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Tethered proteoliposomes containing human ABC transporter MRP3: New perspectives for biosensor application based on transmembrane proteins

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ABSTRACT

While transmembrane proteins and transporters comprise one of the largest protein families, their use in biosensors like biochips or lab-on-a-chip devices has so far been limited by their demanding requirements of a stable and compartmentalized lipid environment. A possible remedy lies in the tethering of proteoliposomes containing the reconstituted transmembrane protein to the biosensoric surface. As a proof of concept, we reconstituted the human ABC transporter MRP3 into biotinylated proteoliposomes and tethered those to a gold surface coated with streptavidin on a biotinylated self-assembled thiol monolayer. The tethering process was investigated by quartz crystal microbalance with dissipation monitoring. The final assembly of tethered proteoliposomes exhibited biological activity in terms of drug-stimulated ATP hydrolysis and substrate translocation. The presented facile immobilization approach can be easily extended to other transmembrane proteins as it does not require any modification of the protein and will open up transmembrane proteins for future application in biosensors.

1. Introduction

Biosensors based on soluble proteins are an established technique with widespread analytical and biomedical applications ranging from quantification of small molecules in complex matrices over high-throughput ligand and inhibitor screening to fundamental kinetic and thermodynamic studies on the protein itself [1]. However, examples of transmembrane proteins used in biosensors (f.e. [2,3]) are still rare because they require a lipid or lipid-like environment, carefully optimized for both supporting the native protein conformation and long-term stability of the biosensor. Transport proteins, particularly ABC transporters, pose even more difficulties because a compartmentalized lipid bilayer environment of low permeability is necessary to facilitate transport. However, their role in metabolic barriers, excretion of toxins and multidrug resistance of cancer cells [4] would imply numerous biosensoric applications if these difficulties could be overcome.

The simplest conceivable model for coupling a transport protein to a biosensoric surface while simultaneously providing stable compartmentalization, is the tethering of proteoliposomes via the biotin–streptavidin interaction which provides irreversible immobilization with a binding constant similar to covalent attachment [5,6]. The most common biosensor surfaces silicon/silica and gold can be readily coated with streptavidin via a biotinylated SAM of either silanes (silicon/silica, [7]) or thiols (gold, [8]). Proteoliposomes containing the membrane protein and doped with the biotinylated lipid DCB can then be tethered to such a surface. Although similar models are well-characterized for liposomes [9,10], they have not yet been extended to actual proteoliposomes containing an active transmembrane transporter.

We therefore investigated the tethering of biotinylated proteoliposomes containing the human ABC transporter MRP3 to a streptavidin-functionalized gold surface (as depicted in Fig. 1) and checked the biological activity of such an assembly in terms of ATP hydrolysis and substrate translocation. MRP3 is supposedly involved in multidrug resistance of cancer cells and features a catalytic cycle with strong positive cooperativity for both ATP hydrolysis and substrate transport [11,12]. Due to its multidomain structure, fragility, intolerance of most covalent modifications and requirements of the lipid environment, MRP3 is a good test candidate for the feasibility of a proteoliposome-based biosensor.

We used the QCM-D as primary tool for the analysis of proteoliposome tethering because it simultaneously provides information

Abbreviations: ABC, ATP binding cassette; CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein; DCB, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl); DDM, *n*-dodecyl β-D-maltoside; DTT, dithiothreitol; eq, stoichiometric equivalents; LUV, large unilamellar vesicle; MRP, multidrug resistance-associated protein; MTX, methotrexate; PL, proteoliposome; QCM(-D), quartz crystal microbalance (with dissipation monitoring); SA, streptavidin; SAM, self-assembled monolayer; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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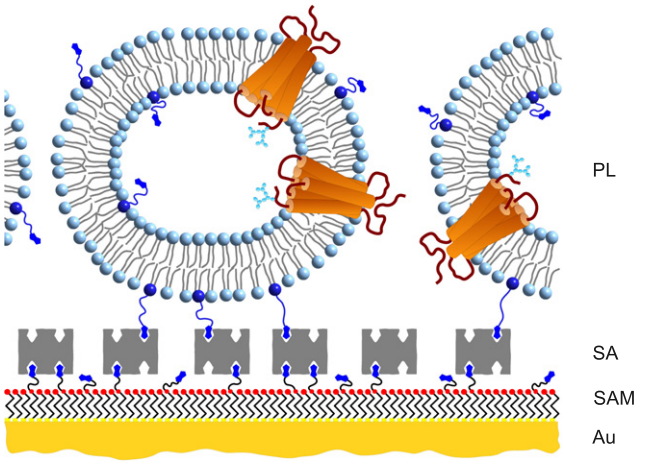

Fig. 1. Schematic illustration of a biosensoric surface with the ABC transporter MRP3 as active biological system. A gold surface (Au) as transducer is coated with streptavidin (SA) on top of a binary biotinylated thiol SAM (SAM). Proteoliposomes (PL) with the reconstituted protein MRP3 are tethered to the streptavidin coating via the biotinylated lipid DCB.

on the mass uptake and the viscoelastic properties of the immobilized mass [13,14].

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (DCB) was purchased from Avanti Polar Lipids, asolectin from Sigma–Aldrich, streptavidin from Rockland, other chemicals from AppliChem or Sigma–Aldrich in the highest available purity. Water was purified and deionized in-house by a Sartorius arium 61316/611VF to resistivity of 18.2 MΩ.

2.2. Preparation of large unilamellar vesicles (LUVs)

Lipids were mixed in $CHCl_3$, dried under a stream of nitrogen at 40 °C and kept in high vacuum over night. The resulting lipid films were resuspended in reconstitution buffer (20 mM TES-NaOH, pH 7.4; 100 mM NaCl; 1 mM $MgCl_2$) at a concentration of 20 mM and extruded 21 times through a polycarbonate membrane with 200 nm pore size (Avestin Liposofast).

2.3. Reconstitution of MRP3

Recombinantly expressed MRP3 was reconstituted into LUVs at a lipid-to-protein ratio of 25/1 (w/w) corresponding to a molar ratio of approximately 5500/1, as described previously [12,15], and resulting PLs were stored flash-frozen at −70 °C. Prior to use, PLs were thawed on ice, incubated with 1 mM DTT and extruded five times through a polycarbonate membrane (Avestin Liposofast, 200 nm pore size).

2.4. Surface cleaning and preparation

5 MHz AT-cut QCM sensors with gold electrodes (Q-Sense QSX 301) were cleaned in an ammonia (25%)/hydrogen peroxide (30%)/water mixture (1/1/5, v/v/v) for 5 min at 80 °C, excessively rinsed with water and dried in a stream of nitrogen. Immediately before use, the sensors were exposed to an argon plasma for 5 min. Self-assembly of a biotinylated thiol monolayer was accomplished by incubation in a chloroformic solution of 16-mercaptohexadecanol and 16-mercapto-(8-biotinamido-3,6-dioxaoctyl)hexadecanamide (50/1 (n/n); 0.1 mM) as described previously [8].

2.5. QCM measurements

A Q-Sense E4 QCM-D equipped with four temperature controlled flow cells in parallel configuration and a four-channel peristaltic sucking pump (Ismatec IPC) monitored frequency and dissipation changes of odd overtones 3 to 13. Flow rates in µL/min were 100 during rinses, 50 during adsorption and 10 during thermal equilibration. Frequency and dissipation shifts are reported normalized to the overtone and relative to a sensor with a thiol SAM in pure reconstitution buffer.

2.6. LUV/PL tethering

QSX 301 QCM sensors bearing a biotinylated thiol SAM were rinsed with reconstitution buffer at 20 °C until the baseline stabilized. SA (10 µg/mL in reconstitution buffer) was allowed to adsorb to the SAM in circular flow for 15 min followed by a 5 min rinse. Subsequently, LUVs/PLs (diluted to 1 mM in reconstitution buffer) were applied for at least 30 min in circular flow followed by a rinse until readings stabilized.

2.7. ATPase assay

After warming tethered LUVs/PLs to 37 °C and equilibration in transport buffer (50 mM TES-NaOH, pH 7.4; 41 mM NaCl; 10 mM KCl; 12 mM $MgSO_4$; 10 mM Na_2ATP), the flow was stopped for 1 h. Subsequently, the liquid in the flow chamber (300 µL) was harvested, flash-frozen in liquid nitrogen, lyophilized and redissolved in 40 µL 200 mM H_2SO_4 containing 2 mM DDM. Inorganic phosphate from ATP hydrolysis was quantified according to van Veldhoven and Mannaerts [16] using a total assay volume of 56 µL.

Stimulation of ATPase activity was achieved by supplementing the transport buffer with 2 mM MTX. LUVs without MRP3 served as negative control for unspecific ATP hydrolysis.

2.8. CDCF uptake assay

After warming tethered LUVs/PLs to 37 °C and equilibration in transport buffer containing 20 µM CDCF, the flow was stopped for 1 h. The tethered (proteo)liposomes were washed with reconstitution buffer at a flow of 100 µL/min for 12 min before lysis with Triton X-100 (0.1% (w/v) in reconstitution buffer) at the same flow rate, collecting fractions of 100 µL. Fluorescence of CDCF was detected at 485 nm excitation and 535 nm emission in a Berthold Mithras LB 940 plate photometer.

3. Results and discussion

3.1. Tethering of proteoliposomes to biosensoric surfaces

We first performed QCM-D measurements on the tethering of LUVs made from asolectin, which is a suitable lipid mixture for the reconstitution of MRP3 and other ABC transporters, and doped with 0.1 mol% of the biotinylated lipid DCB. Fig. 2A shows typical time courses for frequency and dissipation shifts from a QCM-D measurement during immobilization of streptavidin and subsequent tethering of DCB-doped asolectin LUVs to a gold surface functionalized with a biotinylated SAM. In QCM-D experiments, the negative frequency shift correlates with the mass that is acoustically coupled to the surface. While this correlation is linear for rigid layers as shown by Sauerbrey [17], it becomes non-linear for

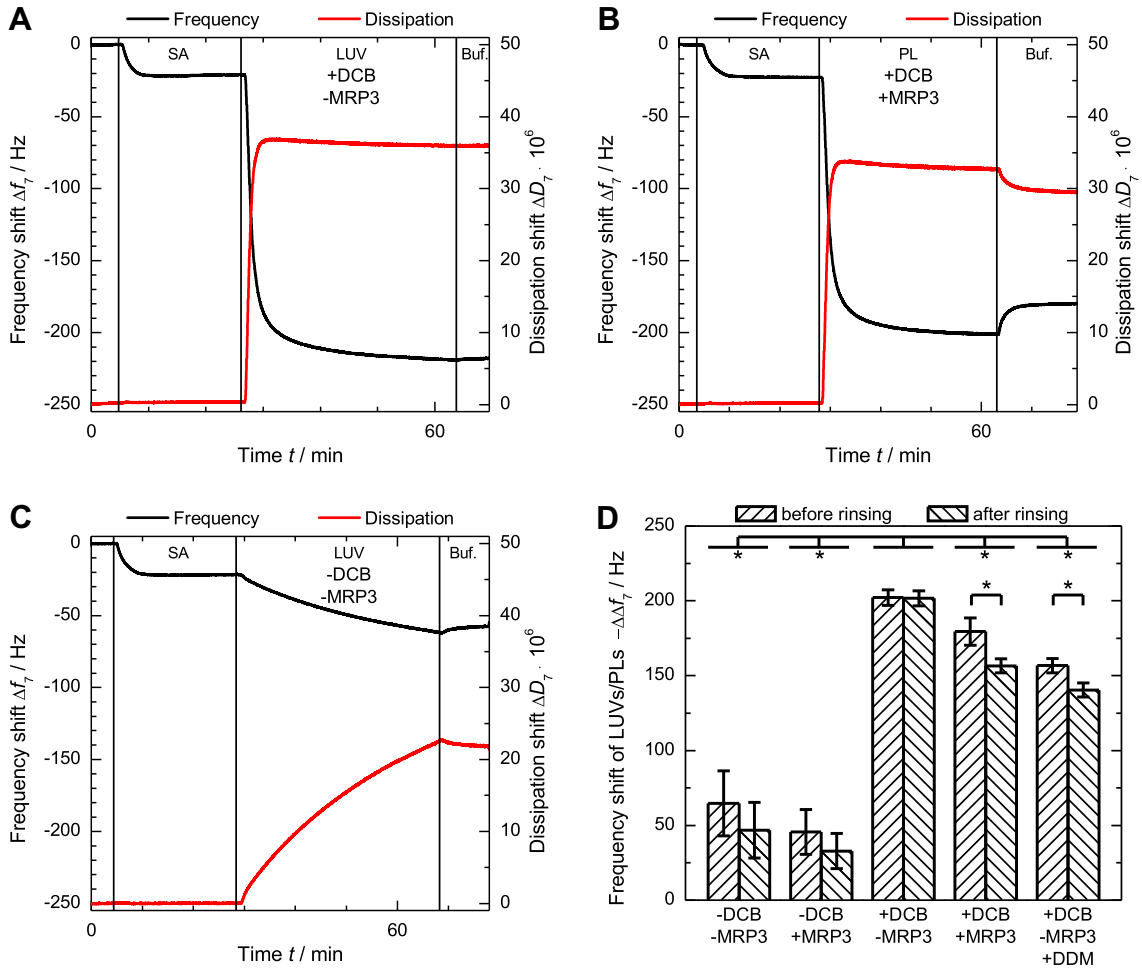

Fig. 2. Tethering of (proteo)liposomes to streptavidin coated thiol monolayers on gold surfaces observed by quartz crystal microbalance. Normalized frequency (black, left axis) and dissipation (red/gray, right axis) shifts of seventh overtone for typical immobilizations of streptavidin onto biotinylated thiol SAM (SA) and subsequent tethering of (proteo)liposomes (LUV/PL) with terminal buffer rinse (Buf.) for (A) LUVs with DCB anchor but without MRP3, (B) PLs with DCB anchor and MRP3, and (C) LUVs with neither DCB anchor nor MRP3. The negative frequency shift correlates with the amount of coupled material whose viscoelasticity is indicated by the dissipation shift (low = rigid, high = viscoelastic). (D) normalized negative frequency shift of seventh overtone for tethering of (proteo)liposomes of different composition before and after buffer rinse. Data presented as mean ± SD of at least eight independent experiments. * Statistically significant ($P \leqslant 0.05$) difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

viscoelastic systems like tethered vesicles precluding the calculation of quantitative loads in ng/cm^2 here. The dissipation correlates with the viscoelasticity of the coupled mass, where viscoelastic loads cause larger dissipation shifts. Upon streptavidin incubation, a frequency shift of −22 to −25 Hz and a negligible dissipation shift below 0.1×10^{-6} were observed. There was no change after a buffer rinse. This agrees well with the formation of a stable, two-dimensional SA monolayer on the SAM in accordance with published literature [8]. Subsequent application of DCB-doped LUVs led to an additional frequency shift of −200 Hz with a distinct dissipation increase of 36×10^{-6}. There was again no detectable change upon rinsing. The marked increase in dissipation is caused by the immobilization of a viscoelastic load and clearly indicates the tethering of intact vesicles [10].

The tethering of DCB-doped PLs containing four mass% MRP3 showed a similar overall behavior (Fig. 2B) but with a slightly lower immobilization and an obvious loss of material (+25 Hz frequency and -4×10^{-6} dissipation shifts) during the terminal buffer rinse. As will be shown later, this loss of material is most likely due to residual detergent from the reconstitution procedure. However, compared to LUV and PL tethering, the unspecific adsorption of LUVs without the biotinylated lipid DCB (Fig. 2C) proceeded much slower and yielded significantly less immobilized material with a higher relative dissipation shift. The ratio of dissipation shift and negative frequency shift during adsorption can be interpreted as a structural parameter of the immobilized layer [18,19]. This ratio with a value of 0.18 ± 0.01 MHz^{-1} was the same for tethered LUVs and PLs after rinsing, indicating that the tethered layers have similar structural properties. In contrast, LUVs without DCB yielded a ratio of 0.61 ± 0.05 MHz^{-1} after the terminal rinse in agreement with different structural properties caused by the unspecific adsorption that lacks a strong coupling to the surface. It should be noted, that unspecifically attached vesicles could be efficiently removed by three alternating buffer-water rinses (data not shown). Although this treatment had little effect on the tethered vesicles, it severely decreased MRP3 activity in proteoliposomes and was hence not applied.

Fig. 2D summarizes the frequency shifts before and after rinsing for vesicles of different composition regarding DCB and MRP3 and clearly shows that the vesicle tethering is a DCB-mediated effect. Additionally, we checked the tethering of DCB-doped LUVs that were supplemented with 0.08 eq DDM. This detergent is regularly used at more than 0.8 eq during reconstitution of MRP3, and residual DDM is most likely present in PL preparations despite careful

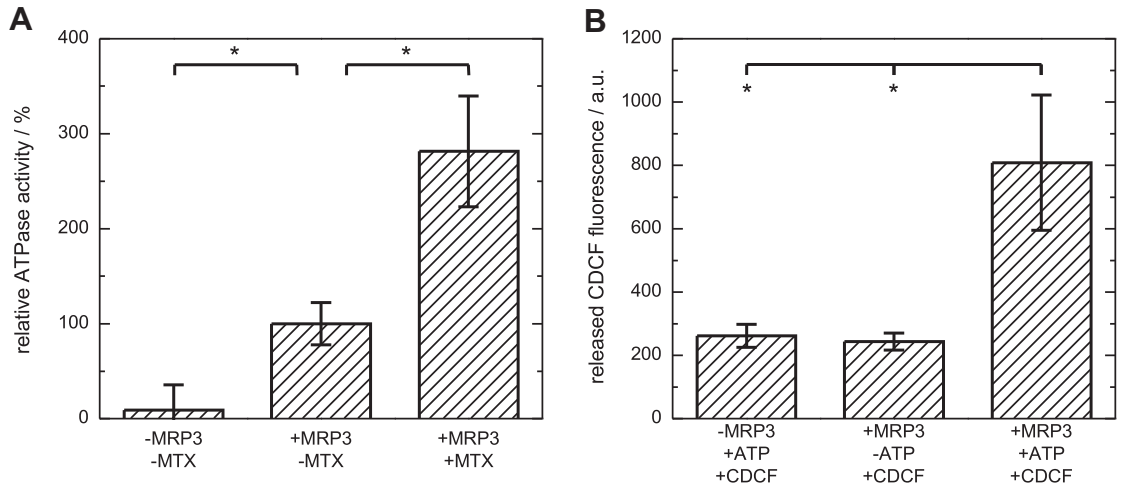

Fig. 3. ATPase and transport activity of MRP3 in tethered proteoliposomes. (A) Relative ATPase activity of tethered (proteo)liposomes. Unspecific ATP hydrolysis by LUVs without MRP3 was negligible compared to basal ATPase activity of MRP3-containing PLs in the absence of substrate. The anti-cancer drug MTX significantly stimulated ATP hydrolysis by MRP3 2.8-fold. (B) Fluorescence intensity of CDCF from liposomal lumen after MRP3-mediated uptake for 1 h. ATP-dependent transport of CDCF by MRP3 was more than 3-fold higher than passive permeation in the absence of ATP or MRP3. Data presented as mean ± SD of four (A), respectively nine (B) independent experiments. * Statistically significant ($P \leqslant 0.05$) difference.

detergent removal. As DDM-treated LUVs also showed a significantly lower frequency shift during tethering and a significant loss of material during rinsing, we attribute these effects to the presence of DDM in the liposomal bilayer. During the buffer rinse, the detergent is washed out from the vesicles thus causing the observed loss of adsorbed material. However, this effect is not detrimental to a biosensoric application of the tethered PLs.

3.2. Activity of MRP3 in tethered liposomes

To prove that tethering PLs with reconstituted MRP3 to a functionalized gold surface does not inactivate MRP3, we checked both drug stimulated ATP hydrolysis and substrate translocation to the liposomal lumen.

ATPase activity was assayed by quantification of inorganic phosphate released from ATP after 1 h incubation at 37 °C in an ATP-containing buffer and is reported relative to the basal ATPase activity of tethered proteoliposomes (Fig. 3A). The unspecific ATP hydrolysis in absence of MRP3 was below 10% of the basal activity. Stimulation with the anti-cancer drug MTX led to a 2.8-fold increase of ATP hydrolysis and is in good agreement with the up to 4-fold stimulation reported previously for MRP3 in free PLs [15].

To asses the MRP3-mediated transport of the fluorescent substrate CDCF, tethered PLs were incubated with 20 μM CDCF for 1 h at 37 °C, subsequently rinsed with buffer containing neither ATP nor substrate, and the fluorescence released from the liposomal lumen after lysis was quantified (Fig. 3B). In presence of both MRP3 and ATP, the uptake of CDCF was more than 3-fold higher than if either ATP or MRP3 were absent, showing the transport activity of MRP3.

One can conclude that the tethering of PLs and the proximity to a functionalized gold surface does not inactivate MRP3.

4. Conclusion

We successfully tethered proteoliposomes containing the human ABC transporter MRP3 to streptavidin coated gold surfaces while retaining ATPase and transport activity of MRP3. Additionally, we showed by QCM-D measurements that tethering of PLs is less efficient than of LUVs due to residual detergent from the protein reconstitution but nonetheless specific, stable to rinsing and efficient enough for further use.

We believe that this system can be easily adapted to other transmembrane proteins and will open up the use of transmembrane transporters for biosensoric applications.

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References

[1] Y. Liu, Z. Matharu, M.C. Howland, A. Revzin, A.L. Simonian, Affinity and enzyme-based biosensors: recent advances and emerging applications in cell analysis and point-of-care testing, Anal. Bioanal. Chem. 404 (2012) 1181–1196.

[2] T. Christopeit, G. Stenberg, T. Gossas, S. Nystroem, V. Baraznenok, E. Lindstroem, U.H. Danielson, A surface plasmon resonance-based biosensor with full-length BACE1 in a reconstituted membrane, Anal. Biochem. 414 (2011) 14–22.

[3] A. Calo, M. Sanmarti-Espinal, P. Iavicoli, M.-A. Persuy, E. Pajot-Augy, G. Gomila, J. Samitier, Diffusion-controlled deposition of natural nanovesicles containing G-protein coupled receptors for biosensing platforms, Soft Matter 8 (2012) 11632–11643.

[4] P. Borst, R.O. Elferink, Mammalian ABC transporters in health and disease, Annu. Rev. Biochem. 71 (2002) 537–592.

[5] L. Chaiet, F.J. Wolf, The properties of streptavidin, a biotin-binding protein produced by streptomycetes, Arch. Biochem. Biophys. 106 (1964) 1–5.

[6] C. Grunwald, A brief introduction to the streptavidin-biotin-system and its usage in modern surface based assays, Z. Phys. Chem. 222 (2008) 789–821.

[7] Y. Li, J.C. Niehaus, Y. Chen, H. Fuchs, A. Studer, H.-J. Galla, L. Chi, Patterning of proteins into nanostripes on Si-wafer over large areas: a combination of Langmuir-Blodgett patterning and orthogonal surface chemistry, Soft Matter 7 (2011) 861–863.

[8] M. Seifert, M.T. Rinke, H.-J. Galla, Characterization of streptavidin binding to biotinylated, binary self-assembled thiol monolayers—influence of component ratio and solvent, Langmuir 26 (2010) 6386–6393.

[9] E. Boukobza, A. Sonnenfeld, G. Haran, Immobilization in surface-tethered lipid vesicles as a new tool for single biomolecule spectroscopy, J. Phys. Chem. B 105 (2001) 12165–12170.

[10] A.R. Patel, C.W. Frank, Quantitative analysis of tethered vesicle assemblies by quartz crystal microbalance with dissipation monitoring: Binding dynamics and bound water content, Langmuir 22 (2006) 7587–7599.
[11] P. Borst, C. de Wolf, K. van de Wetering, Multidrug resistance-associated proteins 3, 4, and 5, Pfluegers Arch. 453 (2007) 661–673.
[12] P. Seelheim, A. Wüllner, H.-J. Galla, Substrate translocation and stimulated ATP hydrolysis of human ABC transporter MRP3 show positive cooperativity and are half-coupled, Biophys. Chem. 171 (2013) 31–37.
[13] M.C. Dixon, Quartz crystal microbalance with dissipation monitoring: enabling real-time characterization of biological materials and their interactions, J. Biomol. Tech. 19 (2008) 151–158.
[14] M. Rodahl, B. Kasemo, A simple setup to simultaneously measure the resonant frequency and the absolute dissipation factor of a quartz crystal microbalance, Rev. Sci. Instrum. 67 (1996) 3238–3241.
[15] B. Zehnpfennig, I.L. Urbatsch, H.-J. Galla, Functional reconstitution of human ABCC3 into proteoliposomes reveals a transport mechanism with positive cooperativity, Biochemistry 48 (2009) 4423–4430.
[16] P.P. Van Veldhoven, G.P. Mannaerts, Inorganic and organic phosphate measurements in the nanomolar range, Anal. Biochem. 161 (1987) 45–48.
[17] G. Sauerbrey, Verwendung von schwingquarzen zur wägung dünner schichten and zur mikrowägung, Z. Phys. A Hadrons Nucl. 155 (1959) 206–222.
[18] D. Johannsmann, I. Reviakine, R.P. Richter, Dissipation in films of adsorbed nanospheres studied by quartz crystal microbalance (QCM), Anal. Chem. 81 (2009) 8167–8176.
[19] E. Tellechea, D. Johannsmann, N.F. Steinmetz, R.P. Richter, I. Reviakine, Model-independent analysis of qcm data on colloidal particle adsorption, Langmuir 25 (2009) 5177–5184.