

## Publication I

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# An electrochemical method for the determination of liposome–water partition coefficients of drugs

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## Abstract

An electrochemical method for the determination of the liposome–water partition coefficients of drugs was developed. Interactions of liposomes and  $\beta$ -blockers were studied using square wave voltammetry (SWV) at a water–DCE interface created at the tip of a micro-pipette. Five different  $\beta$ -blockers, propranolol, timolol, carteolol, nadolol and metoprolol were encapsulated in liposomes prepared using the extrusion method. The liposomes were made in three different sizes by varying the size of the pores in the polycarbonate membrane used in extrusion. The partition of five  $\beta$ -blockers between the cavity and the membrane of the liposome was determined.

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*Keywords:* Partition coefficient; Liquid–liquid interface; Drug; Electrochemistry; Liposome

## 1. Introduction

Liposomes are phospholipid vesicles that form spontaneously in aqueous environments. The interaction of liposomes and drug molecules has been of interest mainly for two reasons: because of their resemblance with biological membranes, liposomes have been used as model membranes to study interactions of drugs and phospholipids at cellular level. Also, biocompatibility has allowed the use of liposomes as delivery systems in drug targeting.

The properties that affect the permeation of drugs through biological membranes are lipophilicity, charge, size and hydrogen bonding properties. Of these, the most important and the most widely used property to assess drug's performance is lipophilicity. Traditionally the partitioning between membrane and drug has been assessed using *n*-octanol–water partition coefficient, which is defined as the ratio of the activity of a species dissolved at equilibrium between two immiscible solvents. The octanol–water partition coefficient can be determined by measuring the

concentrations at equilibrium [1], calculating from the HPLC retention time [2], using a potentiometric titration technique [3], or using electrochemical methods [4]. Also computational methods based on molecular structure of a drug have been used [5].

Liposomes have been used as an alternative to octanol in drug partitioning studies. Because of the superior biomimetic properties of liposomes, the liposome–water partition coefficient offers better opportunities to assess drug–membrane interactions [6]. The advantage of liposome–water partition coefficient over traditional octanol–water system is its ability to take into account the ionic interactions, which are of great importance since many drugs are in their ionised form in the body. The correlation of partition coefficient measured in octanol–water and liposome–water systems are good for neutral species. For the ionised species, however, the measured liposome–water coefficients have been considerably higher compared to octanol–water partition coefficient, which suggests that ionised drugs partition into the membrane [7]. This has been explained by the formation of ionic bonds with the negatively charged phosphate groups. The liposome–water partition coefficients have been measured using distribution

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studies [8], potentiometric titration [7,9,10], dialysis [11], and NMR-spectroscopy [12].

Electrochemical methods, such as cyclic voltammetry, have been used to determine partition coefficients for ionisable drugs [4,13]. Especially electrochemistry at liquid/liquid interfaces is a good tool for determination of  $\log(P)$ , since it allows for the control of the interfacial potential and thus the ionic distribution of the two phases [14]. The traditional solvent used in the partition studies, *n*-octanol, cannot be used in these studies, since electrolytes cannot be dissolved in it. However, it has been suggested that the water/DCE system used in liquid/liquid electrochemistry is, in fact, a more useful system than water/alkane in determining the interactions of solutes with biological membranes [15]. Also NPOE has been used as an organic solvent because of its suitable viscosity, vapour pressure and hydrophobicity [13].

In this study, interactions of liposomes and  $\beta$ -blockers were studied using square wave voltammetry (SWV) at a water–DCE interface created at the tip of a micropipette. Five different  $\beta$ -blockers, propranolol, timolol, carteolol, nadolol and metoprolol were encapsulated in liposomes prepared using the extrusion method. The liposomes were made in three different sizes, with the pore diameters of 100, 400, and 800 nm of the polycarbonate membrane used in extrusion. The partition of five  $\beta$ -blockers between the cavity and the membrane of the liposome was determined using liquid–liquid electrochemistry.

## 2. Materials and methods

### 2.1. Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) were from Avanti Polar Lipids. Propranolol hydrochloride, timolol maleate, nadolol and metoprolol tartrate were purchased from Sigma and carteolol was received as a gift from University of Kuopio. The physicochemical properties of the drugs are shown in Table 1. Tetraphenylammonium tetrakis(4-chlorophenyl)borate (TPAsTPBCl) was prepared by metathesis of tetraphenylarsonium chloride (TPAsCl, Sigma, p.a.) and potassiumtetrakis(4-chlorophenyl)borate (KTPBCl,

Aldrich, p.a.) as described earlier [16]. 1,2-Dichloroethane (DCE) (Sigma, p.a.) was used as received and aqueous solutions were prepared using MQ<sup>®</sup> water. All other chemicals used were analytical grade or better.

### 2.2. Preparation of liposomes

Liposomes composed of POPC and POPG in mass ratio 7:3 and were prepared using the extrusion method. Solid POPC and POPG were dissolved in chloroform and 1:1 chloroform/methanol mixture respectively. Total of 10 mg of lipids in stock solutions was put in a test tube and the organic solvent was evaporated to dryness under a stream of nitrogen. The lipid film formed was hydrated with 1 ml of aqueous buffer solution of 15 mM or 10 mM  $\beta$ -blocker in 2 mM Hepes and 15 mM LiCl at pH 7.4, thus encapsulating the  $\beta$ -blocker in the liposomes. The 15 mM drug solution was used for all other  $\beta$ -blockers except for nadolol, for which 10 mM solution was used because of the low solubility of the drug. The hydration was performed at room temperature. In order to achieve a certain size distribution, the liposome solution was extruded through two-stacked polycarbonate membranes with pore sizes 100, 400 or 800 nm. Unencapsulated  $\beta$ -blocker was separated using a Sephadex G-50 column. The liposome size distribution was measured using dynamic light scattering (Zetasizer Nano, Malvern Instruments). The concentration of liposomes was determined by measuring the amount of phosphorus in the samples using ICP.

### 2.3. Preparation of micropipettes

The micropipettes for the electrochemical measurements were made from borosilicate glass capillaries (outer diameter 1.0 mm, and inner diameter 0.5 mm from Sutter Instrument) and pulled using a laser-based pipette puller (model P-2000, Sutter Instrument). The shape of a micropipette and the diameter of its orifice were controlled by proper choice of the puller's five parameters (heat, filament, velocity, delay, and pull) [18]. To minimize the ohmic resistance due to the shaft leading to the orifice, the pulling program was developed to produce short pipettes. The orifice radius was measured microscopically. The diameter of the orifice varied from 30 to 60  $\mu$ m. The inner wall of the pipette was

Table 1  
Physicochemical properties of the drugs [17]

Drug	Molecular weight (g mol <sup>-1</sup> )	Dissociation constant (pK <sub>a</sub> )	Log( <i>P</i> ) <sub>oct</sub> <sup>a</sup>	Log( <i>P</i> ) <sub>oct</sub> <sup>b</sup>	Log( <i>P</i> ) <sub>lip</sub>
Carteolol	292.38	–	–0.46	1.17	–
Metoprolol	267.37	9.7	1.88	1.20	1.54 [10]
Nadolol	309.41	9.39	0.71	0.23	0.80 [8]
Propranolol	259.35	9.45	3.56	2.75	2.96 [10]
Timolol	316.43	9.21	1.91	1.63	1.50 [10]

Comparison of  $\log(P)$  values of octanol and liposome systems.

<sup>a</sup> Determined experimentally.

<sup>b</sup> Calculated using CLOGP version 3.54.

silanized to make it hydrophobic. This was achieved by dipping the pipette in trimethylchlorosilane to allow the solution to penetrate the pipette. The solution was removed from the pipette after 30 min with a syringe and the silanized pipette was left to dry in the air overnight.

#### 2.4. Electrochemical measurements

Electrochemical measurements were carried out using a micropipette set-up, where the liquid/liquid interface was formed at the tip of the micropipette. A two-electrode cell was used where each electrode serves as a counter and reference electrode for each phase. The potential across the interface was controlled with Ag/AgCl electrode placed in the aqueous phase and Ag electrode in the organic phase. The reference electrode was inserted into the pipette from the rear and the pipette was filled with organic solution using a syringe. A 10 mM solution of TPAsTPBCl served as the organic phase. The aqueous phase of 15 mM LiCl and 2 mM Hepes buffer solution containing the liposomes was outside the micropipette and the liquid/liquid interface was formed at the tip of the pipette. The cell used is shown in Scheme 1 and a schematic illustration of the experimental set-up in Fig. 1.

The measurements were carried out using square wave voltammetry with a computer-controlled potentiostat (Autolab PGSTAT100, Eco Chemie, The Netherlands). The positive current was defined to correspond to the transfer of a positively charged species from the aqueous phase to the organic phase. Within the available potential window, which is determined by the supporting electrolytes used, the  $\beta$ -blocker was the only species transferring across the liquid/liquid interface. The overall process consists of (i) diffusion of the liposomes to the interface, (ii) their decomposition at the contact with the organic phase, (iii) the subsequent release of the drug from liposomes, and (iv) the transfer of the drug across the interface. The rate determining step is diffusion of liposomes, but the height of the current peak is proportional to their drug load.



Scheme 1. The cell used in the electrochemical measurements.

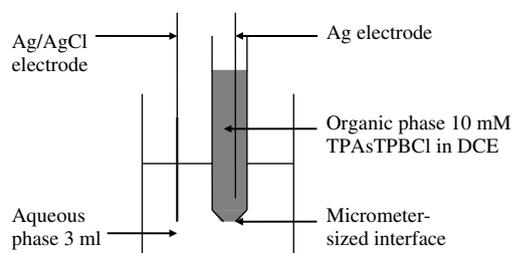


Fig. 1. A schematic illustration of the experimental set-up.

The electrochemical measurements were done either on the same day or the day after the preparation and the size measurement of the liposomes to prevent the possible leakage of the drug out of the vesicle. No significant leakage, however, was ever detected.

### 3. Results

#### 3.1. Liposome size

The size distribution of the liposomes was determined using dynamic light scattering. The average size for each liposome sample is shown in Table 2. It can be seen that the size of the liposomes extruded through 100 nm membrane is of the same magnitude as that of the pore size in the membrane, but as the pore size increases, the liposomes produced are smaller than the pore. The tabulated values are  $z$ -average diameter values, which is the mean diameter determined from the sum of light scattered. For monodisperse systems the  $z$ -average diameter compares well with the mean diameters determined by other methods, but for polydisperse systems this is not the case. In this work, the polydispersity indices of the samples ranged from 0.1 to 0.3, and the  $z$ -average diameter was assumed to describe the dimensions of the liposomes accurately enough.

#### 3.2. Voltammetric measurements

The relationship between the peak current and the concentration of the ionic species for hemispherical diffusion is given by the Eq. (1) [19]

$$\Delta i_p = \frac{zFAD^{1/2}c}{\pi^{1/2}t_p^{1/2}} \Delta\psi_p \quad (1)$$

where  $\Delta i_p$  is the peak current,  $F$  is the Faraday constant,  $A$  the area of the micropipette tip,  $D$  the diffusion coefficient,  $\Delta\psi_p$  a dimensionless peak current,  $t_p$  the pulse width,  $c$  is the bulk concentration of the transferring species, and  $z$  is the charge. The simplest method for determining the dimensionless peak current is to compare the measurement with that of a known solution. In this study, the system was calibrated by measuring the square wave voltammogram of 0.1 mM TEACl in 15 mM Hepes and 2 mM LiCl buffer solution. The frequency used in all measurements was 10 Hz. All the experimental curves were base line corrected

Table 2  
 $z$ -Average diameter of the liposomes containing  $\beta$ -blockers

Drug	Extrusion through 100 nm membrane $d$ (nm)	Extrusion through 400 nm membrane $d$ (nm)	Extrusion through 800 nm membrane $d$ (nm)
Carteolol	97	180	271
Metoprolol	105	225	318
Nadolol	93	193	270
Propranolol	91	172	272
Timolol	86	176	270

using the moving average application included in the potentiostat software (GPES version 4.9). A typical square wave voltammogram representing the transfer of a drug in a liposome across the water–DCE interface is shown in Fig. 2.

The observed peak currents varied from 10 pA to 0.5 nA depending on the encapsulated drug and the size of the liposome. Comparison of the peak currents measured for different sizes of propranolol liposomes is shown in Fig. 3.

The peak current measured can be related to the amount of the drug in the liposome when the size of the liposome and the liposome concentration is known. The concentration of liposomes was determined using the Eq. (2)

$$c_l = \frac{A_p c_p}{2A_l} \quad (2)$$

where  $A_p$  is the area of one phospholipid molecule,  $c_p$  is the phosphorus concentration, and  $A_l$  is the area of the liposome. The measured phosphorus concentrations, which are equal to the lipid concentration, are shown in Table 3. The factor 2 in the denominator accounts for the bilayer structure of the liposome. The area of the phospholipid molecule was taken as  $68 \text{ \AA}^2$  [20].

The diffusion coefficient of the liposomes can be calculated from the Stokes–Einstein relation:

$$D = \frac{kT}{6\pi\eta a} \quad (3)$$

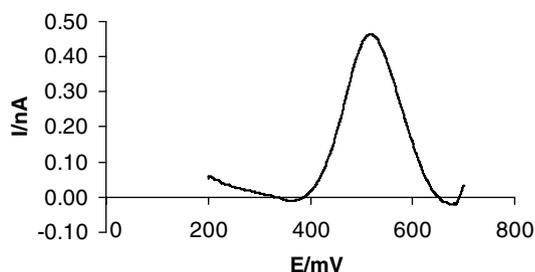


Fig. 2. Measured voltammogram of propranolol liposomes extruded through 400 nm polycarbonate membranes.

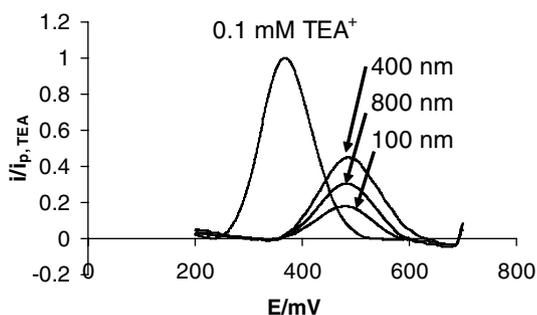


Fig. 3. Measured voltammograms of propranolol encapsulated in liposomes extruded through polycarbonate membranes of different pore sizes. The voltammograms are normalized by the peak current of tetraethylammonium ion.

Table 3  
The phosphorus concentration of the liposome samples

Drug	Extrusion through 100 nm membrane $c \times 10^7/\text{mol cm}^{-3}$	Extrusion through 400 nm membrane $c \times 10^7/\text{mol cm}^{-3}$	Extrusion through 800 nm membrane $c \times 10^7/\text{mol cm}^{-3}$
Carteolol	4.17	1.51	3.11
Metoprolol	2.73	4.34	2.78
Nadolol	3.84	5.19	4.67
Propranolol	2.48	1.40	2.48
Timolol	9.69	2.48	9.58

where  $k$  is Boltzmann constant,  $1.38065 \times 10^{-23} \text{ J/K}$ ,  $T$  is temperature,  $\eta$  is viscosity and  $a$  is the radius of the liposome. The value of viscosity was taken to be that of water at  $25 \text{ }^\circ\text{C}$ ,  $0.89 \text{ cP}$ . The amount of each  $\beta$ -blocker in the liposome is shown in Table 4.

### 3.3. Determination of $\log(P)$

The number of drug molecules in the liposome can be related to the partition coefficient of the drug. Partition coefficient is defined as the ratio of the activity a species in two immiscible phases in equilibrium. In this work, drugs partition between the liposome's aqueous cavity and the lipid bilayer. For simplification, the activities can be approximated by concentrations:

$$P = \frac{c^l}{c^w} \quad (4)$$

where  $c^l$  is the concentration of drug in the lipid bilayer and  $c^w$  is the concentration inside the cavity. The concentration inside the cavity can be assumed to be that of the solution used in the preparation of the liposomes, and the concentration of drug in the lipid layer can be solved as the total amount of drug in a liposome is known:

$$c^l = \frac{n^l}{V^l} = \frac{z - z_c}{N_A V^l} \quad (5)$$

where  $c^l$  is the concentration in the bilayer,  $n^l$  the amount of drug in the bilayer,  $z$  the total number of drug molecules in a liposome measured using square wave voltammetry, and  $N_A$  is Avogadro's constant. The amount of drug in the cavity,  $z_c$ , can be calculated from the drug concentration inside the liposome and the size of the cavity:

$$z_c = N_A c^w \frac{4}{3} \pi a^3 \quad (6)$$

Table 4  
The amount of drug in the liposome

Extrusion pore size (nm)	Carteolol $N \times 10^{-4}$	Metoprolol $N \times 10^{-4}$	Nadolol $N \times 10^{-4}$	Propranolol $N \times 10^{-4}$	Timolol $N \times 10^{-4}$
100	1.10	6.79		9.97	2.79
400	5.60	44.3	3.81	111	71.9
800	12.8	118	7.35	231	73.9

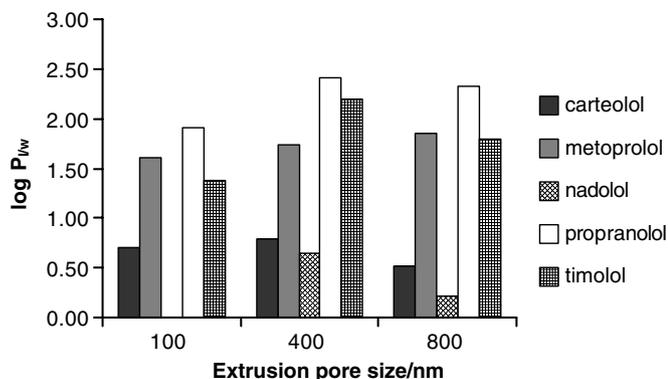


Fig. 4. Partition coefficients of the five  $\beta$ -blockers.

where  $c^w$  is the concentration of the drug used in the preparation of liposomes and  $a$  is the radius of the liposome.  $V^l$  is the volume of the lipid bilayer given by

$$V^l = 4\pi a^2 h \quad (7)$$

where  $h$  is the thickness of the bilayer, which was taken to be 5 nm [21].

The  $\log(P)$  values determined for each  $\beta$ -blocker are shown in Fig. 4. The results shown in Fig. 4 clearly demonstrate that the partition coefficient of ionisable drugs can be determined using the method described in this work. Fig. 4 shows that the partition coefficient of lipophilic drugs such as propranolol and timolol are significantly higher than those of more hydrophilic drugs. Compared to partition coefficients determined by other methods, the method presented gives  $\log(P)$  values of the same order of magnitude as liposome–water partition coefficients determined using distribution studies using UV analysis [8] and HPLC [10]. As observed in earlier studies, these results also suggest that the liposome–water partition coefficients are somewhat lower than the respective octanol–water partition coefficients. The comparison of the octanol–water partition coefficients and liposome–water partition coefficients is shown in Fig. 5. The measured values shown are average values of the determined partition coefficients.

The results show also that the method can be used to determine the partition coefficients of molecules with wide

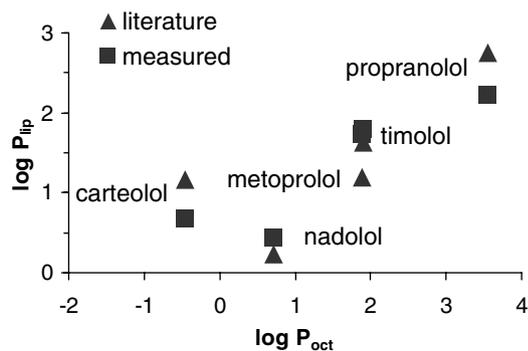


Fig. 5. Comparison of  $\log(P)_{\text{octanol}}$  values and  $\log(P)_{\text{liposome}}$  values.

range of lipophilicity. However, the  $\log(P)$  value of the nadolol liposomes extruded through 100 nm pore size could not be determined because of the hydrophilic nature of the molecule. Due to the hydrophilicity, the liposomes contained such a minute amount of the drug that it could not be observed using SWV. The variability of the  $\log(P)$  values determined using larger nadolol liposomes is also explained by the same fact.

The unsuccessful measurements using nadolol liposomes, nevertheless, confirmed that the possible transfer of anionic lipids does not have a detectable contribution to the measured currents, as the potential window measured with these samples resembled that without lipids at all. Also, the electrostatic binding of drug molecules to the anionic lipids can be neglected based on the unpublished  $\zeta$ -potential measurements of some of liposome samples. These measurements gave typical values of 50–60 mV, from which the charge number of a liposome can be calculated to be of the order of 100. Compared to the number of drug molecules, which range from the order of  $10^4$  to  $10^6$ , it can be seen that the electrostatic binding is rather insignificant.

The precision of the results is not as good as it should be taking into account the accuracy of the electrochemical measurement. The reproducibility of the determination of phosphorus content was not the best possible, which results in the poor precision of the  $\log(P)$  determination. Because of the low lipid concentration and the small sample size, the phosphorus determination was done at the detection limit of ICP. Unfortunately no better option for phosphorus determination was available.

#### 4. Conclusion

The method presented in this work is a fast and easy way to determine the partition constant for ionisable drugs. It combines the rapid and easily controllable electrochemical method with the biomimetic capabilities of liposomes. The method can be applied to wide range of ionisable molecules with varying lipophilicity. In addition, the precision of the method can still be improved greatly using a more accurate method for the determination of the phosphorus content of the samples. Thus the method provides many advantages for studying drug–membrane interactions compared to traditional methods.

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#### References

- [1] A. Leo, C. Hanch, D. Elkins, Chem. Rev. 71 (1971) 526–616.
- [2] P. Boudeville, M. Bona, J.L. Burgot, J. Pharm. Sci. 85 (1996) 990–998.
- [3] A. Avdeef, J. Pharm. Sci. 82 (1993) 183–190.
- [4] K. Kontturi, L. Murtomäki, J. Pharm. Sci. 81 (1992) 970–974.

- [5] G. Klopman, J.-Y. Li, S. Wang, M. Dimayuga, *J. Chem. Inf. Comput. Sci.* 34 (1994) 752–781.
- [6] G.V. Betageri, J.A. Rogers, *Int. J. Pharm.* 36 (1987) 165–173.
- [7] A. Avdeef, K.J. Box, C. Comer, C. Hibbert, K.Y. Tam, *Pharm. Res.* 15 (1998) 209–215.
- [8] G.V. Betageri, J.A. Rogers, *Int. J. Pharm.* 46 (1988) 95–102.
- [9] K. Balon, B.U. Riebesehl, B.W. Müller, *J. Pharm. Sci.* 88 (1999) 802–806.
- [10] A. Taillardat-Bertschinger, C.A.M. Martinet, P.-A. Carrupt, M. Reist, G. Caron, R. Fruttero, B. Testa, *Pharm. Res.* 19 (2002) 729–737.
- [11] S.D. Krämer, H. Wunderli-Allenspach, *Pharm. Res.* 13 (1996) 1851–1855.
- [12] D.A. Middleton, D.G. Reid, A. Watts, *J. Pharm. Sci.* 93 (2004) 507–514.
- [13] S.M. Ulmeanu, H. Jensen, G. Bouchard, P.-A. Carrupt, H.H. Girault, *Pharm. Res.* 20 (2003) 1317–1322.
- [14] F. Reymond, D. Fermin, H.J. Lee, H.H. Girault, *Electrochim. Acta* 45 (2000) 2647–2662.
- [15] G. Steyaert, G. Lisa, P. Gaillard, G. Boss, F. Reymond, H. Girault, P.-A. Carrupt, B. Testa, *J. Chem. Soc., Faraday Trans.* 93 (1997) 401–406.
- [16] V.J. Cunnane, D.J. Schiffrin, C. Beltran, G. Geblewicz, T. Solomon, *J. Electroanal. Chem.* 247 (1988) 203–214.
- [17] C.J. Drayton, Cumulative subject index and drug compendium, in: C. Hansch, P.G. Sammes, J.B. Taylor (Eds.), *Comprehensive Medicinal Chemistry*, vol. 6, Pergamon Press, Oxford, 1990.
- [18] Model P-2000. Micropipette puller, Instruction Manual, Sutter Instrument Co., 2000.
- [19] A.J. Bard, L.R. Faulkner, *Electrochemical Methods. Fundamentals and Applications*, John Wiley & Sons, Inc., New York, 2001.
- [20] G. Beschiaschvili, J. Seelig, *Biochemistry* 29 (1990) 10995–11000.
- [21] H. Binder, K. Gawrisch, *J. Phys. Chem. B* 105 (2001) 12378–12390.