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Application of Bio-Layer Interferometry for the analysis of protein/liposome interactions

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ABSTRACT

The development of biosensor technologies for the investigation of biomolecular interactions has markedly advanced over the last years. One promising biosensor platform, the Bio-Layer Interferometry (BLI), was developed by ForteBio with the main focus to qualify and quantify protein/protein interactions in research and routine applications. Here, a method to characterize protein/liposome binding interactions based on the biophysical principles of this platform is described. Three different liposome formulations and the protein hormone, recombinant human erythropoietin (rh-Epo) were used as models in the test system. Rh-Epo was immobilized on disposable optical fiber streptavidin (SA) biosensor tips and binding of different liposome formulations under certain conditions was measured. The assay performance was evaluated, followed by calculating the kinetic rate and affinity constants. The results showed that all liposome formulations formed extremely stable complexes with the immobilized protein. Nevertheless, liposome specific differences in binding affinities were determined. Furthermore, a liposome concentration dependent binding pattern was demonstrated. The combination of simple sample preparation, the opportunity of automation with high throughput in an acceptable time range and excellent reproducibility, makes this assay suitable for basic research as well as for drug discovery and drug screening to estimate drug/membrane interactions.

1. Introduction

The interaction of biological relevant molecules and cell membranes plays a key role in drug discovery, drug screening and development for pharmaceutical applications [1–3]. In order to study such mechanisms in more detail, appropriate cell based assays and artificial cell membrane models, combined with sensitive analytical techniques are required [4,5]. Artificial membrane systems, such as liposomes, are widely used models to mimic lipid bilayers of biological cell membranes. They are excellent tools to study the biophysical properties and alterations of membranes since lipid composition, lipid ratio and vesicle sizes can be adjusted according to the requirements of the investigation [6,7]. Also a large repertoire of analytical in vitro methods exists to study protein/liposome interactions. This includes microscopy, calorimetry, electrophoresis, analytical ultracentrifugation, circular dichroism, mass-spectroscopy and immobilized artificial membrane chromatography. However, these techniques have several disadvantages, such as they provide only low-resolution data and low throughput. One promising approach to overcome these drawbacks is the application of biosensor technologies [8]. The most commonly applied techniques are Surface Plasmon Resonance (SPR) and Bio-Layer Interferometry (BLI) [9]. For SPR, lipids or small, unilamellar liposomes are coated on dextran surfaces prior pumping the sample solution across the surface [10,11]. In contrast to micro-fluidic SPR, which commonly delivers samples to a stationary sensor chip, BLI technology is established in an open shaking micro-well plate format without any micro-fluidics. In this work we wanted to evaluate a feasible method to study protein/liposome interactions and considered the BLI technology to feature significant advantages as compared to SPR: (i) the BLI technique offers a unique high throughput platform; (ii) a wide range of disposable optical fiber biosensors; coated with a proprietary biocompatible matrix are commercially available; (iii) samples can be recovered and used in other assays (iv); there is no risk for clogging effects as described for SPR; (v) association phase with SPR is limited by approximately 100 s, while the association phase in BLI experiments is only restricted by evaporation of the sample [12–14].

Based on preliminary experiments, recombinant human erythropoietin (rh-Epo) as a membrane-interacting model protein

Abbreviations: BLI, Bio-Layer Interferometry; rh-Epo, recombinant human erythropoietin; b-rh-Epo, biotinylated rh-Epo; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; egg-PC, egg phosphatidylcholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; PBS, Phosphate-buffered Saline; DLS, dynamic light scattering; MLV, multilamellar vesicle; ULV, unilamellar vesicle.

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was used and it was shown that rh-Epo not only induces receptor-mediated signal transductions but also interacts with lipid membranes [15]. Rh-Epo, a highly glycosylated protein hormone with a molecular weight of 30.4 kDa is well known for its haematopoietic but also neuro-protective activity [16,17]. In the present study, biotinylated rh-Epo (b-rh-Epo) was immobilized on the surface of a streptavidin (SA) biosensor tip followed by interaction to well-characterized liposomes of variable compositions. After attachment of three different liposome formulations to the protein coated surface, the thickness of the layer on the surface increased. This increase could be measured since BLI directly correlates the spectral shift ($\Delta\lambda$) with the change in thickness (nm) of the biological layer on the streptavidin (SA) biosensor tips. Thus, a change in optical mass thickness of 1 nm results in a 1 nm shift in the interferometry wave pattern [18]. According to this principle, association was detected by a positive shift caused by the increase in thickness of the layer while dissociation correlated with a negative shift thus providing the basics for calculating the binding kinetics.

However, to the best of our knowledge, protein/liposome interaction studies based on BLI have not yet been published and thus, our results demonstrate for the first time the suitability of BLI as an appropriate analytical tool for protein/membrane interaction studies.

2. Materials and methods

2.1. Liposomes

For the preparation of lipid vesicles POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) was obtained from Sigma (Vienna, Austria). Egg phosphatidylcholine (egg-PC) was kindly provided by Lipoid (Ludwigshafen, Germany).

DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Chloroform (Merck, Vienna, Austria) was used in analytical grade quality. For downsizing of the preformed liposomes an extruder from Avestin (Mannheim, Germany), equipped with polycarbonate Whatman membranes with different cut-offs of 400, 200 and 100 nm (GE Healthcare, Vienna, Austria), was used. Phosphate buffered saline (PBS) was obtained from PAA (Pasching, Austria).

Multilamellar liposomes (MLVs) were prepared by the classical film rehydration method [19]. Three different liposome formulations were prepared consisting of either pure egg-PC, pure POPC or pure DPPC. The individual lipids, in a total lipid concentration of 20 mM, were dissolved in 5 ml chloroform, evaporated in a rotating round bottom flask at 40 °C (50 °C for DPPC) in a water bath at reduced pressure until a dried lipid film was generated. Each film was rehydrated in 5 ml PBS at 40 °C (50 °C for DPPC). Unilamellar vesicles were prepared by downsizing the MLVs by extrusion through 400, 200, and finally 100 nm polycarbonate membranes at 40 °C (50 °C for DPPC). Extrusion was performed 6 times through 400 and 200 nm and 10 times through 100 nm membranes at a working pressure between 2 and 30 bar [20].

2.2. Dynamic light scattering (DLS)

The liposome mean diameter and the homogeneity were measured by DLS on a Zetasizer Nano-ZS with software version 6.01 (Malvern Instruments, Malvern, UK) at 25 °C. Liposomal suspensions were diluted 100-fold with double distilled water prior the measurement. Each suspension was measured five times. Size distribution was calculated by the intensity report. The homogeneity of the liposomes was determined by the polydispersity index (PDI).

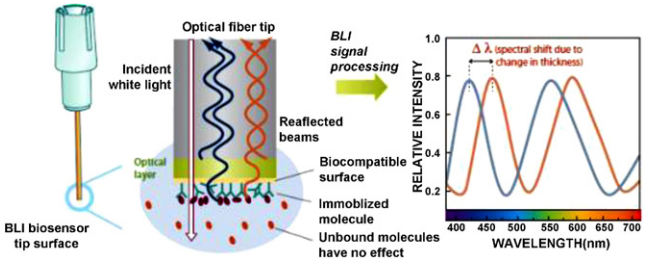

Fig. 1. Principle of the ForteBio BLI technique: on the left side a single biosensor-tip is shown followed by a detailed illustration of the analytical technique that analyze the interference pattern of white light reflected from two biosensor tip surfaces: a layer of immobilized molecules on the biosensor tip, and an internal reference layer. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time. The wavelength shift ($\Delta\lambda$) is a direct measure of the change in thickness of the biological layer as shown on the right side. Unbound molecules and changes in the refractive index of the surrounding medium do not affect the interference pattern.
Cited with permission from ForteBio Inc., Menlo Park, CA, USA.

2.3. Biotinylation of rh-Epo (b-rh-Epo)

Recombinant human erythropoietin (rh-Epo) was provided by Polymun Scientific GmbH (Klosterneuburg, Austria). Biotinylation was performed with NHS-LC-LC-BIOTIN (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Briefly, rh-Epo (3600 μg/ml) in 50 mM phosphate buffer (pH 6.5) was used. A 10-fold molar excess of biotin was added to achieve a preferable N-terminal biotinylation of the rh-Epo [21]. Incubation in the dark at room temperature for at least 1 h was performed. Finally, unbound biotin was removed using a PD-10 desalting column (GE Healthcare, Vienna, Austria).

2.4. BLI

Octet QK (ForteBio, Menlo Park, CA, USA) was used for BLI studies (Fig. 1). Assay was performed in black 96 well plates (Nunc F96 MicroWell™ Plates, Thermo Fisher Scientific, Langenselbold, Germany). The total working volume for samples or buffer was 0.21 ml per well and the rpm setting for each equilibrium, loading, association and dissociation step was 1000 rpm. The test was performed at 37 °C. Prior each assay, streptavidin (SA) biosensor tips (ForteBio) were pre-wetted in 0.21 ml PBS for at least 10 min followed by equilibration with PBS for 100 s (s = seconds). Afterwards streptavidin (SA) biosensor tips were non-covalently loaded with b-rh-Epo in PBS (1:100), followed by an additional equilibration step (100 s). Subsequently, association of b-rh-Epo with the individual liposome formulations in a concentration range of 2.5 mM, 5.0 mM, 7.5 mM and 10 mM was performed. Association at each studied concentration was carried out for 8000 s. Finally, the dissociation was monitored with PBS for 1500 s. All measurements were performed in triplicates.

2.5. Data analysis

The association and dissociation responses were baseline corrected processed with the Octet Software (Version 6.4, ForteBio) and exported to SigmaPlot Version 10.0 (Systat Software Inc., USA). The individual signal responses at each concentration as well as the maximum responses (R_{max}) were calculated as an average of three independent measurements.

The reproducibility of R_{max} was determined with $n = 7$.

Interferometry data were globally fit to a simple 1:1 Langmuir model calculating the affinities and rate constants (Octet software, Version 6.4, ForteBio). The association rate constant (k_a) is defined as the rate of complex formation per second in a 1 molar solution of

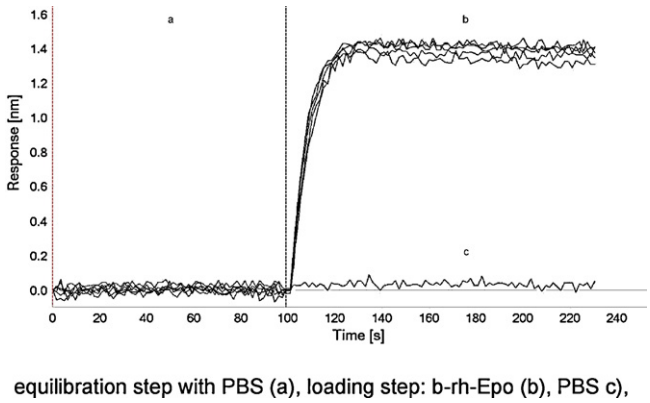

Fig. 2. Typical loading and equilibration curves showing the equilibration step (100 s) with PBS (a), the loading step with b-rh-Epo (b) and the reference curve equilibrated and loaded with PBS (c), simultaneous measurement of seven individual sensor tips.

two reaction partners. The dissociation rate constant (k_d) indicates the stability of this complex. The affinity constant K_D is calculated by the ratio of the k_d/k_a. However, the k_d can only be calculated if a decay in the signal of 5% or more is observed as described by Katsamba et al. [22]. Hence, the k_d (s^{-1}) is given by $k_d < -\ln(0.95)/t_d$, where t_d is the amount of the dissociation time in seconds [22,23].

3. Results and discussion

3.1. BLI–protein/liposome assay

A typical BLI assay involves four essential steps: an equilibrium step, immobilization of the ligand, association of the analyte and a dissociation step. To establish this technology for protein/liposome interaction analysis the assay performance was adjusted. The assay circle started with the equilibration of the streptavidin (SA) biosensor tips with PBS buffer to measure the baseline signal.

For the immobilization step, the protein concentration and loading performance of b-rh-Epo were optimized. In the end, streptavidin (SA) biosensor tips were captured to saturation with b-rh-Epo to obtain a loading signal between 1.2 and 1.5 nm. Typical loading curves are shown in Fig. 2. Subsequently, an additional equilibration step with buffer was applied to remove the excess of b-rh-Epo and to obtain a constant loading baseline. The equilibration time was 100 s to achieve a sufficient baseline signal. Moreover, the protein coated streptavidin (SA) biosensor tips were incubated with various liposomes at different concentrations (2.5–10 mM) to measure the corresponding association and dissociation profiles of DPPC, egg-PC and POPC liposomes, diluted in PBS. In preliminary experiments, different concentrations were tested for each liposome formulation. The specific response at the lowest concentration (2.5 mM) was 0.28 nm for POPC, 0.76 nm for egg-PC and 1.73 nm for DPPC. At lower concentration, no valid response of POPC was detected. A concentration of DPPC above 10 mM resulted in a signal plateau and thus, no further increase of the response signal could be measured. In order to perform comparable experiments, a concentration range between 2.5 mM and 10 mM was defined. The individual association and dissociation curves are shown in Fig. 3. Each selected liposome suspension interacted in a concentration dependent but also lipid dependent manner. The individual surface thickness of DPPC liposomes was significantly higher as compared to POPC and egg-PC. The calculated R_{max} values for all liposomes at each concentration were compared and are shown in Fig. 4.

The binding strength between b-rh-Epo and the tested liposome formulations could be related as follows: DPPC > egg-PC > POPC. DPPC liposomes had the highest affinity constant k_a of 0.124 ($M^{-1}\,s^{-1}$) while POPC liposomes had the smallest k_a 0.034 ($M^{-1}\,s^{-1}$). In Table 2 the calculated rate and affinity constants of the different liposomes are summarized.

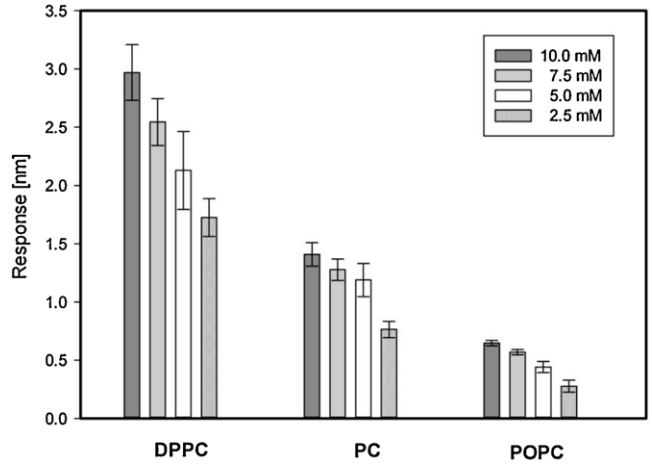

Fig. 4. Bars and error bars represent the mean R_{max} values and standard deviation for triplicate determinations of DPPC, egg-PC and POPC liposomes at 10, 7.5, 5, 2.5 mM.

Table 1
Mean size and homogeneity of the liposome formulations.

Liposome formulation	Mean size (nm)	PDI
POPC	92.9 (±0.39)	0.068 (±0.023)
Egg-PC	94.8 (±0.38)	0.056 (±0.016)
DPPC	90.1 (±0.19)	0.078 (±0.013)

The mean size (nm) was calculated from 5 independent measurements ($n = 5$). The polydispersity index (PDI) was calculated by M_w/M_n, whereas M_w is the weight average molecular weight and M_n is the number average molecular weight. The standard deviation for the mean size and the PDI was determined for 5 independent measurement ($n = 5$).

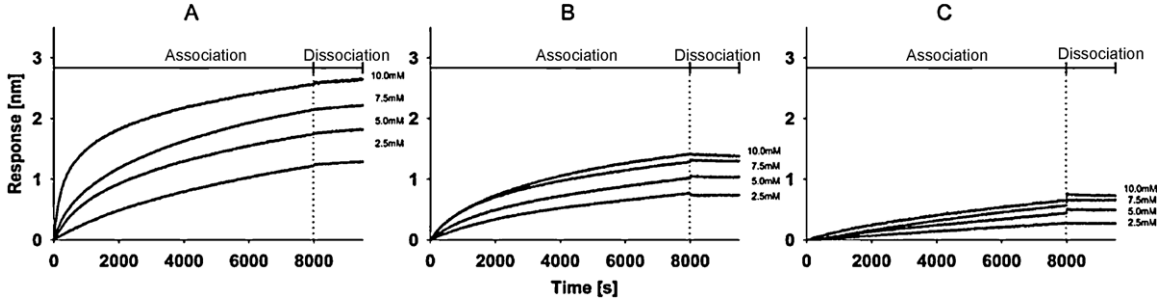

Fig. 3. Association and dissociation curves of DPPC (A), egg-PC (B) and POPC (C) liposomes in a concentration range between 10 mM and 2.5 mM. B-rh-Epo was immobilized on Streptavidin sensor tips prior association. Curves represent the mean values of triplicate measurements of each concentration.

The reproducibility of the test performance was evaluated with egg-PC liposomes (5 mM, $n = 7$) to explore the reliability of the BLI based assay The corresponding R_{max} during the association phase was 0.9878 nm with a relative standard deviation (R.S.D.) of 5.6%, while the R.S.D. was defined as the standard deviation divided by the mean and multiplied by 100%. These results demonstrated that the established assay is appropriate for protein/liposome interaction analysis. After optimization, this method enabled the immobilization of b-rh-Epo on streptavidin (SA) biosensor tips in a reproducible manner under standardized conditions (Fig. 2). No interaction of the prepared liposomes with uncoated streptavidin (SA) biosensor tips occurred, confirming that the streptavidin itself showed no cross reactivity with the artificial membranes (data not shown). In general, the biotin-streptavidin bond is known as one of the strongest non-covalent biological interactions and forms very rapidly stable complexes in wide ranges of pH and temperature [24]. All measurements were performed with PBS at 37 °C to mimic physiological conditions in natural biological systems. The individual binding kinetics of the liposomes with b-rh-Epo are shown in Table 2, demonstrating a lipid and concentration dependent binding behavior (Fig. 3). The resulting R_{max} values and the corresponding standard deviation for triplicate determinations of DPPC, egg-PC and POPC liposomes at all concentrations proved the excellent reproducibility (Fig. 4), which was additionally proven by the standard deviation measured from 7 sensors with egg-PC liposomes. Although, the plates were continuously agitated, one potential risk applying the BLI was the sedimentation of liposomes and loss of homogeneity. However, the obtained good reproducibility of the assay and the individual association curves indicated that this was not the case. In general, it could be demonstrated that the interaction occurred very slowly. Due to this fact, an extended association phase (8000 s) was applied. Thus, BLI technology provides an essential advantage compared to SPR, where the association phase can only be conducted for approximately 100 s (150 μl sample with a flow rate of 100 μl/min) due to technical limitations [14]. Longer measurements with SPR can only be performed with additional equipment, while BLI experiments can be conducted over a significant longer interval and is only limited by the evaporation of the sample volume at higher temperatures or longer measurements.

Based on the establishment of a reliable, simple and novel application for BLI, it is possible to scrutinize protein/membrane interactions in more detail and to enable additional examinations of complex membrane interactions.

3.2. Protein/liposome interactions

Liposomes, containing either synthetic saturated (DPPC, 16:0), synthetic unsaturated (POPC 16:0/18:1) or a natural lipid (egg-PC) were used because phospholipids are the predominant membrane lipids in mammalian cell membranes [25].

It was necessary to ensure that the liposome preparations had the same mean size and a low polydispersity, to prevent that different sizes of the liposomes influence the interaction binding behavior to b-rh-Epo. Therefore, the quality of the preparations was determined with DLS and the mean diameter and homogeneity were calculated. All liposomes had a mean size of 90 ± 10 nm and the calculated polydispersity index (PDI) indicated that all liposomes were homogeneously distributed (Table 1). Various liposomes with different characteristics related to the acyl chain length and saturation of their acyl chains showed significantly different association profiles with b-rh-Epo as shown in Fig. 3. Therefore, the different association responses were related to the lipid composition. In general, it is well accepted that proteins associate with charged lipid head groups of membrane surfaces by electrostatic attraction. This association is predominantly reversible without the destruction of proteins or membranes [26,27]. All liposomes, tested with the established BLI assay, had phosphatidylcholine as head groups, which is a neutral molecule as a whole [28]. This implied that the surface characteristics of the lipid membranes were equal. Based on the data obtained by BLI, it has been demonstrated that rh-Epo also associated with uncharged surfaces of membranes. However, due to the observed kinetics, interactions between rh-Epo and liposomes are not exclusively induced by electrostatic attraction, otherwise rapid association and detectable dissociation should be obtained as shown previously [8]. Once the liposomes had interacted with the protein no dissociation under native conditions within the observation time occurred. This indicated that very stable complexes were formed and therefore, we assumed that after adsorption of rh-Epo at the lipid interface a certain protein domain is able to penetrate into the lipid core [29]. In preliminary studies, the responsible membrane interacting domain of rh-Epo, spanning Met 54 to Lys 97 of the helix B of the rh-Epo molecule has been identified [15]. For a long time the biological function of this helix was unexplained. In addition to the membrane penetrating property of this helix, this domain was identified to be involved in the neuroprotective signal transduction [30]. Considering these facts, we suggest that the slow binding kinetics corresponded to the protein diffusion into and across the lipid bilayer, which indicated that bilayers were interdigitated by the membrane penetrating domain of rh-Epo. This irreversible phenomenon was seen in all experiments. Thus, these results indicated that the lipid head group did not play a dominant role in the interaction behavior between rh-Epo and liposomes.

POPC and DPPC consist of one defined phospholipid, while typical lots of egg-PC have various fatty acid compositions such as 33% C16:0, 13% C18:0, 31% C18:1 and 15% C18:2 [31]. Hence, these results indicated the more saturated lipid chains were present in the bilayer the more liposomes were bound to the immobilized protein molecules.

Table 2
Kinetic rate constants and affinities were determined for the b-rh-Epo/liposome interactions. In all experiments, b-rh-Epo was immobilized on Streptavidin (SA) biosensor tips prior incubation with the different liposome formulations.

Analyte	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (μM)
DPPC	0.124 (0.04) ($n = 12$)[a]	$<6.4 \times 10^{-6}$	<51.66
Egg-PC	0.070 (0.03) ($n = 12$)[a]	$<6.4 \times 10^{-6}$	<91.15
POPC	0.034 (0.02) ($n = 12$)[a]	$<6.4 \times 10^{-6}$	<184.47

[a] Full global fit (mean and standard deviation for n-independent experiments).

4. Conclusion

The proof-of-concept study of protein/liposome interaction measurements using BLI demonstrated that this technique is in principle appropriate for the analysis of protein/liposome interactions in a simple and reproducible manner. These results clearly demonstrated that the interaction between rh-Epo and selected liposomes was strongly related to the biophysical properties of the membranes. The major advantage of this assay is the simple, reliable and reproducible test performance with the ability to estimate affinity constants and monitor dissociation rates of protein/liposome interactions. Various proteins of interest can be easily coated and tested with different liposome formulations in a short but also in an extended time span. Thus, it was confirmed that the application of BLI for protein/liposome interaction analysis is a promising and high throughput tool to obtain data around the binding behavior between liposomes and proteins.

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