e^{-\frac{t}{\tau}}, \quad (1)$$

where I_{max} is the saturation intensity, τ – characteristic time, t – time (in min), A – constant. The results of fitting are presented in Table S1. Comparison of mean values of all fitting parameters shows, that the adsorption of the cationic protein into the film does not depend on the type of the terminating "layer". This may presumably be related to the short incubation times in either PLL or HA solutions before protein loading.

The amount of Lys^{FITC} in the film almost reached a saturation value (Fig. 2, Part 1: Loading of Lys^{FITC}). In a separate experiment the amount of the loaded Lys^{FITC} was determined by dissolution of films loaded with Lys^{FITC} and measurement of the fluorescence intensity of the resulting solution. Using a calibration curve (Fig. S3), the concentration of Lys^{FITC} was found to be in the range of 30–100 mg/ml, which corresponds to 2–7 μg of Lys^{FITC} adsorbed by a film with an approximate surface area of 0.7 mm^2 and volume of 0.07 μl.

The thicknesses of the films were ~20 ± 7 μm at the beginning of Lys loading, and they did not change with time (Fig. 2, Part 1: Loading of Lys^{FITC}). It is important to note, that these values are several times higher than the thicknesses of the films assembled on flat substrates (of the order of 0.6 μm, Fig. S5). This difference could be related to a more efficient polymer supply in case of the cylindrical microfibre. Noteworthy, the PEM growth is very much influenced by the polymer mass transport [26].

3.2. Lys retention in the film during post-treatment

After studying the loading process, the measurements described in the previous section were extended in order to gain information about Lys^{FITC} retention during adsorption of additional "layers" of PEs on the film following the sequence HA-PLL-HA or PLL-HA-PLL (Fig. 2, Part 2: Post-treatment). Four series of experiments were performed which differed in the type of the terminal PE (HA or PLL) both before and after the loading of Lys (see Table 1). There were no differences in the fluorescence intensity and film thickness in dependence of the terminating "layer" of the film (PLL or HA) prior to Lys loading. Therefore, we will not further distinguish the terminating "layer" of the film before protein loading. Thus, only two of the four experiments differing in the sequence of post-treatment are presented in Fig. 2. Incubation in the PLL solution induces considerable changes within the film such as changes of fluorescence intensity, and an increase of the film thickness by 20–30 % (Fig. 2). In contrast, incubation in the HA solution leads to the decrease of the film thickness by 5–10 % (Fig. 2).

The fluorescence intensity increase at the beginning of the PLL deposition could be related to the following effect. The fluorescence properties of FITC are dependent on the protonation of FITC. Thus, change of the pH leads to a shift of the maximum of the fluorescence spectrum of FITC, and, as a result, the quantum yield of FITC changes [28]. It is known that PLL molecules (in contrast to HA molecules) are able to diffuse into the film [29]. The amino groups of the PLL chains ($-NH_2$ groups getting protonated to $-NH_3^+$ groups) may induce an increase of the local pH in the film, leading to an increase of the quantum yield of FITC and a consequent increase of fluorescence intensity. Although these fluorescence effects are hindering quantitative analysis, we can conclude from Fig. 2 that post-treatment of the film loaded with the protein does not lead to the complete release of the protein. After more than 3 h of the post-treatment and deposition of additional "layers" onto the film, more than 60 % of the initial signal is still present (Fig. 2).

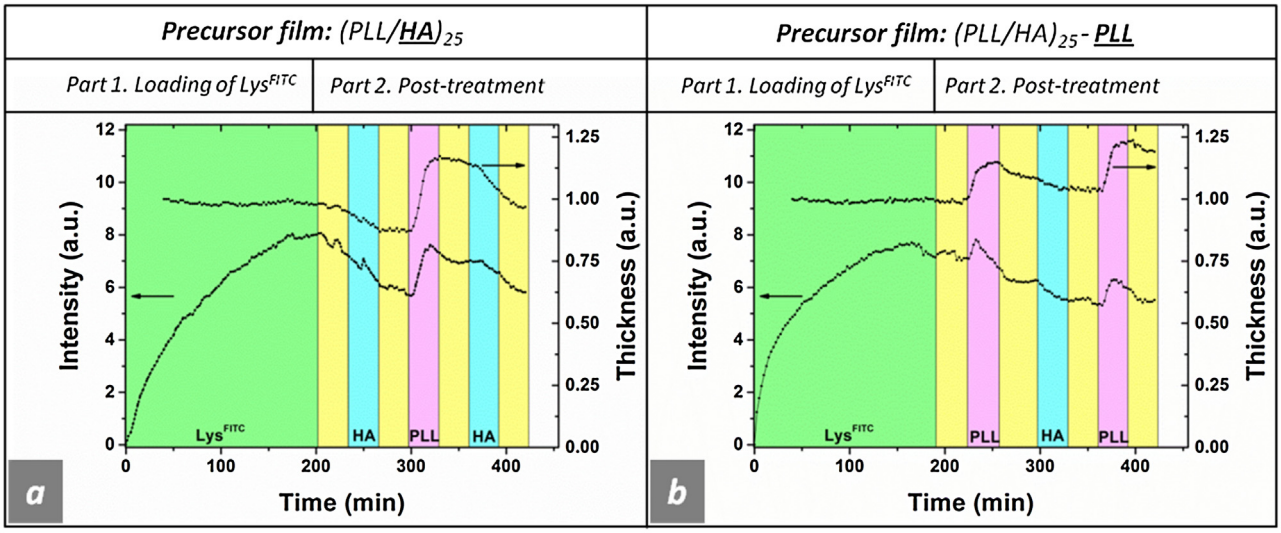


Fig. 2. Temporal fluorescence intensity changes of Lys^{FITC} in the PEM and the film thickness changes. Two separate measurements are shown, differing in the sequence of pre- and post-treatment (relative to Lys loading): $(PLL/\underline{HA})_{25}$-**Lys**-HA-PLL-HA (a) and $(PLL/HA)_{25}$-PLL-**Lys**-PLL-HA-PLL (b) with intermediate washing steps in buffer solution.

Table 1
Sequences of pre- and post-treatment (relative to Lys loading) for four separate measurements.

Nr.	Precursor film	Protein loading	Post-treatment with specified firstly deposited "layer"
1	$(PLL/\underline{HA})_{25}$ –	-Lys-	- HA – PLL – HA (Fig. 2a)
2	$(PLL/\underline{HA})_{25}$ –	-Lys-	- PLL – HA – PLL (not shown)
3	$(PLL/HA)_{25}$ – PLL –	-Lys-	- PLL – HA – PLL (Fig. 2b)
4	$(PLL/HA)_{25}$ – PLL –	-Lys-	- HA – PLL – HA (not shown)

The observed increase and decrease of the thickness of the films during incubation in PLL and HA solution respectively (Fig. 2, Part 2: Post-treatment) are most probably related to the following processes. During incubation in PLL solution hydrated PLL chains diffuse into the film inducing swelling (hydration) of the films. In contrast to PLL, HA of higher molecular weight is not able to diffuse into the film (or at least requires considerably more time for this process). It adsorbs on the surface of the film, forming a negatively charged layer that draws positively charged molecules into the interfacial region between film and solution and favours their release into the solution or formation of a new "layer" by binding to the adsorbed HA chains [25,30,31]. The release of the hydrated PLL chains from the film could be a reason for shrinkage (dehydration) of the film. Thus, the swelling/shrinkage behaviour plays a crucial role in the observed changes.

The observed variations of the swelling extent depending on the sequence (or "history") of PE adsorption after Lys loading can be explained in the following way. Directly after the loading of Lys the film is highly positively charged, and has a high capacity for HA adsorption. That is why when HA is deposited after Lys (Fig. 2a), the film allows more HA to adsorb and becomes highly negatively charged. As a result, in the next step, an increased uptake of PLL can lead to the maximum swelling (up to 30%). However, if PLL is deposited directly after Lys (Fig. 2b), the swelling is around 10% lower than in case when HA deposition separates the stage of Lys loading and PLL deposition (Fig. 2a). This is likely because PLL carries a positive charge like Lys (Lys net charge is meant). The subsequent 30 min incubation with HA (Fig. 2b) increases the negative charge density, which in turn increases the uptake capacity of PLL in the subsequent deposition step. That is why the second deposition of PLL (Fig. 2b) leads to the higher extent of swelling than the first one.

3.3. *Diffusion of Lys during loading into the film*

For the characterization of protein-film interaction we studied the mobility of Lys^{FITC} in the film. FRAP measurements were carried out at different time points during the film incubation in Lys solution. The results of the "mean" diffusion coefficient (D) calculations and the increase in fluorescence intensity during Lys uptake are presented in Fig. 3. Additionally, a multifractional analysis of the FRAP-data [24] was performed, resulting in a distribution of diffusion coefficients. We found three Lys fractions with different diffusion coefficients: lower than 0.04 $\mu m^2/s$, between 0.5 and 1.8 $\mu m^2/s$ and higher than 17 $\mu m^2/s$. The fractions were classified into three arbitrary groups ("slow", "middle" and "fast") and reflected by the split bars in Fig. 3. Fig. 3 shows that the "mean" diffusion coefficient of Lys^{FITC} decreases upon adsorption into the PEM. The multifractional analysis shows presence of at least two differently diffusing fractions at each moment of time during the protein loading. Within the first 30 min of incubation the "fast" fraction is present, which disappears after 1 h of incubation. The found diffusion coefficient values of the "fast" fraction are of the same order of magnitude as values reported for Lys diffusion in solution (of the order of 10^2 $\mu m^2/s$, i.e. 10^{-6} cm^2/s [16]) The "slow" fraction appears at the beginning of the second hour of incubation and increases with time (Fig. 3, Fig. S6). Thus, according to our observations the mobility of Lys^{FITC} in the film correlates with its concentration.

A comparison of our results with those reported in literature for the constituents of PLL/HA PEMs and loaded biomolecules would be interesting. In order to prove that the properties of our films are similar to those described in literature [11,21,23] in respect to molecular diffusion we measured mobility of PLL in our PEMs. "Mean" diffusion coefficient of PLL was found to be around 0.14 ± 0.03 $\mu m^2/s$ and, thus, in agreement with reported values for PLL populations of 0.04–0.2 $\mu m^2/s$ [23] and 0.1–2 $\mu m^2/s$ [21].

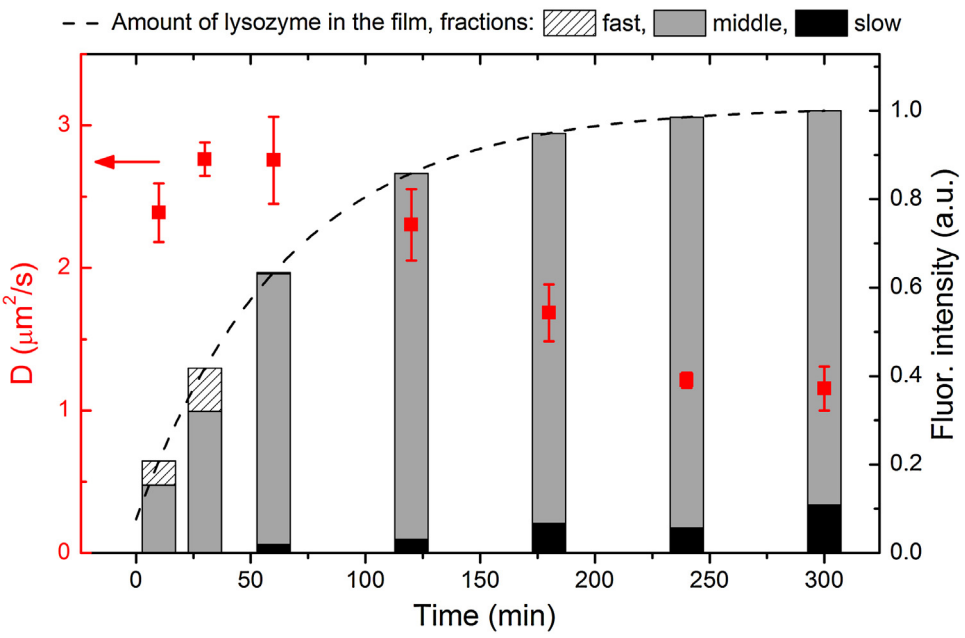


Fig. 3. Dependence of Lys^{FITC} "mean" diffusion coefficient *D* (squares) and fractional distribution (bars) on the period of time the PEM spent in contact with Lys solution. The fractions correspond to the ranges of diffusion coefficients: "slow" – lower than 0.04 $\mu m^2/s$; "middle" – between 0.04 and 17 $\mu m^2/s$; "fast" – higher than 17 $\mu m^2/s$. The trend (dashed line) of fluorescence intensity change (from the experimental data described in Section 3.1) reflects increase in the amount of Lys^{FITC}. .

Our results correlate with mobility studies on the anionic protein, human serum albumin (HSA), in $(PLL/HA)_{24}$ films [11]. The former provide additional evidence for the findings of Vogt et al. that increasing concentration of protein in the PEM leads to the occurrence of an increasing fraction of slowly diffusing protein. We show that this effect is not limited to anionic proteins. On the other hand, at internal concentrations of ~20 mg/ml, the major part (~70%) of HSA was diffusing with the diffusion coefficient $D < 0.1\ \mu m^2/s$ [11], lower than that of the major part of Lys (0.5–1.8 $\mu m^2/s$). This difference in interaction of Lys and HSA [11] with the film could be related to their different charge at the pH used.

The overall decreasing protein mobility during loading (Fig. 3) might be related to the growing amount of the "slow" diffusing fraction (Fig. 3). The "slow" diffusing fraction may represent a more tightly bound part of the protein to HA molecules, which are incompletely screened by counterions. The observation that the "slow" fraction is absent at the beginning of incubation in Lys solution could mean that the loaded protein needs time to adopt to such a conformation within the film. Apart from this, the formation of Lys aggregates may play a role enhanced at higher Lys concentrations. Additionally, interaction between Lys in the film with positively charged PLL molecules due to the negative charges on the protein may contribute substantially.

3.4. Diffusion of Lys during post-treatment

In a separate experiment we studied the influence of adsorption of additional PE "layers" on the mobility of Lys loaded in the film. The sequences of adsorbed PEs were similar to those presented in Table 1. Before every solution exchange step, FRAP measurements were performed. In Fig. 4 the change of the diffusion coefficient is presented in relative percent. Additionally, the composition of the last two or three deposited PE "layers" (PLL or HA) is specified. From this diagram we conclude that PLL leads to an increase of *D*, whereas HA leads to the decrease of *D*. Moreover, the history of adsorbed PE "layers" has a pronounced influence on the diffusion coefficient of the loaded protein. If the PEM is immersed into the same polymer solution two times in a row (with exposure to a Lys solution in between) the diffusion coefficients only slightly change (Fig. 4, two left bars). However, if the type of polyelectrolyte is changed (HA to PLL or PLL to HA) the mobility of Lys is affected to a higher extent (two bars in the middle of the diagram, Fig. 4). Notably, if the PE (PLL or HA) is adsorbed on a film that was previously covered two times with the oppositely charged polyelectrolyte (HA or PLL respectively, the right two bars of the diagram, Fig. 4) the diffusion coefficient of Lys changes even more considerably. It is worth noticing that PLL has a more pronounced influence on the diffusion coefficient of the protein than HA.

Since the duration of incubation in the PE solution could have an influence on the diffusion coefficient of Lys, we measured the diffusion coefficient continuously during longer incubation periods of the film in either PLL or HA solution. The results are presented in Fig. 5. We observe a gradual increase of diffusion coefficient during the incubation in PLL solution. In contrast, the diffusion coefficient of Lys decreased during incubation in the HA solution.

During incubation in PLL solution the PEMs swell up to 30% (Fig. 2) due to penetration of hydrated PLL chains. This should result in an increase of the pore size of the PEMs (noteworthy, the pore size of the PEMs should lay in the range of 10 nm) [8,9]. This could be a reason for a reduction of the steric hindrance of diffusing Lys (Scheme 1). Vice versa, during the incubation in HA the film shrinks, which may cause an increase of steric hindrance (Scheme 1). In addition, the electrostatic interactions between the protein and the PEM may change due to the charge increase during incubation in PE solutions. Noteworthy, for the case of a solution Sela et al. [32] demonstrated the disruption of the complexes between Lys and HA by polylysine, and complexation of HA with polylysine. PLL, entering the film preloaded with positively charged Lys, would preferentially bind to HA replacing protein molecules (Scheme 1), since PLL shows a higher charge density compared to that of the protein. This process should lead to an increase of Lys mobility during incubation in the polycation solution.

However, if the film is incubated in the polyanion solution, an opposite process may take place. As was discussed in Section 3.2, in contrast to PLL, HA of higher molecular weight is not able to

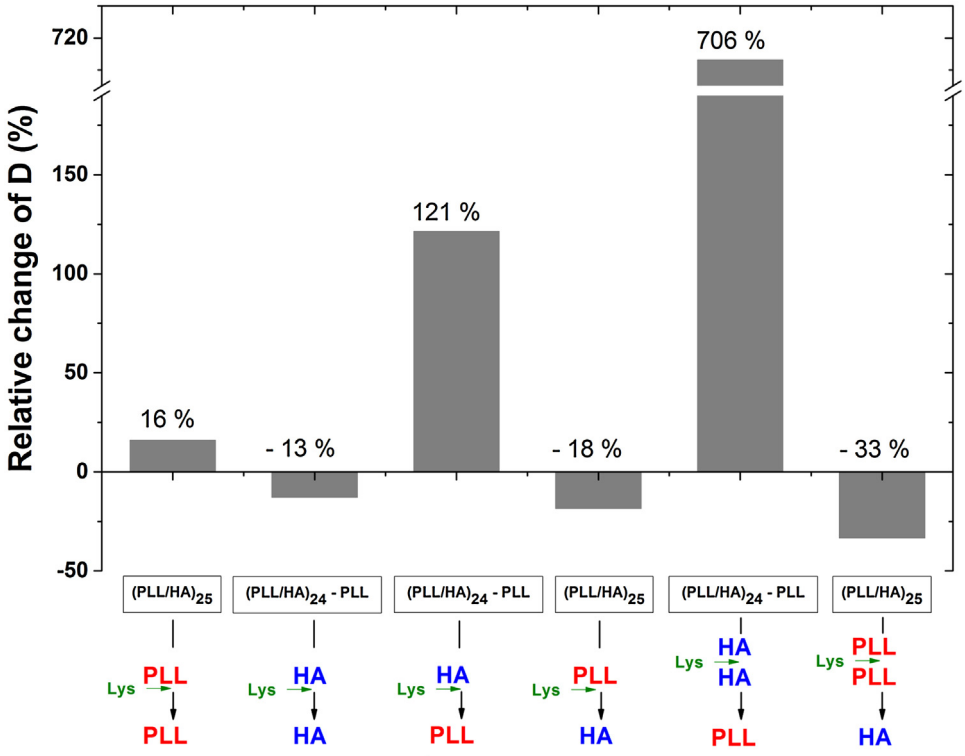
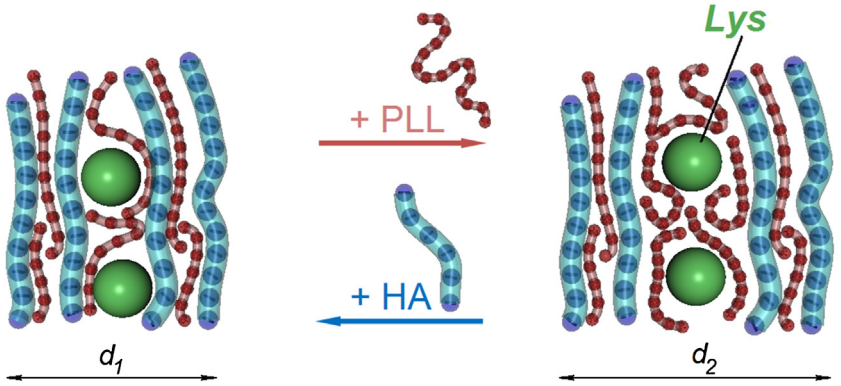


Fig. 4. Relative change of Lys^{FITC} "mean" diffusion coefficient D as a function of sequence of pre- and post-treatment (relative to Lys loading) of the films. Relative change of D designates the difference between D measured before and after deposition of the terminating "layer". The value of D measured before deposition of the terminating "layer" is taken as 100% for each particular column.



Scheme 1. Schematics of the interior of the PEM composed of rigid, matrix-forming HA chains and flexible PLL chains, the latter able to penetrate the film. The spheres represent Lys molecules. d stands for the thickness of the film which is increasing during the incubation in PLL and decreasing during the incubation in HA.

migrate inside the film. It adsorbs on the surface of the film drawing positively charged molecules and enabling their release into the solution (Scheme 1) [25,30,31]. This process eventually drives Lys molecules into more bound states and consequently reduces the protein mobility.

3.5. *Influence of assembly conditions on mobility of Lys*

The main parameters which can be varied during the assembly of the films are pH [33], ionic strength [4] and temperature [34]. We assembled the films under standard conditions and changed one of the assembly parameters (see Table 2). To assemble the films at a changed temperature (room temperature instead of 37 °C, Fig. S7), planar substrates were used instead of microfibres to provide a comparison with other studies on diffusion of loaded biomolecules and constituents of PEMs [11,21,23]. The observed values of the "mean" diffusion coefficient were within a range of ~0.2–0.9 μm^2/s (Table 2). Two to four times higher diffusion coefficients of Lys were observed in case of changed conditions compared to the standard ones. However, these values lay in a seemingly characteristic range of mobile species in the PLL/HA films of up to several μm^2/s

Table 2
The "mean" diffusion coefficient D of Lys^{FITC} in the PEM depending on the assembly conditions. The text in bold represents the conditions which during a particular film assembly process deviated from the ones used in a standard case (specified in raw Nr. 1).

Nr.	pH	[NaCl], mM	Temperature, °C (substrate)	D, μm^2/s
1	7.2–7.4	15	37 (microfibre)	0.24 ± 0.01
2	**9**	15	37 (microfibre)	0.85 ± 0.02
3	7.2–7.4	**150**	37 (microfibre)	0.69 ± 0.06
4	7.2–7.4	15	**24 (coverslip)**	0.42 ± 0.18

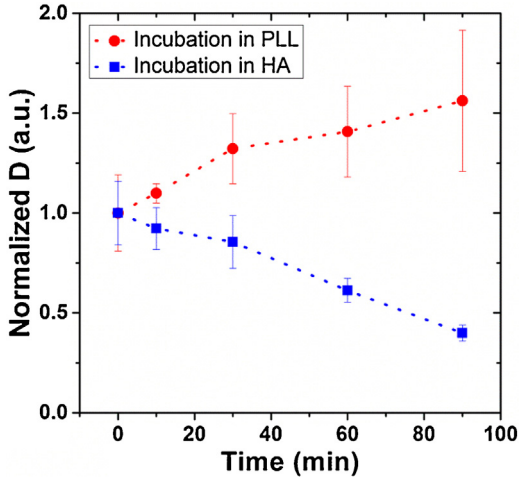


Fig. 5. Normalized Lys^{FITC} "mean" diffusion coefficient *D*as a function of time during incubation in PLL or HA solution. The data for PLL and HA were normalized by the first "mean" diffusion coefficient value gotten during the incubation in PLL or HA solution respectively.

[9,11,21,23,27]. Thus, the varied parameters during PEM assembly did not have a pronounced influence on the mobility of Lys in the PEMs.

4. Conclusions

In this study, we investigated the interaction of a model cationic protein, Lys, with the PLL/HA PEMs performing a step towards understanding complex intermolecular relations existing in multicomponent biochemical systems [35]. Our study opens new facets of previously suggested concept of implementation of microfibres as substrates for deposition of PEMs [9]. We show that this approach can be conveniently combined with microfluidic experiments. Monitoring the protein distribution within the PEM during loading and release together with the film thickness changes was successfully performed.

We observed a high loading capacity of Lys in the PLL/HA films, which allowed us within a relatively short period of time (three hours) to achieve internal concentrations in the range of 30 to 100 mg/ml that is up to two orders of magnitude higher than the concentration of the stock solution (around 1 mg/ml). From fluorescence measurements, no dependence of the amount of loaded protein on terminating "layer" of the films could be concluded. Post-treatment of PEMs with additional adsorption of PEs lasting for several hours did not lead to a complete release of the loaded protein. Thus, Lys seems to strongly interact with the PEMs. These observations point to exceptionally good reservoir properties of the studied PEMs on the time scale, relevant for most cell culture applications [36].

FRAP data were treated to determine diffusion coefficients of Lys fractions and "mean" diffusion coefficient. We observed a decrease of protein mobility upon adsorption into the film. Our results provide additional support to the findings of Vogt et al. [11] that upon adsorption into the film the fraction of the slowly diffusing protein increases, and that this effect is not limited to anionic proteins. However, the predominant fraction of Lys is diffusing faster compared to that of human serum albumin. This difference may be related to the difference in charge of the studied proteins.

The PLL/HA PEMs exhibit stable properties in terms of protein diffusion even at different but constant environmental characteristics. Indeed, variation of pH, ionic strength and temperature during film assembly did not have a pronounced influence on protein mobility in the film. In contrast, the system responses by transition of the PEM from pure buffer environment to PE solutions. PLL and HA adsorption always led to an increase and decrease of the diffusion coefficient of Lys respectively. The extent of change of the diffusion coefficients was dependent on the "history" of the PEs adsorption and presumably related to the extent of change of the charge of the film. These results are promising for development of systems with adjusted biomolecules availability.

In addition, understanding of molecular diffusion through variation of a change balance in multilayers opens new ways to assemble tailor-made functional multilayers and capsules with polymer exchange [39], multilayers with cellular response controlled by the polymer charge, nature, and density [40] as well as more complex multilayer architectures such as polymer scaffolds made of interconnected multilayer shells [41,42].

Last but not least, swelling/shrinkage of the PEM was observed during the incubation in PLL and HA solutions respectively. The latter finding combined with deposition of films on microfibres may inspire one to develop non-destructive sensing applications such as that for thickness change measurements of various coatings [37]. Additionally, the reported swelling/shrinkage behaviour might be of interest for flow control applications inside biocompatible microfluidic networks [38].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2016.07.055.

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Further reading

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