A study of Cobalt (II) complexes involved in marine biogeochemical processes: Co(II)-1,10-Phenanthroline and Co(II)-1,10-Phenanthroline-L-α-Phosphatidylcholine

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Abstract

The cell membrane is structured so that the surface layer is composed of lipid molecules with selective permeability for micronutrients and organic ligands. Binding of Co (II) to natural lipid phosphatidylcholine (PC) has been studied to identify a possible mechanism of Co (II) entry through the cell membrane of the biota in detail, by voltammetry followed by checking the system at the air-water boundary, by Langmuir method. Binding of cobalt (II) ions to the PC molecules was enabled by the Co(II)-1,10-Phenanthroline (Phen) complex formation as an intermediate. Co(II)-Phen-PC complex reduction was recorded in the pH range from 5 to 9.5. The reduction was identified as a two-electron irreversible reaction at about -1.5 V, with the reactant adsorption followed dissociation (EC mechanism). The Co(II)-Phen-PC complex electrode surface concentration (Γ) was calculated to be (1.45 ± 0.12) x 10^{-10} mol cm^{-2}. Conditional stability constants $K_{Co(II)Phen2PC}$ = 23.02 ± 0.26 and log $K_{Co(II)Phen2PC2}$

= 29.31 ± 0.17 (l_c = 0.55) were calculated by CLE/ACSV method. Pressure-area (π-A) isotherms obtained at water-air interface by Langmuir monolayer technique indicated penetration of Co(II)-Phen into the PC monolayer, supporting electrochemical results. The equilibrium constants of the Co (II)-PC system (1:1) at the air-water interface was calculated to be K_1 = 2.4 x 10^{-2} m^3 mol^{-1}, while for Co(II)-Phen-PC K_2 = 4.86 x 10^{10} m^2 mol^{-1}.

Keywords: Cobalt (II), 1,10-Phenanthroline, Phosphatidylcholine, Voltammetry, Langmuir trough

1. Introduction

Cobalt is an essential trace element important for the functioning of all organisms, including biota in the marine environment [1]**.** Micronutrient ions play a variety of important roles in biota and complexation by organic ligands drives their chemical speciation. Namely, cobalt is an important co-factor in vitamin B12-dependent enzymes [2]. Studies on cobalt ions in the marine environment, which define their behavior associated with interactions with cell membranes, have been poorly described in the literature. Identification of the formation mechanism of natural organic ligands is fundamental for determining the micronutrients speciation and determining their bioavailability [3,4]. Cobalt ion express the nutrient profile and shows high reactivity in seawater related to the functioning of the biota. The complexation of cobalt ions in the marine environment is predominant by organic ligands and it suggests the biological control through the production of organic cobalt complexes [5,6]. Studies have shown that cyanobacteria produce cobalt complexing

ligands [7]. For some strains such as *Synechococcus,* cobalt is necessary for their growth and development, however, some can substitute zinc for cobalt in trace metal limited areas, such as *Prochlorococcus*. The best-known ligands that bind cobalt are vitamin B12 and coenzyme B12 [8,9]. The stability constants for these ligands are extremely high (log*K*>17). 1,10-Phenanthroline ($C_{12}H_8N_2$, Phen) is a heteroaromatic compound whose nitrogen atoms are arranged perfectly to bond various cations [10], is often used as humic/fulvic acid model ligand in natural waters [11], as well as vitamin B12.

Two general features of phospholipid bilayers are critical to membrane function [12]. First, the structure of phospholipids is responsible for the basic function of membranes as barriers between two aqueous compartments. Hydrophobic fatty acid chains occupy the interior of the phospholipid bilayer making the membrane impermeable to water-soluble molecules, including metal ions and most biological molecules. Second, bilayers of naturally occurring phospholipids are viscous liquids [13]. The fatty acids of most natural phospholipids have one or more double bonds that introduce hooks into the hydrocarbon chains and make them difficult to pack together. Therefore, the long hydrocarbon chains of the fatty acids move freely inside the membrane, making it soft and flexible [14]. In model solutions metal ions are most often coordinated with carboxylate and NH groups [15,16]. Phospholipid monolayers absorbed at model hydrophobic interfaces, such are the mercury electrode surface and air-water interface, actually represent a biological membrane model system [17,18]. Some studies reported interactions of metal ions, drugs, toxins, and biologically important species with model phospholipid monolayers [19-22]. Additionally, liposomes are frequently used as unilamellar or multilamellar spherical structures in studies of biological model membranes [23]. Adsorption of organic

molecules to the phospholipid membrane causes a change in membrane surface potential and/or modifies its dipole potential. Interactions of micronutrient ions with cells through membranes in the marine environment are of particular interest as they are critical for the maintenance of cellular function [24].

The aim of this study was to investigate the interaction/binding mechanism of Co(II) with phospholipids, as a vital component of hydrophobic cell membranes, following the formation of their complexes through an intermediate, Co(II)-Phen complex.

2. Experimental

2.1. Chemicals and solutions

L-α-phosphatidylcholine (PC; *Sigma-Aldrich*, egg yolk, type XVI-E, ≥ 99% (TLC)) as lyophilized powder was used in this study. The components of the PC commonly present in natural seawater, contain approximately 33% 16:0 (palmitic acid), 13% 18:0 (stearic acid), 31% 18:1 (oleic acid) and 15% 18:2 (linoleic acid) (other fatty acids in a smaller amounts), resulting in an average molecular weight of approximately 768 g mol^{-1}. The original stock solution was kept at 253.15 K. PC solution for electrochemical measurements was prepared by dissolution in methanol (1×10^{-2} mol dm^{-3}) (p.a. *Kemika d.d.,* Zagreb, Croatia). Methanol stock solution of PC was added in to the NaCl solution (1×10^{-5} mol dm^{-3}) and by the accumulation monolayer was formed by the accumulation at E_{acc} = 0.0 V vs Ag/AgCl (Fig S1A,B). For the monolayer experiments by Langmuir method the PC stock chemical (0.5 mg cm^{-3}) was dissolved in hexane of HPLC grade (Merck) and monolayer was formed at the NaCl surface by the syringe applying. A stock solution (1×10^{-3} mol dm^{-3}) of 1,10-Phenanthroline ($C_{12}H_8N_2$,Phen) (*p.a.*, Merck), was dissolved in ultraclean water.

NaCl (Suprapur®, Merck, Germany) as electrolyte solution (I_c = 0.55 mol dm^{-3}) was prepared by dissolution in Milli-Q water. A standard solution of Co(II) nitrate (1.69 x 10^{-2} mol dm^{-3}) (Fluka Chemie GmbH, Buchs, Switzerland), as the source of cobalt (II) in all experiments, was used.

2.2. Electrochemical measurements

Voltammetry studies were performed by μ-AUTOLAB multimode potentiostat (ECO Chemie, Utrecht, the Netherlands) using Metrohm 663 VA stand (Metrohm, Herisau, Switzerland). The instrument was computer-controlled using GPES 4.9 control software, while a static mercury drop electrode (SMDE, size 2, i .e. 0.40 mm^2) was working electrode, a glassy carbon stick counter electrode and Ag/AgCl (sat. NaCl) (+0.197 V *vs*. SHE) reference one. Experiments were done in quartz cell at 25±1 °C in NaCl of I_c = 0.55 mol dm^{-3} (I_c = ionic strength). Voltammetric methods used were alternating current (AC), square-wave (SWV) and cyclic technique (CV). Solutions were deaerated by bubbling with extra pure nitrogen for about 20 min with stirring (3000 rpm) before measurements, while during measurements, nitrogen circulated above the solution. pH was measured by glass-Ag/AgCl electrode linked to ATI Orion PerpHecTMeter, model 320 (Cambridge, USA). All relevant measurements were repeated for three times, at least.

2.3. Surface pressure measurements

Surface pressure-area (π-A) measurements were performed in a Fromherz-type round Teflon trough (Mayer-Feintechnik, Germany), equipped with two movable barriers. Surface pressure was performed at 21 °C with a precision ± 0 .1 mN m^{-1}

using a Wilhelmy plate (Whatman filter paper, No. 1) as pressure sensor. On the initial surface area (A_{max} = 160 cm^2), the PC monolayer was formed by carefully applying aliquots (V = 0.02 cm^3) of the PC solution in hexane (0.5 mg cm^{-3}) onto the subphase using a microsyringe (Hamilton−Bonaduz, Switzerland). The supporting monolayer was left for 3 h to allow the incorporation of the subsurface components within the monolayer. The monolayer was further compressed at a rate of 60 cm^2 min^{-1}, and changes in surface pressure due to changes in molecular packing of the formed monolayer were followed while π-A isotherms were recorded. The monolayer was compressed up to the compression limit of 40 mN m^{-1}, prior to collapse. Consistency and the reproducibility of results were ensured by repeating each measurement at least five times. The limiting area per molecule, A_{lim}, was determined conventionally by extrapolating the linear part of the obtained π - A isotherm to π = 0 mN m^{-1}, which corresponds to the cross-sectional area of the maximally ordered molecule for a given monolayer. The trough and barriers were cleaned after each run with hexane and Milli-Q water.

3. Results and Discussion

3.1. Voltammetric characterization of Co (II)-1,10-Phen and Co (II)-1,10-Phen-PC complex

Lipid membranes attached to the mercury drop electrode surface are highly promising biomimetic membranes for the elucidation of structure-function relationships micronutrients in the environment [25]. Investigations of the potential interactions of Co(II) with PC were carried out using square wave (SW), alternating current (AC) out of phase mode (tensametry, phase angle 90°) and cyclic voltammetry (CV). We anticipated a simple binding mechanism between PC with

Co(II) via Co(II)-PC complexes formation, however, our results advocated rather more complex reaction pathway. The introduction of 1,10-Phenanthroline (Phen) as an intermediate ligand led to the formation of Co(II) mixed ligand complex with PC. According to our previous investigations, Co(II) complexes bind mainly to nitrogen-containing organic ligands [16]. Its entropic advantage indicates fast formation of very stable complexes with metal ions, especially with transition metals. The Phen ligand behaves like a weak base in aqueous solution with pK_a ~ 4.9. Alternating current voltammograms (ACV) indicate moderate adsorption of Phen at the mercury drop electrode surface due to aromatic structure in the potential range from -0 .1 to -1.6 V. By adding Co (II) to the Phen solution complex SW reduction peak appears at about -1.0 V (pH = 8.2). pH dependence of the Co(II)-Phen complex reduction current showed an increase in the pH range from 3.5 to 5.0, while it remained constant at higher pH values (up to pH 9). By titration of Phen (1 x 10^{-6} mol dm^{-3}) with Co(II) in the range from 1 x 10^{-7} to 1 x 10^{-6} mol dm^{-3}, the reduction current of the complex (E_p ≈ -1.0 V) increased until c_{Co} = 8 x 10^{-7} mol dm^{-3} (Fig.1). With higher Co(II) concentrations reduction current decreased due to saturation of the mercury drop surface with Co(II)-Phen molecules, due to a reduction process inhibition. The reduction potential of Co(II)-Phen changed slightly towards negative values (Fig 1).

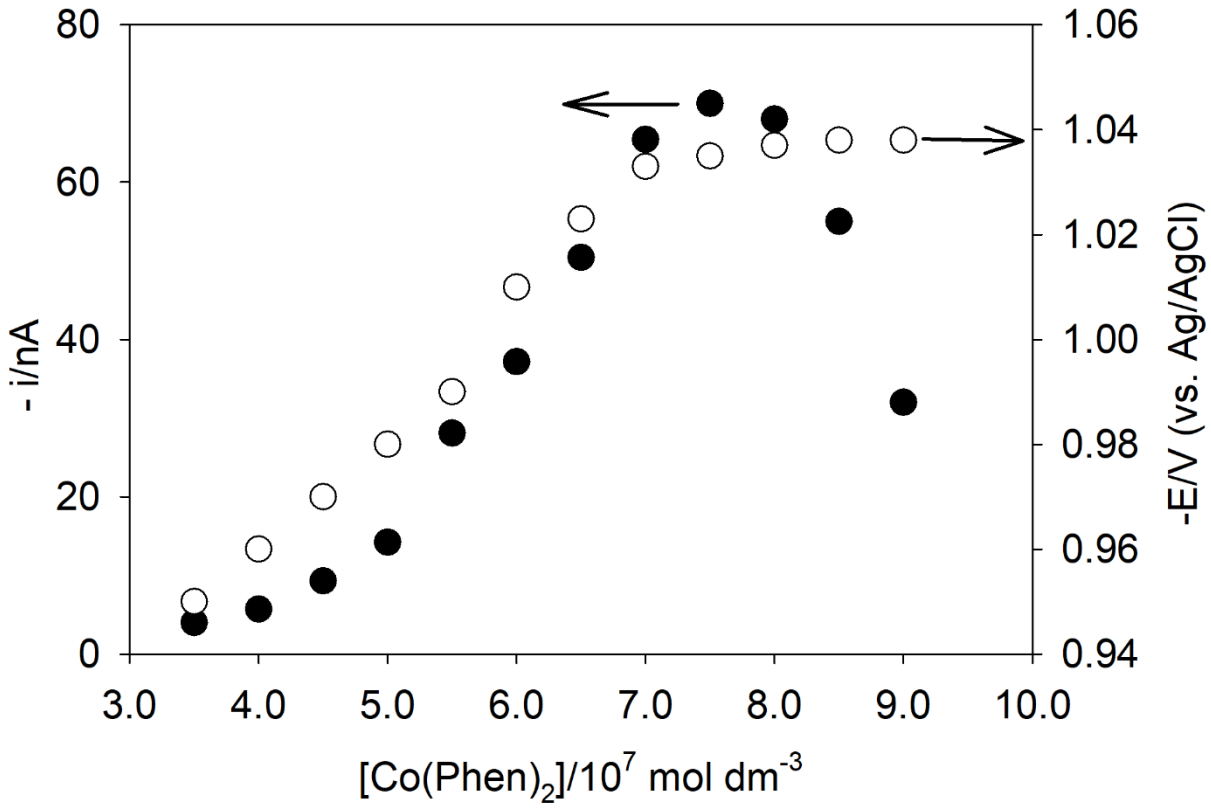

Fig 1. Dependence of (●) peak current and (○) peak potential of the $Co(Phen)_2$ complex on $c_{(Co)}$ in range from $1x10^{-7}$ to $1x10^{-6}$ mol dm^{-3}; $c_{(Phen)}$ = $1x10^{-6}$ mol dm^{-3}, E_{acc}= 0 V, t_{acc} = 180 s, f = 50 s^{-1}, a = 25 mV.

Furthermore, interaction of Co(II) with PC was investigated. The ACV point to PC adsorption in the potential range from about -0.1 V and desorption at potential > -1.65 V (Fig. S1A). The capacitive peak registered at about -1.1 V represents the reorientation process of PC molecules as the consequence of charging current, revealing structural/electrostatic changes in the adsorbed lipid layer from flat to perpendicular position [16, 24-26]. No interaction of PC with Co (II) ions was recorded on SW voltammograms (Fig 2, curve 2). The sequence of the ligands (Phen, PC) addition to the solution revealed to be very important. Namely, the hydrophobic ligand – PC adsorbs at the mercury drop surface by the accumulation, and added Phen adhere to the PC layer and only slightly affects the surface

organization of its monolayer (Fig. 2, curve 3). With the addition of Co(II) to the solution of both ligands, neutral hydrophilic Co(II)-Phen complex forms in the solution and by the accumulation at the electrode surface creates hydrophobic mixed ligand complex. Namely, PC molecules replace the remaining water molecules from the Co(II) ion coordination sphere. As described in the literature, the ion channels open in response to the initiating stimulus [27]. In ligand-gated channels, ligand-binding domains are attached to the pore in the adsorption layer at the electrode surface. In neutral ligand channels attached to the electrode surface, the parts that bind the hydrophilic complex are in the pores of the adsorption layer. Therefore, the Co(II)-Phen complex as intermediate assisted Co(II) mixed ligand complex formation with lipid. Co(II)-Phen-PC complex in the adsorption layer allows the electron path from the electrode to Co(II) ion through the ligand gated channel. A sharp reduction signal at about −1.5 V was registered as Co(II) was added to the solution of both ligands present (Fig. 2, curve 4) and it is assigned to two-electron reduction of Co(II)-Phen-PC complex. After reduction process mixed ligand complex dissociate (Fig. 2, CV inset), only the PC layer remains on the electrode surface, as evident when two subsequent scans were performed at the same Hg drop.

Accumulation time as well as accumulation potential variation influenced on the amount of adsorbed mixed ligand complex at the electrode. The reduction peak of Co(II)-Phen-PC complex increased with the variation of the accumulation potential between -0.3 and -1.3 V. With E_{acc} = −1.3 V for 3 minutes or more, it was calculated that by SWV measurable Co (II) concentrations would be down to 10^{-10} mol dm^{-3}.

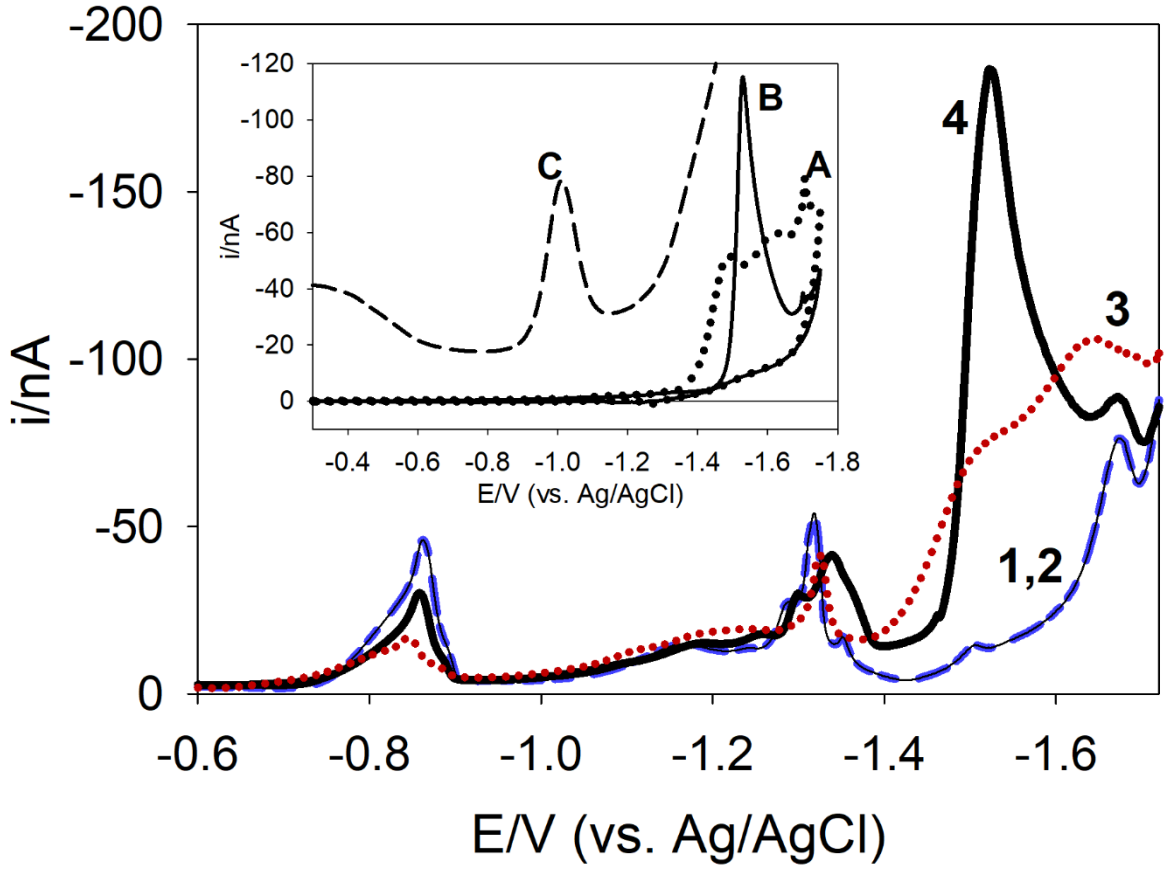

Fig 2. SW voltammograms of c_{PC} = 1x10^{-5} mol dm^{-3}, c_{Phen} = 10^{-6} mol dm^{-3}, c_{Co} = 5x 10^{-7} mol dm^{-3}; 1) PC, 2) PC+Co, 3) Phen+PC, 4) Phen+PC+Co; E_{acc}= -0.3 V, t_{acc}=60 s, E_{step} = 2 mV, a = 25 mV, f =25 s^{-1}; inset: CV of Co(II)-Phen-PC, 2 scans at the same mercury drop; **A** first plot, **B** second plot; **C** Co(II)-Phen linear scan voltammogram; c_{Phen} = 10^{-6} mol dm^{-3}, c_{Co} = 1x10^{-6} mol dm^{-3}; E_{acc} = -0.3 V, t_{acc}= 180 s, v=50mV s^{-1}; 0.55 mol dm^{-3} NaCl, pH = 8.2.

The Co(II)-Phen-PC complex was registered in the pH range from 5 to 9, largely corresponding to the pH range in which Co(II)-Phen is present in solution. In the pH range from 5 to 8 the reduction current of Co(II)-Phen-PC complex increased, while at higher pH values current decreased due to the formation of Co (II) hydroxides. Simultaneously, the Co(II)-Phen-PC the reduction potential shifts to more negative

values due to the thicker layer of adsorbed complex molecules. Co(II)-Phen-PC complex reduction peak current increased linearly in the Co(II) concentration range 1 × 10^{-9} mol dm^{-3} - 5 × 10^{-7} mol dm^{-3} with the slope 20.91 ± 0.21 nA/μM (Fig. 3; Fig. S2). With further additions of Co(II) to the solution, the reduction current decreased due to the redox process inhibition.

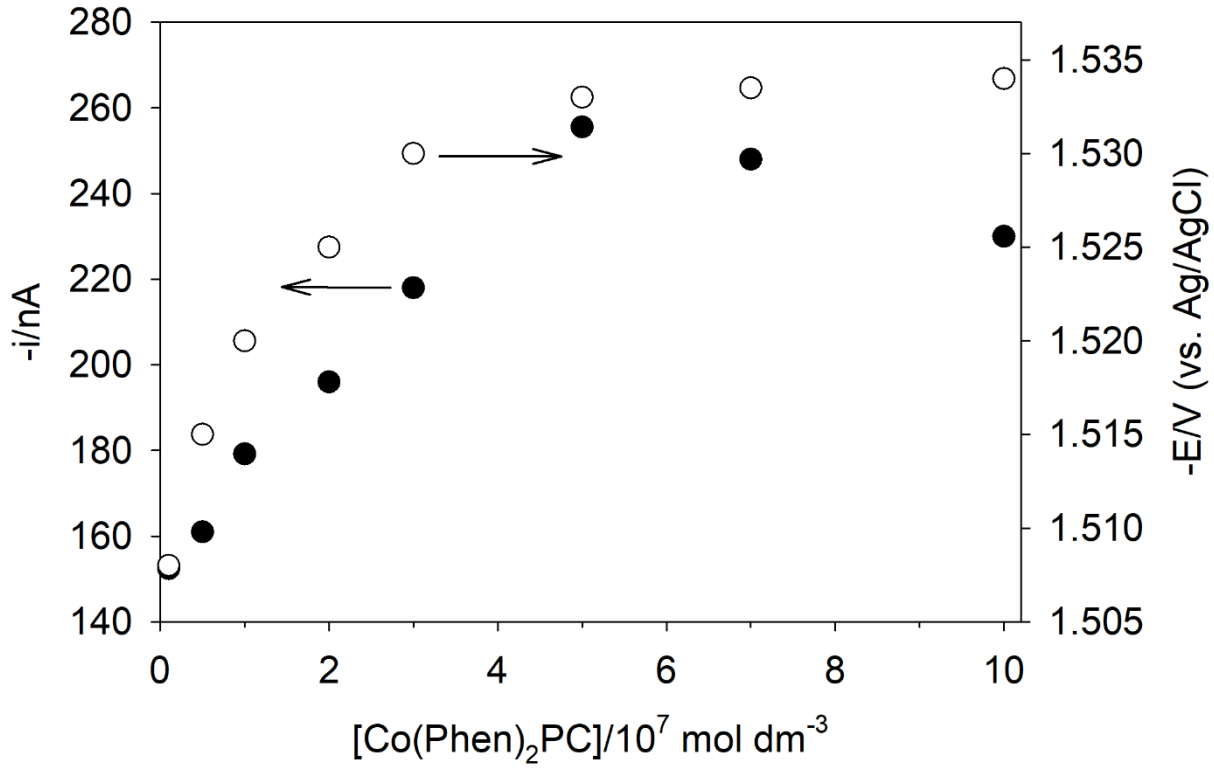

Fig. 3. Dependence of (●) peak current and (○) peak potential of the Co-Phen-PC complex on $c_{(Co)}$ in range from 1x10^{-9} to 1x10^{-6} mol dm^{-3}. $c_{(Phen)}$ = 1x10^{-6} mol dm^{-3}, c_{PC} = 1x10^{-5} mol dm^{-3}; E_{acc}= -0.3 V, t_{acc} = 180, f = 50 s^{-1}, a = 25 mV.

3.1.1. Estimation of the electrochemical redox process mechanisms and calculated conditional stability constants

By SWV and CV was examined reduction process mechanisms and electrochemical characteristics of described Co (II) complexes. Their reduction peak currents and

potential dependencies on SW frequency, SW amplitude and CV scan rate, were measured with c_{Phen} = 1 × 10^{-6} mol dm^{-3}, c_{Co} = 1 × 10^{-6} mol dm^{-3} pH = 8.2 for Co (II)-Phen complex. Mixed ligand complex measurements were done with c_{PC} = 1 × 10^{-5} mol dm^{-3}, c_{Phen} = 10^{-6} mol dm^{-3}, c_{Co} = 5 × 10^{-7} mol dm^{-3} at E_{acc} = -0.3 V with t_{acc} = 180 s. As SWV technique discriminates faraday and capacitive currents, and provides an insight into both half-electrode reactions, it is particularly suitable for studying the mechanisms of electrode processes [28]. The SWV response separation into forward current measured before the "down" pulse and backward, and reverse current measured at the "down" pulse of staircase, showed their reduction characteristics. SW forward-backward (f-b) showed that Co-Phen reduction process is reversible (Fig. 4 A), while of Co(II)-Phen-PC mixed ligand complex completely irreversible (Fig. 4.B).

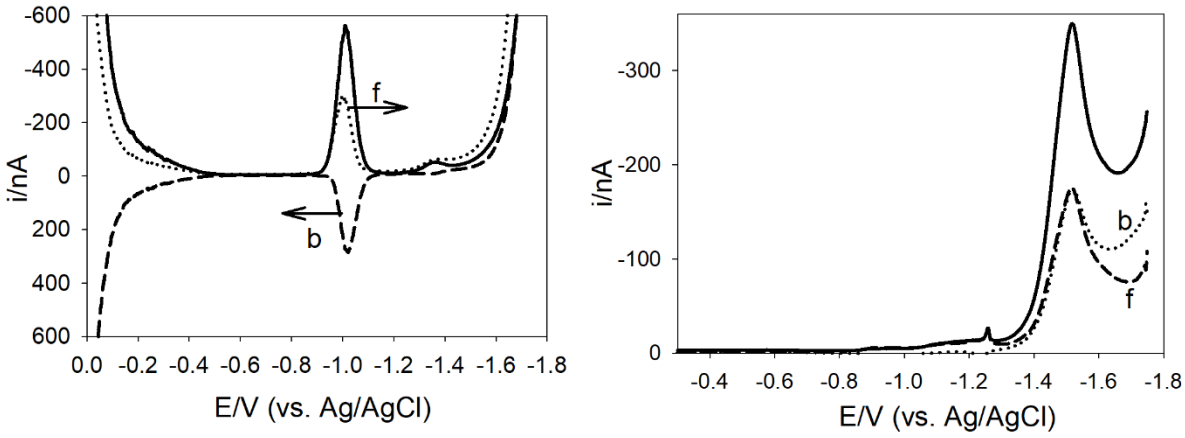

Fig 4. backward-forward SW voltammograms of **A**) Co(II)-Phen; **B**) Co(II)-Phen-PC complexes; c_{PC} = 1 × 10^{-5} mol dm^{-3}, c_{Phen} = 1 × 10^{-6} mol dm^{-3}, c_{Co} = 10^{-6} mol dm^{-3}.

Amplitude dependence (in the range from 5 to 50 mV) on the reduction current of the Co(II)-Phen complex increased linearly with a slope of 24.18 ± 0.11 nAV^{-1}, while with A >50 mV reduction peak split in two what is characteristic for reversible reduction

processes from adsorbed state [29]. Reversible reduction with reactant reduction Co(II)-Phen reduction current linear dependence on the scan rate (CV) in the range from 1 to 100 mV/s with the slope of 1.65 ± 02 nAV^{-1}, and shift of the reduction potential towards negative values, is characteristic for reversible reduction processes with the reactant adsorption [16].

Impact of the SW frequency and amplitude on the net reduction current (i_p) and potential (E_p) on Co(II)-Phen-PC complex reduction was investigated as well. Co(II)-Phen-PC complex reduction current (E_p ~ -1.5 V, t_{acc} = 60 s) depends linearly on f in the range 8 –300 s^{-1}, with the slope 21.25 ± 0. 12 nA, while reduction peak potential dependence was linear with log f (slope 20.07 ± 0.08 mV/d.u.) implying the irreversible reactant reduction from adsorbed state [30]. Co(II)-Phen-PC complex reduction peak current dependence on SW amplitude was linear in range 5-60 mV with the slope 89.52 ± 0.14 AV^{-1}.

By of the CV reduction current dependence inspection of the Co(II)-Phen-PC complex on the scan rate (v) in the range 1-100 mV/s, linear dependence of the reduction current with the shift of the reduction potential towards negative values confirmed that mechanism is irreversible with the reactant adsorption. Mixed ligand complex reduction current increased exponentially with $v^{1/2}$.

Furthermore, reactant adsorption of the irreversible reduction process revealed that the reduction peak width at half-height, satisfied the relationship $\Delta E_{p/2}$ (mV) = (63.5 ± 0.5)/αn, where n = 2 (number of simultaneously transferred electrons) and where α is the average transfer coefficient [29,30]. Form the experimental data calculated peak width at half-height of Co(II)-Phen was $E_{p/2}$ = 73 mV and relates to α = 0.5 ± 0.05, while for Co(II)-Phen-PC complex was $E_{p/2}$ = 85 mV and relates to α = 0.37 ± 0.02.

According to equation:

$i_p = (5\pm1) \times 10^2 q\alpha n^2 F \alpha f \Delta E \Gamma$ (1)

from the slope i_p/α using values $\alpha_{Co(II)\text{-}Phen\text{-}PC} = 0.37$, n = 2 and q = 0.004 cm^2 (the surface of the mercury drop), F = 96 485 s A mol^{-1} (Faraday constant) and ΔE = 20 mV (square-wave scan increment) the amount of the adsorbed reactant (Γ) was calculated. Co(II)-Phen-PC complex maximum concentration adsorbed at the mercury drop electrode amount to $\Gamma = (1.45 \pm 0.12) \times 10^{-10}$ mol cm^{-2}.

Conditional stability constants and apparent stoichiometry of Co(II) complexes were determined by CLE/ACSV method [31]. The method was described as competitive ligand equilibrium (CLE) followed by adsorptive cathode stripping measurements (ACSV). The conditional stability constant for Co(II)-Phen was determined using Nitrilotriacetic acid (NTA) as a competitive ligand [16, 32], while for Co(II)-Phen-PC complex Phen. Conditional stability constant was calculated based on following equations.

$c_{Co} = [Co'] + [(CoPhen)_n] + [(CoNTA)]$ (2)

or for the mixed complex:

$c_{Co} = [Co'] + [(CoPhen_xPC_y)_n] + [(CoPhen)_n]^*$ (3)

where [Co´] is the sum of concentrations of all inorganic species $(Co(OH)^+$ and $Co(OH)_2$ and $Co(Phen)_n$ $Co(Phen_xPC_y)_n$ (complex of Co with n molecules of ligand). $[(CoNTA)_n]$ represents the concentration quantity of all present Co(II) species with NTA or Phen* as a competitive ligand at given pH. Peak currents were directly related to $[(CoPhen)_n]$ or $[(CoPhen_xPC_y)_n]$ through the proportionally factor S:

$I_p = S\,[Co(CoPhen)_n]$ (4)

$I_p = S\,[Co(CoPhen_xPC_y)_n]$ (5)

295 The ratio, *X*, of cobalt peak current in presence of 1,10-Phenanthroline as a
296 competitive ligand ($i_{p,i}$) to one without it, $i_{p,0}$ can be written as

297 $X = i_p/i_{p,0} = [(CoPhen)_n] / [(CoPhen)_n] + [CoNTA]$ (6)

298 $X = i_p/i_{p,0} = [(CoPhen_xPC_y)_n] / [(CoPhen_xPC_y)_n] + [(CoPhen)_n]$* (7)

299 Using expressions for $[(CoPhen)_n]$ and $[(CoPhen_xPC_y)_n]$

300 $X = K^{cond}_{CoPhen}[Phen]_n / K^{cond}_{CoPhen2}[Phen]_n + K_{CoOHNTA}[NTA]_n$ (8)

301 $X = K^{cond}_{\mathrm{CoPhenxPCy}}[CoPhen_xPC_y]_n / K^{cond}_{\mathrm{CoPhenxPCy}}[CoPhen_xPC_y]_n + K_{CoOHPhen}[Phen]_n$* (9)

302 From the mean value of the experimentally obtained values it was calculated log
303 $K_{Co(II)\text{-}1,10\text{-}Phen2}$ to amount to 22.75 ± 0.48. Obtained conditional constants amount to
304 log $K_{Co(II)Phen2PC}$ = 23.02 ± 0.26 and log $K_{Co(II)Phen2PC2}$ = 29.31 ± 0.17. The
305 stoichiometry was determined so that the calculated curve agreed well with our
306 experimental data (Fig. S2 A, B, C).

307

308 **3.2. Monolayer surface pressure - area measurements**

309 Monolayer studies at the air-water interface were performed to complement the
310 voltammetric conclusions of the mixed ligand complex Co(II)-Phen-PC formation at a
311 model hydrophobic interface. An aqueous solution of 0.55 mol dm^{-3} NaCl (pH = 8.2; 1
312 mol dm^{-3} borate buffer) was used as pure subphase, while pure subphase with
313 additions of Co(II) ions (1×10^{-5} mol dm^{-3}), Phen (1.2×10^{-5} mol dm^{-3}), and/or both
314 Co (II) ions and Phen was further used for penetration experiments. The compression
315 behavior of the PC monolayer on the pure subphase was firstly evaluated (Fig. 4,
316 curve 1). At A_{max} (> 60 $Å^2$), PC molecules were in the liquid-expanded state with
317 some degree of cooperative interaction but still not closely packed, as previously well
318 described [33-36]. As the area per molecule decreased during the compression of the

monolayer, the surface pressure steadily increased due to the coexistence of liquid expanded and liquid condensed phases, reaching the maximal surface pressure. Determined A_{lim} of PC monolayer on pure subphase was 58 Å^2, which is in the range of the previous results. Penetration experiments were further performed during 3h at the constant A_{max} where PC monolayer was in the liquid-expanded phase, facilitating the interactions of the different subphase constituents with supporting monolayer. A comparison of the specific π-A isotherms of PC monolayer on pure subphase as a reference with those where additions of Co(II) ions (1×10^{-5} mol dm^{-3}) and both Co(II) ions and Phen, are present in subphase (Fig.5), enables information on the intermolecular interactions within the reference monolayer.

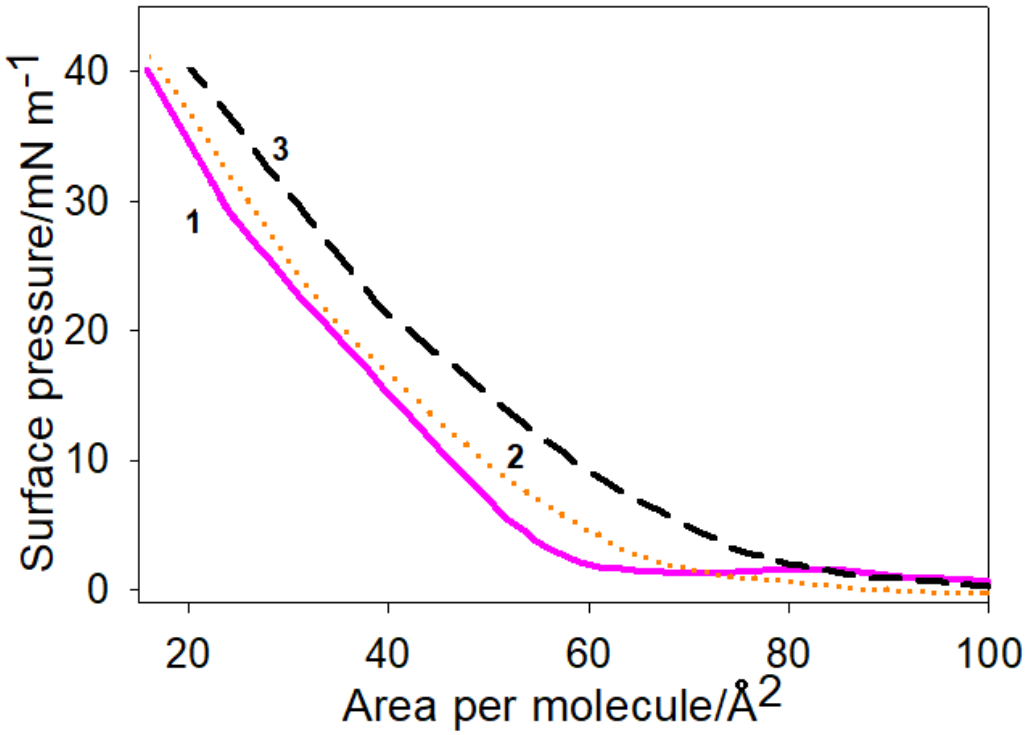

Fig 5. Surface pressure (π) - area (A) compression isotherms of the PC monolayer on a pure subphase (0.55 mol dm^{-3} NaCl; 1 mmol dm^{-3} borate buffer) (1); pure subphase containing c_{Co} 1x10^{-5} mol dm^{-3} (2); 1x10^{-5} mol dm^{-3} Co(II) + 1.2 × 10^{-5} mol dm^{-3} Phen (3); pH=8.2; T = 294 K.

The *π-A* isotherms of the PC monolayer on a subphase containing Co(II) ions (Fig. 5, curve 2) showed that a weak interaction take place between the PC molecules and the Co(II), allowing the PC molecules to pack more densely, i.e. to occupy a smaller surface area, A_{lim} = (53.0 ± 0. 5) Å^2, relative to PC monolayer on pure subphase (Fig.5, curve 1). The observed condensation effect is noticed for the dipalmitoylphosphatidylcholine (DPPC) monolayers affected by the increasing concentrations of Zn^{2+} in the subphase where dehydration of phosphate groups of the DPPC occurred through direct interaction with metal ions [37]. The neutralization processes within the lipid head-group moiety may change the local conformation and have a general electrical screening effect [38-41]. The strength of the interaction between the divalent ion and the lipid monolayer most likely increase in the area of the lipid head-group, where the dielectric permittivity of the environment and, thus, the electrostatic screening of the charges was reduced.

Compared to PC monolayer on a subphase containing Co(II) ions, an increase of A_{lim} to (64.0 ± 0.8) Å^2 was observed when of Co(II) and Phen were present in the subphase (Fig. 5, curve 3). Both components present in the subphase, thus, affected the surface organization of the PC monolayer, resulting in a more expanded film. The observed expansion effect was different from the previously detected condensation effect due to the presence of Co (II) ions (without Phen) in the subphase. Since Phen was not absorbed on the PC monolayer itself (isotherm identical to PC on pure subphase, not shown), we hypothesized that the observed effect of expansion on the incorporation of Co (II) -Phen complex into the PC monolayer and the formation of Co (II) -Phen-PC complex is the explanation for such behavior. This is supported by the fact that the expansion of PC in the subphase Co (II) + Phen in relation to the reference PC monolayer occurred even

359 at the highest surface pressures. This indicates the strong incorporation of
360 dissolved forms into the carrier monolayer of PC molecules, so that, “extrusion”
361 from the lipid head region was not observed in the tightly packed state of the
362 monolayer [34,37].

363 The expansion of the *π-A* isotherms, as a result of the interaction between the lipid
364 monolayer and solutes from the subphase, was commonly interpreted as
365 penetration or incorporation of the solute forms into the supporting monolayer
366 [42,43]. It is worth noting that the isotherms of the subphase containing only Co(II)
367 ions or Phen in the absence of the PC layer at the surface did not show significant
368 adsorption at the interface itself (data not shown), as reported previously [44]. The
369 observed effects with the monolayer study supported previous electrochemical
370 findings on the Co(II) -Phen complex binding within the PC layer and formation of a
371 mixed Co(II)-Phen-PC complex at the surface.

372 The equilibria of observed system at the air-water interface can be described by
373 following chemical reactions:

374 $PC + Co^{2+} \leftrightarrow PCCo^{2+}$ (10)

375 $PCCo^{2+} + 2Phen \leftrightarrow PCCo^{2+}Phen_2$ (11)

376 and the equilibrium state of the system could be described by following equations:

377 $$K1 = \frac{cPCCo^{2+}}{c\mathrm{PC} * c\mathrm{Co}^{2+}}$$ (12)

378 $$K2 = \frac{cPCCo^{2+}Phen_2}{cPCCo^{2+} * c^2 Phen}$$ (13)

379 $c_{PC}*A_{PC}+c_{PCCo2+}*A_{PCCo2+} + c_{PCCo2+Phen}*A_{PCCo2+Phen} =1$ (14)

380 $c_{PC} + c_{PCCO2+} + 2c_{PCCo2+Phen2} = C$ (15)

where c_{PC}, c_{PCCo2+}, $c_{PCCo2+Phen2}$ (mol m^{-2}) are the surface concentrations of components PC, $PCCo^{2+}$, $PCCo^{2+}Phen_2$; c_{Co2+} (mol m^{-3}) is the concentration of Co^{2+} ions; A_{PC}, A_{PCCo2+}, $A_{PCCo2+Phen}$ (m^2 mol^{-1}) are surface areas occupied by 1 mol of components PC, $PCCo^{2+}$, $PCCo^{2+}Phen_2$. K_1 (m^3 mol^{-1}) K_2 (m^2 mol^{-1}) are the stability constants of systems $PCCo^{2+}$ and $PCCo^{2+}Phen_2$, and C (mol m^{-2}) is the total surface concentration.

Multiple linear regression was performed, following the established procedure described in details previously [45,46], $K1$, $K2$, A_{PC}, A_{PCCo2+}, $A_{PCCo2+Phen}$ were calculated. Equations used are shown following instructions from the literature [34]:

$$A\ \mathrm{PC} = (-m3)/\mathrm{b} \quad (16)$$

$$A\ \mathrm{PCCo} = (-m1)/\mathrm{m4} \quad (17)$$

$$A\ \mathrm{PCCoPhen} = 2\ (A\ \mathrm{PC} * \mathrm{m4} - A\ \mathrm{PCCo} * \mathrm{b})/(\mathrm{m4} - \mathrm{b}) \quad (18)$$

$$K_1 = -\frac{2 * A\ PCCo^{2+} - A\ PCCo^{2+}Phen_2}{m_4 * K_2 * A^2 PCCo^{2+}Phen_2} \quad (19)$$

$$K_2 = -\frac{(2APC - APCCo^{2+}Phen_2) + (2APCCo^{2+} - APCCo^{2+}Phen_2)}{4 + m_5 * A^2 PCCo^{2+}Phen_2} \quad (20)$$

The stability constant of the PC-Co system (1:1) was calculated to amount to K_1 = 2.4 x 10^{-2} m^3 mol^{-1}, while K_2 = 4.86 x 10^{10} m^2 mol^{-1} upon the addition of Phen describing Pc-Co-$Phen_2$ system the air-water boundary. Although the monolayer study at the air-water interface was accomplished to complement the electrochemical results, it is important to note that the obtained complex constants calculated based on the electrochemical and monolayer data, are not comparable due to the different approaches applied. Although a single-layer air-water boundary study was conducted to confirm the electrochemical results, it is important to note that the equilibrium constants obtained based on electrochemical and monolayer data, are not comparable due to the application of different approaches. However, both constant

405 types showed higher stability of the mixed complex than the stability of the Co(II)-
406 Phen or Co(II)-Phen-PC complex.
407

408 Conclusion

409 The penetration of cobalt (II) as one of the crucial micronutrient in numerous
410 biological processes, through the cell membrane is important for understanding its
411 fate and role in the marine environment living organisms. Studies of PC adsorbed on
412 hydrophobic surfaces (mercury drop electrode and air-water interface) used as a
413 model for a cell membrane, showed that direct binding between Co(II) and PC is very
414 weak, and not registered by electrochemical measurements. Conditional Stability
415 Constants of the complexes are essential for assessing the ability of ligands to bind
416 micronutrients under actual conditions. The study of the ligand substitution
417 mechanism in the Co(II) coordination sphere enable to predict the micronutrients
418 behavior in biological processes. Co(II) complexation takes place with Phen forming
419 a stable complex with a stoichiometry of 1:2. Co (II)-Phen complex with conditional
420 stability constant log $K_{Co(II)\text{-}1,10\text{-}Phen2}$ = 22.75 ± 0.48. Co (II)-Phen complex as an
421 intermediate interacts with PC molecules by substituting two remained water
422 molecules in its coordination sphere resulting most probably in the stable octahedral
423 configuration. In that way is formed hydrophobic mixed ligand complex Co(II)-Phen-
424 PC. The mixed ligand complex reduction occurs at about -1.5 V by an irreversible
425 mechanism, followed by dissociation, leaving only PC molecules adsorbed at the
426 electrode surface (EC mechanism). The net stoichiometry of the mixed ligand
427 complexes formed at the mercury drop electrode was calculated to be 1:2:1 and 1:2:2
428 for Co:Phen:PC. The conditional stability constants of mixed ligand complexes were
429 calculated to amount *log* $K_{Co(II)Phen2PC}$ = 23.02 ± 0.26 and *log* $K_{Co(II)Phen2PC2}$ = 29.31±

0.17 (l_c = 0.55). The quantity of adsorbed reactant (Γ) Co (II)-Phen-PC complex was *calculated to be* (1.45 ± 0. 12) x 10^{-10} mol cm^{-2}. The totally irreversible mechanism of reduction with adsorption of the reactant was confirmed by examining the reduction current and potential in dependence on the SW frequency and amplitude, as well as by examining the SW forward and backward current. Our electrochemical measurement results s were supported by experiments at the air-water interface. The change in the parameters of obtained *π-A* isotherms indicated a penetration of Co(II)-Phen complex into the supporting PC monolayer. A weak stability constant of the PC-Co system (1:1) at the air-water interface was determined to be K_1 = 2.4 x 10^{-2} m^3 mol^{-1}. Obtained PC isotherms affected by the addition of both Co(II) ions and Phen in the subphase, indicated a possibility of a mixed ligand complex formation. Furthermore, calculated stability constant for described system amount to K_2 = 4.86 x 10^{10} m^2 mol^{-1}. This study, performed at model hydrophobic surfaces, is a base for a better understanding of the cobalt (II) - lipid association mechanism in biogeochemical processes mediated by cell membranes, with particular relevance to the marine environment.

Acknowledgement

The financial support for this work was provided by BiREADI project IP-2018-01-3105 (Biochemical responses of oligotrophic Adriatic surface ecosystems to atmospheric deposition inputs), funded by the Croatian Science Foundation.

452 References:

453

454 [1] K. W. Bruland, J. R. Donat, D. A. Hutchins, Interactive influences of bioactive
455 trace metals on biological production in oceanic waters, Limnol. Oceanogr., 36(8)
456 (1991) 1555; doi:10.4319/LO.1991.36.8.1555

457

458 [2] E. I. Hamilton, The geobiochemistry of cobalt, Sci. Total Environ., 150 (1–3)
459 (1994) 7.; doi: 10.1016/0048-9697(94)90126-0

460

461 [3] K. N. Buck, M. C. Lohan, S. G. Sander, C. Hassler, and I. Pižeta, Editorial:
462 Organic ligands-A key control on trace metal biogeochemistry in the ocean, Front.
463 Mar. Sci., 4 (2017) 1; doi: org/10.3389/fmars.2017.00313

464

465 [4] T. W. Lane, M. A. Saito, G. N. George, I. J. Pickering, R. C. Prince, F. M. M.
466 Morel, A cadmium enzyme from a marine diatom, Nature, 435 (2005),42;
467 doi: 10.1038/435042a

468

469 [5] M. J. Ellwood, C. M. G. Van den Berg, Determination of organic complexation of
470 cobalt in seawater by cathodic stripping voltammetry, Mar. Chem., 75(1-2) (2001) 33;
471 doi: org/10.1016/S0304-4203(01)00024-X

472

473 [6] M. A. Saito, J. W. Moffett, Complexation of cobalt by natural organic ligands in the
474 Sargasso sea as determined by a new high-sensitivity electrochemical cobalt
475 speciation method suitable for open ocean work, Mar. Chem., 75 (1–2) (2001) 49;
476 doi: org/10.1016/S0304-4203(01)00025-1

[7] J. M. Vraspir, A. Butler, Chemistry of marine ligands and siderophores, Ann. Rev. Mar. Sci., 1 (2009) 43; doi:10.1146/annurev.marine.010908.163712

[8] Deenah Osman, Anastasia Cooke, Tessa R. Young, EvelyneDeery, Nigel J. Robinson, Martin J. Warren, The requirement for cobalt in vitamin B12: A paradigm for protein metalation, BBA - Molecular Cell Research 1868 (2021) 118896; doi: org/10.1016/j.bbamcr.2020.118896

[9] Q. Z. Zhu, Robert Curwood Aller, Robert Curwood Aller, Aleya Kaushik, Analysis of vitamin B12 in seawater and marine sediment porewater using ELISA, 2011 Limnology and Oceanography, Methods 9(10):515-523; doi: org/10.4319/lom.2011.9.515

[10] A. Bencini and V. Lippolis, 1,10-Phenanthroline: A versatile building block for the construction of ligands for various purposes, Coord. Chem. Rev., 254(17–18) (2010) 2096; doi: org/10.1016/j.ccr.2010.04.008

[11] N. Fujisawa, K. Furubayashi, M. Fukushima, M. Yamamoto, T. Komai, K. Ootsuka, Y. Kawabe, Evaluation of the Iron(II)-binding Abilities of Humic Acids by Complexometric Titration using Colorimetry with ortho-Phenanthroline, Humic Sub Res., 8 (2011) 1.

[12] R. Pichot, R. L. Watson, I. T. Norton, Phospholipids at the interface: Current trends and challenges, Int. J. Mol. Sci., 14(6) (2013) 11767; doi: 10.3390/ijms140611767; doi: 10.3389/fimmu.2017.01513

[13] Y. Ma, K. Poole, J. Goyette, K. Gaus, Introducing membrane charge and membrane potential to T cell signaling, Front. Immunol., 8 (2017) 1; doi: 10.3389/fimmu.2017.01513

[14] L. O. Gerlach, J. S. Jakobsen, K. P. Jensen, M. R. Rosenkilde, R. T. Skerlj, U. Ryde, G. J. Bridge, T. W. Schwartz, Metal ion enhanced binding of AMD3100 to Asp262 in the CXCR4 receptor, Biochemistry, 42(3) (2003), 710; doi: 10.1021/bi0264770

[15] J. J. Giner-Casares, G. Brezesinski, H. Möhwald, Langmuir monolayers as unique physical models, Curr. Opin. Colloid Interface Sci., 19(3) (2014) 176; doi: org/10.1016/j.cocis.2013.07.006

[16] A. Bačinić, L. M. Tumir, and M. Mlakar, Electrochemical characterization of Cobalt(II)-Complexes involved in marine biogeochemical processes. I. Co(II)-4-nitrocatechol and Co(II)-Humate, Electrochim. Acta, 337 (2020) 135797; doi: org/10.1016/j.electacta.2020.135797

[17] S. M. Baumler, G. J. Blanchard, The Influence of Metal Ions on the Dynamics of Supported Phospholipid Langmuir Films, Langmuir, 33(12) (2017) 2986; doi: org/10.1021/acs.langmuir.7b00042

[18] A. P. Serro, R. Galante, A. Kozica, P. Paradiso, A. M. P. S Gonçalves da Silva, K. V. Luzyanin, A. C. Fernandes, B. Saramago, Effect of tetracaine on DMPC and

526 DMPC + cholesterol biomembrane models: Liposomes and monolayers, Colloids
527 Surfaces B Biointerfaces, 116 (2014) 63; doi: 10.1016/j.colsurfb.2013.12.042
528
529 [19] V. P. N. Geraldo, F. J. Pavinatto, T. M. Nobre, L. Caseli, O. N. Oliveira, Langmuir
530 films containing ibuprofen and phospholipids, Chem. Phys. Lett., 559 (2013), 99; doi:
531 10.1016/j.cplett.2012.12.064
532
533 [20] J. J. Leitch, C. L. Brosseau, S. G. Roscoe, K. Bessonov, J. R. Dutcher, J.
534 Lipkowski, Electrochemical and PM-IRRAS characterization of cholera toxin binding
535 at a model biological membrane, Langmuir, 29(3) (2013) 965; doi:
536 org/10.1021/la304939k
537
538 [21] A. Akbarzadeh, R. Rezaei-sadabady, S. Davaran, S. W. Joo, N. Zarghami,
539 Liposome : classification , prepNew aspects of liposomesaration , and applications,
540 Nanoscale Res. Lett., 8(102) (2003) 1; doi: org/10.1186/1556-276X-8-102
541
542 [22] M. N. Jones, The surface properties of phospholipid liposome systems and their
543 characterisation, Adv. Colloid Interface Sci., 54 (1995) 93; doi: 10.1016/0001-
544 8686(94)00223-y
545
546 [23] A. Nelson, N. Auffret, J. Borlakoglu, Interaction of hydrophobic organic
547 compounds with mercury adsorbed dioleoylphosphatidylcholine monolayers, BBA -
548 Biomembr., 1021(2) (1990) 205; doi: org/10.1016/0005-2736(90)90035-M
549

[24] L. M. Pfeffer, B. C. P. Kwok B.C.P., F.R. Landsberger., I. Tamm, Interferon stimulates cholesterol and phosphatidylcholine synthesis but inhibits cholesterol ester synthesis in HeLa-S3 cells (human (3-interferon/lipid metabolism/membrane structure/endocytosis/low density lipoprotein). Proc. Nati. Acad. Sci. USA 82 (1985) 2417–2421; doi:10.1073/pnas.82.8.2417

[25] I. Čuljak, M. Mlakar, M. Branica, Synergetic adsorption of the copper-phenanthroline-tributylphosphate complex at a mercury drop electrode, Anal. Chim. Acta, 297(3) (1994) 427; doi: org/10.1016/0003-2670(94)00225-8

[26] X. Liang, D. J. Campopiano, P. J. Sadler, Metals in membranes, Chem. Soc. Rev., 36, (6) (2007) 968; doi: org/10.1039/B617040B

[27] M. Zelić, Reverse Scan as a Source of Information in Square Wave Voltammetry, Croat. Chem. Acta 79 (1) (2006) 49

[28] M. Lovrić and Š. Komorsky-Lovrić, Theory of Square-Wave Voltammetry of Two-Electron Reduction with the Adsorption of Intermediate, International Journal of Electrochemistry, 2012, (2012) 7; doi: org/10.1155/2012/596268

[29] M. Lovrić, Š. Komorsky-Lovrić, R. W. Murray, Adsorption effects in square-wave voltammetry of totally irreversible redox reactions, Electrochim. Acta, 33(6) (1988) 739; doi: org/10.1016/S0013-4686(98)80002-9

574 [30] E. L. Rue, K. W. Bruland. Complexation of iron(III) by natural organic ligands in
575 the central North Pacific as determined by a new competitive ligand
576 equilibration/adsorptive cathodic stripping voltammetric method, Mar. Chem. 50
577 (1995) 117–138; doi.org/10.1016/0304-4203(95)00031-L
578
579 [31] P. Vukosav, V. Tomišić, M. Mlakar, "Iron(III)-complexes engaged in the
580 biochemical processes in seawater. II. Voltammetry of Fe(III)-malate complexes in
581 model aqueous solution, Electroanalysis, 22(19) (2010) 2179; doi:
582 org/10.1002/elan.200900632
583
584 [32] G. L. Gaines, Insluble monolayers at liquid-gas interfaces, Academic press, New
585 York 1966, p. 254; doi: 10.1007/978-94-011-2272-6_3
586
587 [33] A. Aroti, E, Leontidis, M. Dubois, T. Zembb, G. Brezesinski, Monolayers, bilayers
588 and micelles of zwitterionic lipids as model systems for the study of specific anion
589 effects. Colloids Surf A. 303 (2007) 144–158., doi: 10.1016/j.colsurfa.2007.03.011.
590
591 [34] M. Sovago M, W. H. George, M. S. Wurpel, M. Müller, M. Bonn, Calcium-
592 induced phospholipid ordering depends on surface pressure. J Am Chem Soc. 129
593 (2007) 11079–11084. doi: 10.1021/ja071189i.
594
595 [35] A. D. Petelska, Z. A. Figaszewski, Phosphatidylcholine - Mg^{2+} equilibria in a
596 monolayer at the air/water interface. Open Chem. 11 (2013) 424–429; doi:
597 10.2478/s11532-012-0170-7
598

599 [36] P. Laszuk, W. Urbaniak, A. D. Petelska, The Equilibria in Lipid–Lipoic Acid
600 Systems Monolayers, Microelectrophoretic and Interfacial Tension Studies,
601 Molecules, 25(16) (2020), 3678; doi.org/10.3390/molecules25163678
602
603 [37] S. Li, L. Du, N. T. Tsona, W. Wang, The interaction of trace heavy metal with
604 lipid monolayer in the sea surface microlayer, Chemosphere 196 (2018) 323–330;
605 doi: org/10.1016/j.chemosphere.2017.12.157
606
607 [38] M. Bara, A. Guiet-Bara, J. Durlach, Analysis of magnesium membraneous
608 effects:
609 612 binding and screening, Magnes Res 29 (1990) 4121–4128; pmid: 3274925
610
611 [39] Adams, E.M., Casper, C.B., Allen, H.C., Effect of cation enrichment on
612 dipalmitoylphosphatidylcholine (DPPC) monolayers at the air-water interface, J.
613 Colloid Interface Sci. 478(2016) 353–364;doi: 10.1016/j.jcis.2016.06.016
614
615 [40] S. Kewalramani, H. Hlaing, B.M. Ocko, I. Kuzmenko, M. Fukuto Effects of
616 divalent cations on phase behavior and structure of a zwitterionic phospholipid
617 (DMPC) monolayer at the air–water interface. J Phys Chem Lett 1 (2010) 489–498,
618 doi: org/10.1021/jz9002873
619
620 [41] A. D. Petelska, M. Naumowicz, M. The effect of divalent ions on L-α-
621 phosphatidylcholine from egg yolk monolayers at the air/water interface, J Biol Inorg
622 Chem 22 (2017)1187–1195; doi: org/10.1007/s00775-017-1495-7
623

[42] Z. Kozarac, B. Ćosović, R. C. Ahuja, D. Moebius, W. Budach, Interaction of Pyrene-3-sulfonate with Lipid Monolayers // Langmuir, 12(22) (1996) 5387-5392; doi: 10.1021/la950908g

[43] Z. Kozarac, D. Möbius, D. B. Spohn, Investigation of Sea-surface Microlayer and Phytoplankton Culture Samples by Monolayer Techniques and Brewster Angle Microscopy, Croatica Chemica Acta, 71(2)(1998) 285-301

[44] R. Verger, F. Pattus, Lipid-Protein Interactions in Monolayers, Chemistry and physics of lipids 30 (1982) 189-227; doi: org/10.1016/0009-3084(82)90052-4

[45] M. Ivanova, M. G., Verger, R., Bois, A. G., Panaiotov, I. Proteins at the air/water interface and their inhibitory effects on enzyme lipolysis. Colloids Surf. 54 (1991) 279–296; doi: org/10.1016/0166-6622(91)80067-X

[46] M. Mlakar, V. Cuculić, S. Frka, B. Gašparović, Copper-phospholipid interaction at cell membrane model hydrophobic surfaces, Bioelectrochemistry, 120 (2018) 10; doi: org/10.1016/j.bioelechem.2017.11.004