

Elektrokemijska karakterizacija kompleksa Co(II) s organskim ligandima iz mora

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Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET

Anđela Bačinić

**ELEKTROKEMIJSKA
KARAKTERIZACIJA KOMPLEKSA Co(II)
S ORGANSKIM LIGANDIMA IZ MORA**

DOKTORSKI RAD

Zagreb, 2022.



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Mentor: Dr.sc. Marina Mlakar

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Sveučilište u Zagrebu

FACULTY OF SCIENCE

Anđela Bačinić

**ELECTROCHEMICAL
CHARACTERISATION OF Co(II)
COMPLEXES WITH ORGANIC LIGANDS
FROM SEAWATER**

DOCTORAL THESIS

Supervisor: Marina Mlakar, PhD

Zagreb, 2022.

Ovaj doktorski rad izrađen je u Laboratoriju za fizičku kemiju tragova, Zavoda za istraživanje mora i okoliša Instituta Ruđer Bošković u Zagrebu, pod mentorstvom dr.sc. Marine Mlakar, u sklopu Interdisciplinarnog doktorskog studija Oceanologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu.

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ELEKTROKEMIJSKA KARAKTERIZACIJA KOMPLEKSA Co(II) S ORGANSKIM LIGANDIMA IZ MORA

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Sažetak: Kobalt je esencijalan mikronutrijent u morskoj vodi. U morskom okolišu prisutan je u otopljenoj frakciji u vrlo niskim koncentracijama, $<10^{-9}$ mol dm⁻³. Vezan za organske ligande stvara stabilne komplekse u otopljenoj frakciji morske vode koji su slabo istraženi. U karakterizaciji organskih kompleksa kobalta u uvjetima morske vode korištena je adsorpcijska voltometrija katodnog otapanja. Metoda se temelji na adsorpciji formiranih kompleksa kobalta s odabranim organskim ligandima prisutnim u moru (kateholi, humusna kiselina, fosfolipidi) na površini elektrode živina kap, te njihovoj redukciji. Osim elektrokemijski, kompleksi su istraživani i metodom spektrofotometrije. S obzirom da su neki od korištenih liganada (fosfolipidi) amfifilne molekule, kompleksiranje kobalta praćeno je i na granici faza voda/zrak Langmuir-Blodgett metodom te mikroskopijom atomskih sila. Kobalt s ispitanim ligandima stvara stabilne komplekse visokih uvjetnih konstanti stabilnosti $\log K_{Co(II)-4NC2} = 21,86$ (pH = 8,2) i $\log K_{Co(II)-4NC2} = 21,11$ (pH = 6,5), te $\log K_{Co(II)HA2} = 11,32$, $\log K_{Co(II)Phen2PC} = 23,02$ i $\log K_{Co(II)Phen2PC2} = 29,31$ (pH = 8,2) nastale na hidrofobnoj elektrodi živina kap u modelnoj otopini morske vode (NaCl, pH = 8,2, $I_c = 0,55$ mol dm⁻³). Spektrofotometrijski su detektirani kompleksi kobalta topljivi u vodenoj otopini te su izračunate ravnotežne konstante stabilnosti kompleksa $\log K_{Co(II)-4NC} = 5,76 \pm 0,40$ (pH = 6,5), $\log K_{Co(II)-4NC} = 3,98 \pm 0,17$ (pH = 8,2) i $\log K_{Co(II)-HA} = 3,80 \pm 0,29$ (pH = 8,2). Langmuir-Blodgett metoda ukazuje na vezanje 1,10-fenantrolina i kobalta na monosloj PC-a. Određena konstanta stabilnosti takvog sloja je iznimno niska i iznosi $K_1 = 2,4 \times 10^{-2}$ m³ mol⁻¹, dok dodatkom 1,10-fenantrolina dolazi do nastajanja sloja velike stabilnosti i konstante $K_2 = 4,86 \times 10^{10}$ m² mol⁻¹. Mikroskopijom atomskih sila pokazano je kako smjesa fenantrolina i fosfolipida, kako standarda tako i realnih uzoraka, pokazuje stvaranja vezikula, dok dodatak kobalta u smjesu dovodi do stvaranja "terasa" zbog pretpostavljenog povezivanja molekula. Istraživanje provedeno u svrhu izrade ove doktorske disertacije, istaknulo je važnost poznavanja specijacije kobalta i razumijevanja biogeokemijskih procesa u kojim sudjeluje u morskom okolišu.

(106 stranica, 10 slika, 143 literaturna navoda, jezik izvornika: hrvatski)

Ključne riječi: elektrokemija, kobalt, more, katehol, humusna kiselina, fosfolipidi

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ELECTROCHEMICAL CHARACTERISATION OF Co(II) COMPLEXES WITH ORGANIC LIGANDS FROM SEAWATER

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Abstract: Cobalt is an essential micronutrient in seawater. In the marine environment, it is present in the dissolved fraction at very low concentrations, $< 10^{-9}$ mol dm⁻³. Bound to organic ligands, it forms stable complexes in the dissolved fraction of seawater, which have been poorly investigated. Adsorption cathodic stripping voltammetry was used to characterise cobalt complexes with organic ligands under seawater conditions. The method is based on the adsorption of formed complexes with selected organic ligands present in the sea (catechols, humic acid, phospholipids) on the surface of the mercury drop electrode and their reduction. In addition to the electrochemical studies, the complexes were also investigated by the spectrophotometry method. Since some of the ligands used (phospholipids) are amphiphilic molecules, cobalt complexation was also monitored at the water/air phase boundary using the Langmuir-Blodgett method and Atomic force microscopy. Cobalt formed stable complexes with the ligands with high conditional stability constants $\log K_{\text{Co(II)-4NC2}} = 21.86$ (pH = 8.2) and $\log K_{\text{Co(II)-4NC2}} = 21.11$ (pH = 6.5), and $\log K_{\text{Co(II)HA2}} = 11.32$, $\log K_{\text{Co(II)Phen2PC}} = 23.02$, and $\log K_{\text{Co(II)Phen2PC2}} = 29.31$ (pH = 8.2) formed at a hydrophobic mercury drop electrode in a model seawater solution (NaCl, pH = 8.2, $I_c = 0.55$ mol dm⁻³). Cobalt complexes soluble in aqueous solution were detected spectrophotometrically as well, and the equilibrium stability constants of the complex $\log K_{\text{Co(II)-4NC}} = 5.76 \pm 0.40$ (pH = 6.5), $\log K_{\text{Co(II)-4NC}} = 3.98 \pm 0.17$ were calculated. (pH = 8.2) and $\log K_{\text{Co(II)-HA}} = 3.80 \pm 0.29$ (pH = 8.2). The Langmuir-Blodgett method shows the binding of 1,10-phenanthroline and cobalt to the PC monolayer. The determined layer stability constant is extremely low and amounts to $K_1 = 2.4 \times 10^{-2}$ m³ mol⁻¹, while the addition of 1,10-phenanthroline leads to the formation of a layer of high stability with constant $K_2 = 4.86 \times 10^{10}$ m² mol⁻¹. Atomic force microscopy showed that the mixture of phenanthroline and phospholipids of both standard and real samples, shows the formation of vesicles, while the addition of cobalt to the mixture leads to the formation of "terraces" due to the connection of molecules. The studies carried out in this dissertation have shown the importance of knowing the speciation of cobalt and understanding the biogeochemical processes in which it is involved in the marine environment.

(106 pages, 10 figures, 143 references, original in Croatian)

Keywords: electrochemistry, cobalt, seawater, catechol, humic acid, phospholipids

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POPIS KORIŠTENIH KRATICA I SIMBOLA

AC voltometrija izmjenične struje (engl. *alternatic current voltammetry*)

AFM mikroskopija atomskih sila (engl. *atomic force microscopy*)

ASV voltometrija anodnog otapanja (engl. *anodic stripping voltammetry*)

CSV voltometrija katodnog otapanja (engl. *cathodic stripping voltammetry*)

CV ciklička voltometrija

AdCSV adsorpcijska voltometrija katodnog otapanja (engl. *adsorptive cathodic stripping voltammetry*)

DOC otopljeni organski ugljik (engl. *dissolved organic carbon*)

DOM otopljena organska tvar (engl. *dissolved organic matter*)

CDOM obojana otopljena organska tvar (engl. *colored dissolved organic matter*)

EDTA etilendiamintetraoctena kiselina (engl. *Ethylenediaminetetraacetic acid*)

EMZ elektromagnetsko zračenje

FK fulvinska kiselina

HK humusna kiselina

HT humusna tvar

LB Langmuir-Blodgett

MALDI-TOF-MS matricom potpomognuta ionizacija laserskom desorpcijom spektrometrija masa s analizatorom masa s vremenom leta (engl. *Matrix Assisted Laser-Mass Spectrometry-Time-of-Flight*)

NC nitrokatehol (engl. *nitrocatechol*)

NTA nitriloctena kiselina (engl. *nitrilotriacetic acid*)

PC fosfatidilkolin (engl. *phosphatidylethanolamine*)

PE fosfatidiletanolamin (engl. *phosphatidylethanolamine*)

PG fosfatidilglicerol (engl. *phosphatidylglycerole*)

PHEN fenantrolin (engl. *phenanthroline*)

PI fosfatidilinozitol (engl. *phosphatidylinositol*)

PL fosfolipidi (engl. *phospholipids*)

POC čestični organski ugljik (engl. *particulate organic carbon*)

POM čestična organska tvar (engl. *particulate organic matter*)

PSA Potenciometrijska stripping analiza (engl. *potential stripping voltammetry*)

PVV Pravokutno valna voltometrija

UV/Vis spektrofotometrija (engl. *Ultraviolet-visible spectrophotometry*)

Simbol	Fizikalna veličina	Jedinica
A_{UV-V}	apsorbancija	
A_{LB}	površina koje zauzima 1 mol komponente	mol m^{-2}
a_{LB}	površinska koncentracija komponenti na kadi	mol m^{-2}
a	amplituda	V
C_{LB}	ukupna površinska koncentracija	mol m^{-2}
c	koncentracija (ukupna)	mol dm^{-3}
D_i	difuzijski koeficijent vrste	m^2s^{-1}
E	potencijal elektrode	V
E_{ak}	potencijal akumulacije	V
E_p	potencijal vrha vala	V
$\Delta E_{p/2}$	širina vala na polovici visine	V
E°	standardni potencijal	V
ΔE	korak potencijala	V
f	frekvencija	s^{-1}
I_c	ionska jakost (koncentracijska)	mol dm^{-3}
i	jakost struje	A
i_p	jakost struje vrha vala	A
$k_{a,i}$	konstanta brzine asocijacije vrste	$\text{mol}^{-1}\text{dm}^3 \text{s}^{-1}$
$k_{d,i}$	konstanta brzine disocijacije vrste	s^{-1}
K_{ML}	konstanta stabilnosti kompleksa ML	$\text{mol}^{-1} \text{dm}^3$
K'_{ML}	uvjetna konstanta ravnoteže	$\text{mol}^{-1} \text{dm}^3$
n	broj izmijenjenih elektrona	-
P	površina elektrode	m^2
t	vrijeme	s
t_{ak}	vrijeme akumulacije	s
V	volumen	m^3
z	nabojni broj iona	
Γ	površinska koncentracija	mol cm^{-2}
Γ_{\max}	maksimalna površinska koncentracija	mol cm^{-2}
δ	debljina difuzijskog sloja	m
ε	molarni apsorpcijski koeficijent	$\text{mol}^{-1} \text{dm}^3 \text{cm}^{-1}$
J_{ad}	adsorpcijski protok	$\text{mol m}^{-2} \text{s}^{-1}$
λ	valna duljina svjetlosti	m
ν	frekvencija elektromagnetskog zračenja	s^{-1}
τ	vrijeme trajanja impulsa u PVV	s

PROŠIRENI SAŽETAK

Kobalt je bioesencijalan element u tragovima uključen u biološke procese (Swanner *et al.*, 2014). Potreban je za razvoj fitoplanktona, posebno kokolitofora i cijanobakterija (*Prochlorococcus* i *Synechococcus*), te ostalih viših organizama u moru (Bown *et al.*, 2012; Morel i Milligan, 2003). Dok je za prokariote nužan, eukarioti kao što su npr. kokolitofore, mogu ga zamijeniti cinkom (Sunda i Huntsman, 1995). Esencijalan je nutrijent jer izgrađuje molekulu kobalamina (vitamin B₁₂) potrebnu za metabolizam organizama (Osman *et al.*, 2021). Pri niskim koncentracijama esencijalnog cinka kobalt ga može zamijeniti u strukturi enzima kao npr. ugljične anhidraze (Moore *et al.*, 2018). Kobalt u more ulazi iz različitih izvora, kako prirodnih tako i antropogenih, stoga je njegova raspodjela kemijskih vrsta u moru (specijacija) dinamičan proces (Noble *et al.*, 2017). Redoks je aktivan metal koji se u morskom okolišu pojavljuje u redoks stanjima Co(II) i Co(III) ovisno o uvjetima okoliša, u slobodnom obliku, vezan za organske ili anorganske ligande i adsorbiran na čestice (Saito *et al.*, 2004). Otopljen u morskoj vodi prisutan je u vrlo niskim koncentracijama, u rasponu od 10⁻¹⁰ do 10⁻¹² mol dm⁻³ (Ellwood *et al.*, 2005). Koncentracija varira sezonski, a u oceanima raste s dubinom što ukazuje na uključenost u biološke procese u površinskim slojevima (Ellwood i Van den Berg, 2001). Kako bismo razumjeli biološke, geološke i kemijske procese (biogeokemiju) u kojima sudjeluje kobalt u moru nužno je istražiti njegovu specijaciju. Mehanizam potrošnje kobalta od strane vodenih organizama, kao i proces njegova uklanjanja precipitacijom i međudjelovanjem sa suspendiranom tvari (organskom ili anorganskom) dio su njegovog biogeokemijskog ciklusa (Noble *et al.*, 2017). Kobalt stvara komplekse s anorganskim i organskim ligandima u kiselom i neutralnom pH području, dok je u lužnatom prisutan (pH > 8,5) u obliku hidroksida (Ćosović *et al.*, 1982a). Kompleksi kobalta s organskim ligandima su stabilni, s uvjetnim konstantama stabilnosti log *K* > 16 izmjerenim pri određenim eksperimentalnim uvjetima (ionskoj jakosti, pH) (Noble *et al.*, 2017). Poseban afinitet kobalt ima prema organskim ligandima s dušikovim skupinama (Chen *et al.*, 2014; Ferreira *et al.*, 2018; Noble *et al.*, 2017).

Ovaj doktorski rad temelji se na tri objavljena znanstvena rada, a cilj mu je istražiti stvaranje kompleksa kobalta s odabranim organskim ligandima prirodno prisutnim u morskom okolišu. Sukladno prethodnom iskustvu i istraženju literaturi, izabrali smo modelne i prirodne ligande od biološke važnosti za biokemijske procese u moru. Korišteni ligandi su kateholi (katehol, 4-nitrokatehol (engl. 4-*nitrocatechol* – NC)), humusna kiselina (engl. *humic acid* – HA) i fosfolipidi koji izgrađuju stanične membrane. Za dopunu voltametrijske analize Co(II) kompleksa korištene su i druge metode analize. Kompleksi 4-NC i HA s Co(II) analizirani su

voltametrijskim i spektrofotometrijskim metodama u prvom znanstvenom radu (Bačinić *et al.* 2020), a hidrofobni kompleksi s fosfolipidima u drugom i trećem znanstvenom radu (Bačinić *et al.* 2022a, 2022b), voltametrijom katodnog otapanja, Langmuir-Blodgett metodom za analizu amfifilnih molekula, te mikroskopijom atomskih sila. Razvijene su specifične metode za analizu kompleksa, određeni optimalni parametri mjerenja, te uvjetne konstante stabilnosti.

Postavljena je hipoteza da Co(II) kao važan mikronutrijent u moru stvara komplekse s prirodno prisutnim organskim ligandima što utječe na njegovu biodostupnost.

U prvom znanstvenom radu (Bačinić *et al.* 2020) voltametrijski je istraženo nastajanje kompleksa kobalta s 4-NC, te je zabilježena redoks reakcija (dvoelektronski redukcijski val Co(II)) na potencijalu -1,08 V prema Ag/AgCl referentnoj elektrodi. UV/Vis spektrofotometrijom zabilježen je signal kompleksa pri valnoj duljini $\lambda = \sim 350$ nm. Kako bi se odredila stabilnost kompleksa korištena je adsorpcijska voltometrija katodnog otapanja, metoda s kompetitivnim ligandom (Zhang *et al.*, 1990). Za kompeticiju s 4-nitrokateholom (4-NC) korištena je nitrilotriocetna kiselina (NTA), te je izračunata uvjetna konstanta stabilnosti kompleksa pri pH morske vode ((pH = 8,2) i pri pH 6,5 koje iznose: $\log K_{\text{Co(II)-4NC}_2} = 21,86$ (pH = 8,2) i $\log K_{\text{Co(II)-4NC}_2} = 21,11$ (pH = 6,5). Spektrofotometrijski je određena stehiometrija kompleksa Co(II) s 4-NC; 1:1 i 1:2, te izračunate ravnotežne konstante stabilnosti kompleksa u otopini iznose: $\log K_{\text{Co(II)-4NC}_2} = 3,98$ (pH = 8,2) i $\log K_{\text{Co(II)-4NC}_2} = 5,76$ (pH = 6,5). HA je složena, polidisperzna organska molekula prisutna u morskoj vodi (Boggs *et al.*, 1985). U radu Bačinić *et al.*, 2020 prikazano je nastajanje kompleksa kobalta s HA, te je zabilježena redoks reakcija na potencijalu -1,02 V prema Ag/AgCl. Metoda s kompetitivnim ligandom je korištena za određivanje uvjetne konstante stabilnosti kompleksa Co(II) s HA. Za kompetitivni ligand je korištena etilendiamintetraocetna kiselina (EDTA). Određena je uvjetna konstanta stabilnosti kompleksa stehiometrije 1:1 koja iznosi $\log K_{\text{Co(II)-HA}} = 11,32$ (pH = 8,2). Ravnotežna konstanta stabilnosti kompleksa kobalta s HA nastalog u otopini određena je spektrofotometrijski pri valnoj duljini $\lambda = \sim 300$ nm za kompleks stehiometrije 1:1, te iznosi $\log K_{\text{Co(II)-HA}} = 3,80$ (pH=8,2). Vrlo važna skupina liganada koja utječe na specijaciju kobalta u morskoj vodi su fosfolipidi, molekule koje fitoplankton izlučuje prilikom prilagodbe na okolišne uvjete uvjetovane klimatskim promjenama. Ključni fosfolipidi su fosfatidilglicerol (engl. *phosphatidylglycerol* – PG), fosfatidiletanolamin (engl. *phosphatidylethanolamine* - PE) i fosfatidilkolin (engl. *phosphatidycholine* - PC) (Vrana Špoljarić *et al.*, 2021). U druga dva znanstvena rada istražena je interakcija fosfolipida s hidrofilnim kompleksom kobalta i 1,10-fenantrolina (engl. *phenanthroline* - Phen). U drugom radu (Bačinić *et al.* 2022a) prikazana je interakcija sa standardom PC, dok je u trećem radu (Bačinić *et al.* 2022b) istražena interakcija

kobalta s prirodno smjesom fosfolipida iz kulture fitoplanktona *Dunaliella tertiolecta*, uz usporedbu s onim standardnim. PC i PG interagiraju s kobaltom uz uvjet prethodnog stvaranja kompleksa s Phen. Kompleks Co(II)-Phen reducira se na potencijalu -1,0 V u uvjetima morske vode. Reagira s molekulama fosfolipida zamjenjujući preostale molekule vode iz koordinacijske sfere kobalta. Na taj način dolazi do stvaranja stabilnih miješanih kompleksa između Co(II), 1,10-Phen i fosfolipida. Redukcija kompleksa Co(II)-Phen-PC zabilježena je na potencijalu ~ -1,5 V, a miješanog kompleksa s PG-om na -1,4 V. Kompleks s prirodnim uzorkom smjese fosfolipida ekstrahiranim iz uzorka zelene alge *Dunaliella tertiolecte* reducira se na potencijalu od približno -1,6 V, što sugerira stvaranje kompleksa Co(II) s Phen i PC. Pomak signala redukcije prema negativnijim potencijalima uvjetovana je činjenicom da je prirodni uzorak smjese fosfolipida koji se adsorbiraju na površinu elektrode pa je potreban veći napon za redukciju istraživanog kompleksa. Stabilnost miješanog kompleksa na površini živine kapi određena je metodom kompeticije. Određene su uvjetne konstante stabilnosti za miješane komplekse stehiometrije 1:2:1 i 1:2:2 koje iznose $\log K_{\text{Co(II)Phen2PC}} = 23,02$ i $\log K_{\text{Co(II)Phen2PC2}} = 29,31$. Interakcija između Co(II) i PC je istraživana i Langmuir-Blodgettovom (LB) metodom. Praćena je ugradnja kompleksa Co(II) i Phen u fosfolipidni monosloj. LB metodom je zabilježeno direktno vezanje kobalta s PC te je izračunata konstanta stabilnosti nastalog sloja $K_I = 2,3 \times 10^{-2} \text{ m}^3 \text{ mol}^{-1}$, koja ukazuje na formiranje vrlo slabog kompleksa. Izračunata konstanta stabilnosti monosloja kad su prisutne sve tri komponente, Co, Phen i PC, iznosi $4,86 \times 10^{10} \text{ m}^2 \text{ mol}^{-1}$, što pak ukazuje na snažnu interakciju kobalta s fosfolipidnim monoslojem kad su oba liganda u otopini, formirajući miješani kompleks. U trećem znanstvenom radu (Bačinić *et al.* 2022b) istražena je interakcija kobalta s fosfolipidima korištenjem mikroskopije atomskih sila (engl. *Atomic Force Microscopy* - AFM). Rezultati dobiveni za prirodne fosfolipide ekstrahirane iz lipidne smjese zelene alge *Dunaliella tertiolecta* uspoređeni su s rezultatima za fosfolipidne standarde. Razlika u AFM slikama između standarda i smjese ekstrahiranih fosfolipida je očekivana zbog kompleksnosti uzorka. Dodatkom Phen dolazi do stvaranja vezikula kako kod kompleksa sa standardom fosfolipida tako i kod smjese ekstrahiranih fosfolipida. Konačno, dodatkom kobalta u oba slučaja dolazi do stvaranja “terasa”, zbog pretpostavke povezivanja molekula, što pak ukazuje na snažan afinitet PC prema kobaltu.

Na temelju provedenih istraživanja možemo zaključiti da dobiveni rezultati daju novi uvid u biogeokemijske procese kobalta u moru.

THESIS SUMMARY

Cobalt is a bioessential trace element involved in biological processes (Swanner *et al.*, 2014). It is necessary for the growth of phytoplankton, especially coccolithophores and cyanobacteria (*Prochlorococcus* and *Synechococcus*), and other higher organisms in the ocean (Bown *et al.*, 2012; Morel and Milligan, 2003). While cobalt is essential for prokaryotes, some eukaryotes such as coccolithophores can substitute it with zinc (Sunda and Huntsman, 1995). Cobalt is an essential nutrient as present in the structure of cobalamin molecule (vitamin B₁₂), which is necessary for metabolism of organisms (Osman *et al.*, 2021). In cink deficiency, cobalt can replace it in the structure of enzymes such is carbonic anhydrase (Moore *et al.*, 2018). Cobalt enters into the ocean from a variety of natural and anthropogenic sources, therefore, cobalt speciation in the ocean is a dynamic process (Noble *et al.*, 2017). Cobalt is a redox-active metal that occurs in oxidation states Co(II) and Co(III) depending on conditions, in free form or bound to organic or inorganic ligands, or adsorbed on particulate organic matter (Saito *et al.*, 2004). Cobalt ions dissolved in seawater are present in very low concentrations, between 10⁻¹⁰ and 10⁻¹² mol dm⁻³. Concentrations vary seasonally and increase with depth in the ocean, indicating biological activity of cobalt in surface layers (Ellwood and Van den Berg, 2001). However, to understand the biogeochemical processes of cobalt, we need to understand its speciation. The mechanism of cobalt consumption by aquatic organisms and the process of its removal by precipitation and interaction with suspended substances (organic or inorganic) are part of its biogeochemical cycle (Noble *et al.*, 2017). Cobalt forms complexes with inorganic and organic ligands at acidic and neutral pH, while in the alkaline (pH > 8,5) it occurs predominantly in the form of hydroxide (Ćosović *et al.*, 1982a). These complexes are extremely stable with conditional stability constants $\log K > 16$ measured under certain experimental conditions (ionic strength, pH) (Noble *et al.*, 2017). Cobalt shows special affinity for nitrogen groups in organic ligands (Chen *et al.*, 2014; Ferreira *et al.*, 2018; Noble *et al.*, 2017).

This thesis is based on three published scientific papers, and the aim is to study the formation of cobalt complexes with organic ligands naturally occurring in the marine environment. In accordance with previous experience and the reviewed literature, we selected model and natural ligands that are of biological importance for biochemical processes in seawater. Ligands used are catechols (catechol, 4-nitrocatechol (4-NC)), humic acid (HA) and membrane phospholipids. In addition to voltammetric analysis of Co(II) complexes, other methods were used. Complexes of cobalt with 4-NC and HA were analyzed by voltammetry

and spectrophotometry in the first published paper (Bačinić *et al.*, 2020), while the hydrophobic complexes with phospholipids published in the second and third paper (Bačinić *et al.* 2022a, 2022b), were analyzed by cathodic stripping voltammetry, Langmuir-Blodgett method, which was specially developed for the analysis of amphiphilic molecules and by atomic force microscopy. A methods for analyzing these complexes have been developed and certain parameters have been optimized, and conditional stability constants were calculated.

The proposed hypothesis is that Co(II) as an important micronutrient in the ocean that forms dissolved hydrophilic as well as hydrophobic complexes with naturally present organic ligands affect its bioavailability, and it is important for the functioning of marine microorganisms.

In first published paper (Bačinić *et al.* 2020) formation of the cobalt complex with 4-NC was investigated voltammetrically, and redox reaction (two-electron reduction signal of Co(II)) was recorded at the potential of -1.08 V vs. Ag/AgCl reference electrode. By UV/Vis spectrophotometry the wave of the complex was recorded at the wavelength $\lambda = \sim 350$ nm. To determinate stability of the complexes adsorptive cathodic stripping voltammetry with competitive ligand exchange method (CLE-AdCSV) was used (Zhang *et al.*, 1990). Nitrilotriacetic acid (NTA) was used as competition ligand and the conditional stability constant was calculated at pH of seawater (8.2) and 6.5. Calculated values of the conditional stability constants are $\log K_{\text{Co(II)-4NC}_2} = 21.86$ (pH = 8.2) and $\log K_{\text{Co(II)-4NC}} = 21.11$ (pH = 6.5). The stoichiometry of the Co(II) complex with 4-NC 1:1 and 1:2 in the solution was determined spectrophotometrically and the calculated equilibrium stability constants of the complex amount to: $\log K_{\text{Co(II)-4NC}_2} = 3.98$ (pH = 8.2) i $\log K_{\text{Co(II)-4NC}_2} = 5.76$ (pH = 6.5). HA is a complex and polydisperse organic compound widely present in seawater. In the manuscript Bačinić *et al.*, 2020. Is decribed formation of a Co(II) complex with HA. Reduction signal was recorded at -1.02 V according to Ag/AgCl. The method CLE-AdCSV with ethylenediaminetetraacetic acid (EDTA) as a competitive ligand was used to determine the conditional stability constanta and it is calculated for the complex with stoichiometry 1:1 $\log K_{\text{Co(II)-HA}} = 11.32$ (pH = 8.2). The equilibrium stability constant of the complex of stoichiometry 1:1 in solution with HA was also determined spectrophotometrically at $\lambda = \sim 300$ nm and it amount $\log K_{\text{Co(II)-HA}} = 3.80$ (pH = 8.2). A very important group of ligands for cobalt speciation in seawater are phospholipids, molecules that phytoplankton secrete due to adaptation to climate change (Vrana Šoljarić *et al.*, 2021). Main phospholipids are phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Vrana Šoljarić *et al.*, 2021). In the second and third scientific paper interaction interaction of cobalt with phospholipids including an intermediate complex with

1,10-phenanthroline (Phen), was investigated. In the second manuscript (Bačinić *et al.* 2022a), investigation of Co(II) with the phosphatidylcholine standard was presented, while in the third one (Bačinić *et al.* 2022b), with a natural phospholipids extracted from the culture of phytolankton *Dunaliella tertiolecta*. PC and PG as standard phospholipids interact with Co(II) only when in solution is formed an intermediate complex of Co(II) with 1,10-Phen. The reduction signal of the Co(II)-Phen complex was recorded at a potential of -1.0 V. The complex of cobalt and 1,10-phenanthroline reacts with phospholipid molecules replacing the remaining water molecules from cobalt coordination sphere. In this way, stable mixed ligand complexes between cobalt and phospholipids are formed. Reduction peak of the complex Co(II)-Phen-PC is registered at the potential ~ -1.5 V, while with PG at a potential -1.4 V. Complex with natural phospholipids was recorded at a potential -1.6 V, which assumes the formation of a Co(II) complex with PC. The shift of the reduction signal to more negative potentials is caused by the fact that the natural sample is a mixture of several phospholipids adsorbed on the surface electrode, so a higher voltage is required for the reduction of the researched cobalt mixed ligand complex.

The stability of the mixed ligand complex at mercury drop surface was determined by the CLE-AdCSV method. Calculated conditional stability constants of 1:2:1 and 1:2:2 complexes are $\log K_{\text{Co(II)Phen}_2\text{PC}} = 23.02$ i $\log K_{\text{Co(II)Phen}_2\text{PC}_2} = 29.31$. The interaction between cobalt and phosphatidylcholine was also studied using the Langmuir-Blodgett method (LB). The incorporation of the Co(II)-Phen complex into the phospholipid monolayer was monitored. The stability constant of the resulting layer is $K_l = 2.3 \times 10^{-2} \text{ m}^3\text{mol}^{-1}$ - indicating the formation of a weak complex. Calculated stability constant of the monolayer when all three components, Co(II), Phen and phosphatidylcholine, are present is $4.86 \times 10^{10} \text{ m}^3\text{mol}^{-1}$, indicating a strong interaction when both ligands are involved in the interaction with cobalt forming a mixed ligand complex. In the third published paper (Bačinić *et al.* 2022b) the interaction of phospholipids with cobalt was additionally detected using atomic force microscopy-AFM. The results obtained with phospholipid standards were compared with the results obtained with natural phospholipids extracted from the lipid mixture of green algae. The difference in the images captured between standards and real phospholipids is to be expected due to the complexity of the natural sample. The addition of Phen leads to the formation of vesicles both in the model and in the natural sample, and the addition of cobalt to the mixture leads to the formation of "terraces" due to the presumed interactions of the molecules. There was a high degree of similarity in the results between phosphatidylcholine and natural phospholipids.

The proposed research provides insight into the processes controlling the biogeochemicals of cobalt in the oceans.

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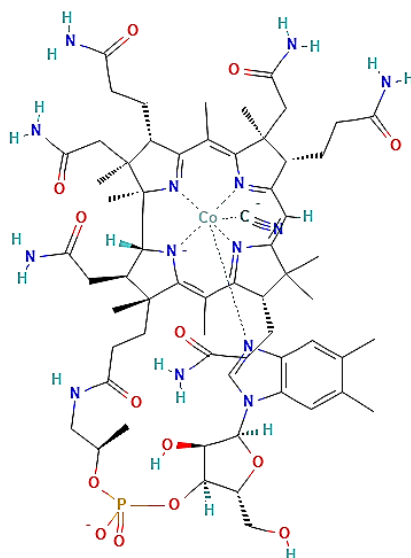
II: Bačinić, A., Frka, S., Mlakar, M., 2022. A study of cobalt (II) complexes involved in marine biogeochemical processes: Co(II)-1,10-Phenanthroline and Co(II)-1,10-Phenanthroline-L- α -Phosphatidylcholine. *Bioelectrochemistry* 144, 108009. <https://doi.org/10.1016/j.bioelechem.2021.108009>

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1. UVOD

1.1. Kobalt u moru

Kobalt je bioesencijalan element u tragovima i limitirajući nutrijent u pojedinim dijelovima oceana (Swanner *et al.*, 2014). Bioesencijalan je, odnosno nužan za razvoj morskih organizama, s naglaskom na plankton i bakterije (*Prochlorococcus* i *Synechococcus*) (Bown *et al.*, 2012a; Morel i Milligan, 2003; Saito i Moffett, 2002). Dok je za prokariote, posebno cijanobakterije kobalt nužan, kokolitofore mogu kobalt zamijeniti s cinkom i obrnuto (Sunda i Huntsman, 1995). Pojam „limitirajući nutrijent“ odnosi se na činjenicu da je koncentracija otopljenog kobalta niža od 1×10^{-12} mol dm⁻³ (u prosjeku od 10^{-10} do 10^{-12} mol dm⁻³) i da je nužan za biosintezu kobalamina (vitamina B₁₂) (slika 1.) neophodnog za metabolizam morskih organizama (Bown *et al.*, 2012b; Bundy *et al.*, 2020; Ellwood *et al.*, 2005).



Slika 1. Struktura vitamina B₁₂ (preuzeto s <https://pubchem.ncbi.nlm.nih.gov/compound/70678590>; 20.9.2022, 17:50)

Vitamin B₁₂ je organometalna molekula koja u svojoj strukturi sadrži kobalt (slika 1.). U morskom okolišu sudjeluje u mnogim enzimskim reakcijama algi i bakterija (Panzeca *et al.*, 2008). Eukarioti koriste kobalamin za sintezu metionina, dok ga bakterije i arheje koriste u enzimima za anaerobni metabolizam, uključujući fermentaciju, dehalogenaciju i prijenos elektrona s jednim ugljikom (Swanner *et al.*, 2014). Kobalt veže i nitrilna hidrataza koja sudjeluje u metabolizmu amida (Huertas *et al.*, 2014). U uvjetima niskih koncentracija cinka, tzv. limitirajućim uvjetima, kobalt ga može zamijeniti u strukturi enzima ugljične anhidraze

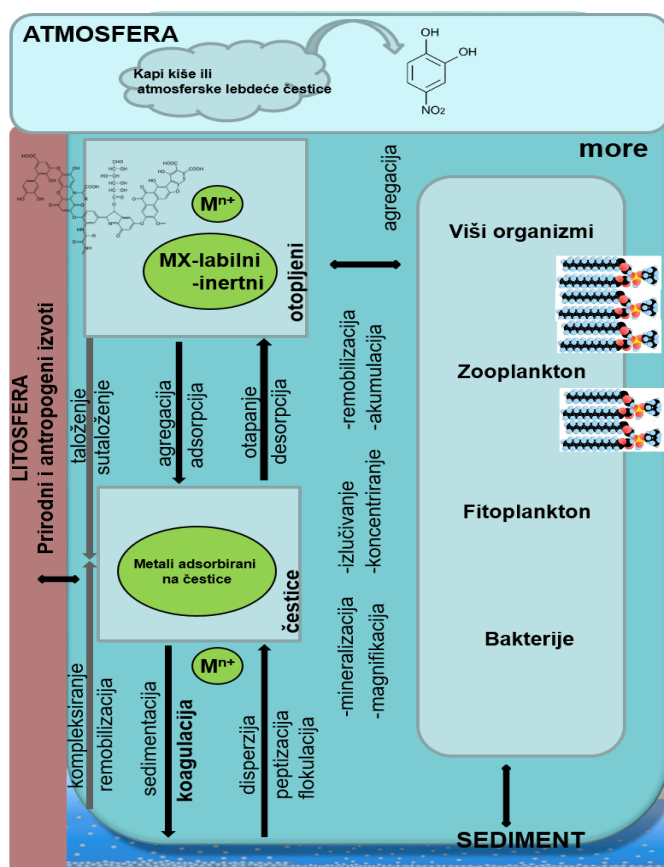
(Morel i Milligan, 2003) koji fiksira ugljikov dioksid (CO₂) iz zraka u more, uravnotežuje proces prijelaza bikarbonata u karbonate, te sudjeluje u kruženju ugljika (Swanner *et al.*, 2014).

Važnost kobalta za fiziologiju i ekologiju cijanobakterija je da izlučuju snažne, specifične kobaltne kelatore tzv. kobaltofore, čija je uloga slična ulozi siderofora za vezanje željeza (Morel i Milligan, 2003). Kobalt je biolimitirajući faktor u moru s obzirom da njegov nedostatak ograničava/smanjuje biološku produkciju (Bundy *et al.*, 2020). Promjena koncentracije kobalta u stupcu morske vode je sezonska, a u oceanima raste s dubinom što ukazuje na njegovu važnost u biološkoj aktivnosti u površinskim fotičkim slojevima u kojima se odvija većina biokemijskih procesa. Naime, njegova koncentracija je u površinskom sloju niska zbog fitoplanktonske aktivnosti, svoj maksimum postiže u plićim slojevima termokline, a u dubljim slojevima vodenog stupca ima uglavnom jednoliku raspodjelu (Ellwood i Van den Berg, 2001). Kobalt u vodenom stupcu ima dvojako (hibridno) obilježje, ponaša se kao nutrijent („*nutrient type*“) i/ili kao element koji se uklanja iz vodenog stupca („*scavenging type*“) (Saito i Moffett, 2002). Vrijeme zadržavanja kobalta u oceanima je oko 780 godina što ukazuje na njegovu slabiju biokemijsku reaktivnost u usporedbi s Fe i Mn (30 i 53 godine), ali veću od Cu, Zn ili Cd (Yeats *et al.*, 1978).

Kobalt u more dolazi iz različitih izvora, prirodnih (vulkani, rijeke, stijene, sediment, obala, hidrotermalni izvori, remineralizacija, atmosfera, resuspenzija iz sedimenta) (Noble *et al.*, 2017), i antropogenih (rudarstvo, procesi prerade/proizvodnja legura i kemikalija, otpadne vode i vode s poljoprivrednih područja (Collins i Kinsela, 2010; Noble *et al.*, 2017). Izvori i različite kemijske vrste kobalta posebno su zanimljivi u priobalnim područjima, kao i u plitkim morima zbog intenziteta i brzih promjena fizikalno-kemijskih parametara (Bundy *et al.*, 2020).

1.1.1. Raspodjela kemijskih vrsta kobalta u stupcu morske vode

(Noble *et al.*, 2017). Kobalt je redoks aktivan metal koji se u moru pojavljuje u oksidacijskim stanjima Co(II) i Co(III) ovisno o uvjetima, u slobodnom obliku - hidratiziran ili vezan za organske ili anorganske ligande (Saito *et al.*, 2004). Istraživanja vitamina B₁₂ koji sadrži kobalt unutar molekule, pokazala su mogućnost njegovog pojavljivanja u različitim oksidacijskim stanjima (Osman *et al.*, 2021; Saito i Moffett, 2002). Mehanizam apsorpcije kobalta od strane vodenih organizmima, kao i proces njegovog uklanjanja precipitacijom i međudjelovanjem s česticama (organskim ili anorganskim), važan su dio njegovog biogeokemijskog ciklusa (Geraldo *et al.*, 2013; Noble *et al.*, 2017). Njegovo biogeokemijsko kruženje u moru usporedivo je s ostalim dvovalentnim ionima metala (slika 2.).

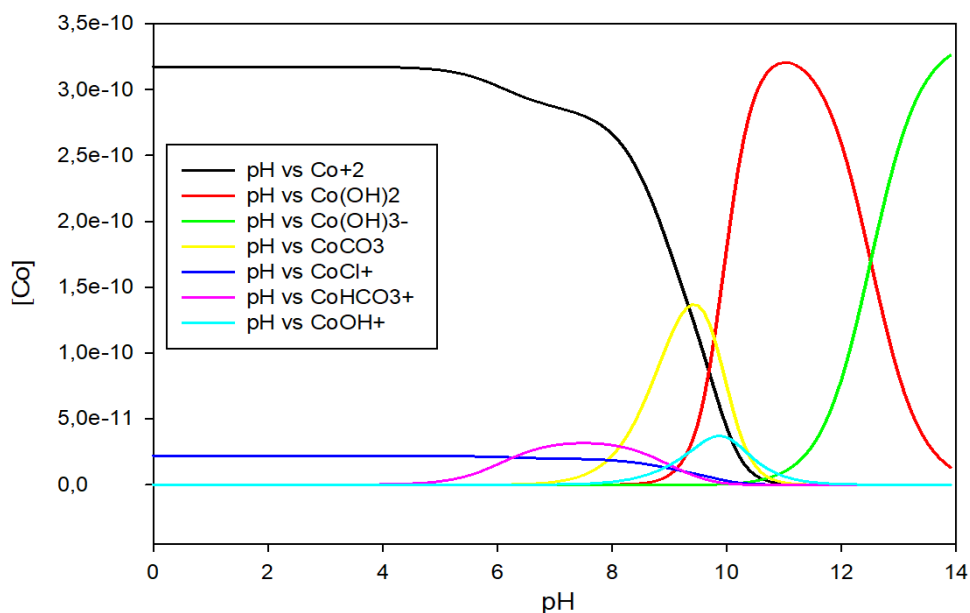


Slika 2. Shematski prikaz kruženja metala u moru (prerađeno prema Stumm i Morgan, 1996)

U stupcu morske vode kobalt je uglavnom u obliku slobodnog Co(II), dok u vrlo oksidnim djelovima vodenog stupca može oksidirati u Co(III), iako je ta reakcija kinetički spora neovisno o povoljnim termodinamičkim uvjetima (Saito *et al.*, 2004). Međutim, istraživanja pokazuju kako su kompleksi s Co(III) stabilniji od kompleksa s Co(II). Pokazano je da su Co(III) kompleksi kemijski inertni i ne sudjeluju u daljnim kemijskim reakcijama

(Ogino i Ogino, 1982; Saito i Moffett, 2001). Oksidacija Co(II) u Co(III) najčešće se odvija u obalnim područjima pod utjecajem mangan oksidirajućih bakterija (Bundy *et al.*, 2020). Mn-oksidirajuće bakterije utječu i na koprecipitaciju kobalta s manganom (Mn). Koprecipitacija s manganom te adsorpcijom Co(II) i Co(III) na površinu Fe(III) oksihidroksida i Mn(III/IV) (Bundy *et al.*, 2020) neki su od ključnih mehanizama ukaljanja otopljenog kobalta iz vodenog stupca. (Sunda i Huntsman, 1988; Swanner *et al.*, 2014).

Kobalt stvara komplekse s anorganskim ligandima (oko 22%) CoCl^+ i CoCO_3 pri kiselim i neutralnim pH vrijednostima te hidrokside CoOH^+ , Co(OH)_2 u lužnatom pH području (Ćosović *et al.*, 1982b) (slika 3.). Koeficijenti raspodjele (omjer vrste i ukupnog kobalta) vrste u vodi ovise o pH, redoks uvjetima, ionskoj jakosti i koncentraciji otopljene organske tvari.



Slika 3. Raspodjela kompleksa s anorganskim ligandima po pH
-raspodjela napravljena korištenje pHab (Hyperquad) programa (Gans *et al.* 1996, 2000) na temelju konstanti iz MINEQL programa (Schecher i McAvoy, 1992)

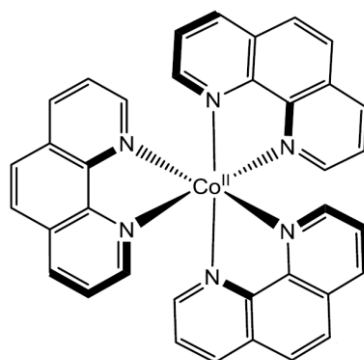
Reaktivnost anorganskih kompleksa kobalta ima važnu ulogu pri njegovom uklanjanju iz vodenog stupca dok kompleksiranje s organskim ligandima ima značajan utjecaj na biodostupnost kobalta organizmima (Ellwood i Van den Berg, 2001).

1.2. Organski ligandi uključeni u prirodne procese u moru

Kompleksiranje metala s organskim ligandima utječe na raspodjelu, mobilnost, biodostupnost, toksičnost, bioakumulaciju, biorazgradivost i geokemijsko kruženje metala u moru (Dulaquais *et al.*, 2014; Ellwood i Van den Berg, 2001).

Kao što je prethodno navedeno, metali u otopljenom obliku u stupcu morske vode prisutni su u obliku slobodnih hidratiziranih iona, ionskih parova, te anorganskih i organskih kompleksa (Abbasse *et al.*, 2003). Međutim, dok je poznato vezanje metala s anorganskim i nekim organskim ligandima manjih relativnih molekulskih masa ($< 10^3$) kao što su siderofori, karboksilne kiseline, aminokiseline, šećeri, itd., interakcije, stehiometrija i uvjetne konstante stabilnosti kompleksa metala s heterogenim prirodnim organskim ligandima kao što su humusne, fulvinske kiseline, itd. velike relativne molekulske mase (10^3 - 10^7) velik su izazov pri istraživanju procesa u morskom okolišu. Izvor organskih liganada u vodenim sustavima najčešće su mikroorganizmi i alge koje stvaraju polisaharide, polipeptide, lipide i ostale složene spojeve koje u uvjetima stresa izlučuju u morski okoliš. Bitni su i razni antropogeni izvori. Organski ligandi u moru mogu se podijeliti u dvije kategorije: ligandi čija su fizikalno-kemijska svojstva određena (aminokiseline, šećeri, lipidi, urea itd.) i ligandi koji ne mogu biti u potpunosti izolirani i analizirani zbog složenosti strukture (fulvinska i humusna kiselina, polisaharidi, proteini, siderofori itd.). Posebno zanimanje za istraživanje organskih kompleksa metala u tragovima pobudilo je saznanje da sudjeluju u biološkim procesima nalazeći se u reaktivnom centru enzima i na taj način stvaraju stabilne otopljene vrste (Keilin, 1997; Hirose, 2022). Stabilnost takvih kompleksa ovisi o aromatičnosti, elementnom sastavu, te funkcijskim skupinama, a opisuje se konstantama stabilnosti kompleksa. Studije kompleksiranja metala u moru često predlažu dvije glavne vrste organskih liganada (Aiken *et al.*, 2011): jaki ligandi ($\log K = 12$ - 14) prisutni u nižim koncentracijama (1 - 40 nmol dm^{-3}) i slabi ligandi ($\log K = 8$ - 10) prisutni u višim koncentracijama (6 - 150 nmol dm^{-3}). U skupinu slabijih liganada ubrajaju se humusna i fulvinska kiselina dok se za jake ligande uobičajeno predlaže biološki izvor, odnosno različite unutarstanične komponente, koje su ispuštene u vodeni medij nakon smrti organizma. Pored mehanizama unutarstaničnog izlučivanja poznato je da biota izlučuje i izvanstanične spojeve kao odgovor na izloženost metalnim ionima (Aiken *et al.*, 2011; Laglera i Van Den Berg, 2009).

Kateholi i fenantrolin su jednostavni organski ligandi koji snažno vežu divalentne ione. Kateholi se nalaze u atmosferi odakle otapanjem u moru ulaze u morski okoliš. Osim iz atmosfere kateholi u more nastaju raspadom kompleksnih aromatskih struktura. Često sudjeluju u stvaranju kompleksa s metalnim ionima ali i u izgradnji-polimerizaciji složenih aromatskih struktura molekula (biomolekula, humusnih tvari, itd.). Kako kobalt pokazuje afinitet vezanja za nitro skupine posebice nam je zanimljiv 4-nitrokatehol. Raspadom kompleksnih struktura može nastati i fenantrolin koji stvara stabilne koordinirane komplekse s metalima (slika 4.).



Slika 4. Kompleks kobalta s 1,10-fenantrolinom (Ferreira *et al.* 2018)

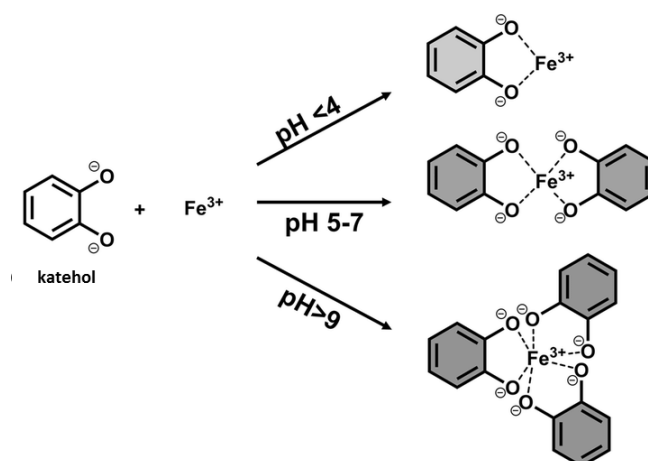
Analiza kompleksa s jednostavnijim ligandima može služiti za predviđanje vezanja na složenim molekulama.

Biomolekule su također važni ligandi prisutni u moru. Posebno su to lipidi točnije fosfolipidi koji izgrađuju staničnu membranu. Interakcija metalnih iona i organskih molekula s fosfolipidima omogućuje mobilnost kroz membranu u unutrašnjost stanice gdje metali sudjeluju u aktivnostima metaboličkih enzima. Iz navedenih razloga bitno je proučavati interakcije metalnih iona s organskim ligandima u morskom okolišu.

1.2.1. 4-nitrokatehol

Katehol (o-dihidroksibenzen) je žuto obojena, kromoforna organska tvar, nastala izgaranjem biomase (Santana-Casiano *et al.*, 2010; Zhang *et al.*, 2015). U moru nastaje kao metabolički međuprodukt u procesima mikrobiološke degradacije različitih kompleksnih aromatskih struktura. Interakcijom s morskom vodom (u obliku atmosferskih lebdećih čestica) otapa se i ulazi u vodeni stupac gdje stvara stabilne komplekse s metalnim ionima ili polimerizacijom stvara kompleksnije organske strukture – polimere procesima oksidativne polimerizacije (Frka *et al.*, 2016). Iznimno je reaktivna molekula koja u moru stvara stabilne komplekse s di- i tri- valentnim metalima (Santana-Casiano *et al.*, 2010). Izrazito je otporan na biodegradaciju i jedno je od veznih mjesta metala u molekuli humusne kiseline. Istraživanje koordinacije kateholnih skupina, omogućava proučavanje interakcija kompleksnijih struktura s metalnim ionima.

Bijlsma *et al.* su u radu iz 2020. prikazali stvaranje kompleksa željeza i hidroksilnih kateholnih skupina (slika 5.) (Bijlsma *et al.*, 2020). Kako pojedini metali pokazuju afinitet prema nitro skupinama u istraživanju je korišten i 4-nitrokatehol (engl. 4-nitrocatechol – NC). 4-NC je poznat kao međuprodukt u procesima degradacije molekula koje sadrže dušik, a vrlo često se koristi kao model za proučavanje fundamentalnih mehanizama odgovornih za mobilizaciju/imobilizaciju metala s organskom tvari (Cornard *et al.*, 2005; Saravanska *et al.*, 2021). Kompleksi kobalta s kateholima do danas nisu istraživani za razliku od kompleksa sa željezom o kojima su objavljeni mnogi radovi (Avdeef *et al.*, 1978; Oess *et al.*, 1999). Ovisno o pH, mogu nastati mono-, di-, ili tri- koordinirani kompleksi (Schweigert *et al.*, 2001).

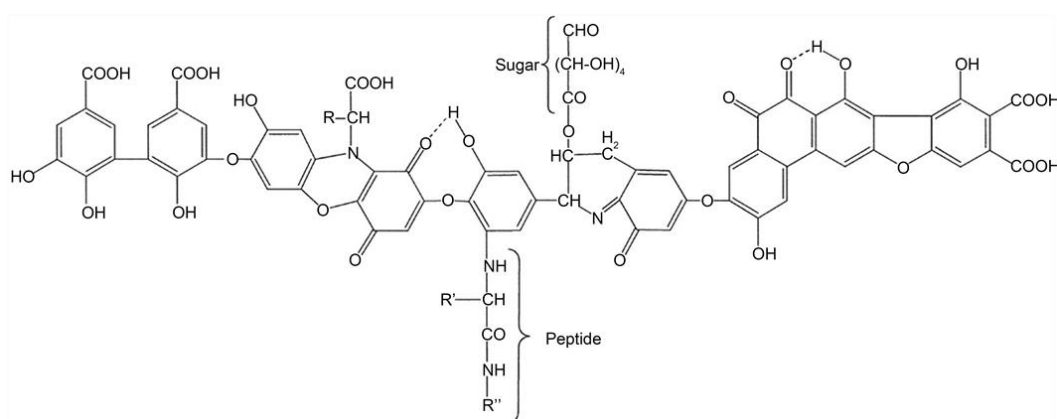


Slika 5. Koordinacija iona željeza pri različitim pH vrijednostima (Bijlsma *et al.*, 2020)

Konstanta stabilnosti kompleksa je veća ukoliko ligand sadrži elektrone akceptorske skupine npr. NO_2 . Saravanska *et al.* u radu iz 2021. prikazali su spektrofotometrijski stvaranje žuto obojane otopine miješanog kompleksa vanadija (V):4-nitrokatehola (4-NC):ksilometazolin klorovodika u omjeru 1:2:1 (Saravanska *et al.*, 2021). Dimitrov *et al.* u radu iz 2007. spektrofotometrijom pokazuje stvaranje kompleksa Mo(VI) s 4-NC i benzalkolijevim kloridom (Dimitrov *et al.*, 2007). Cornard *et al.* prikazuje UV/Vis spektre protoniranog i deprotoniranog 4-NC, te navodi da u fenolnim derivatima nitro skupina pojačava kiselost hidroksilne skupine pa su prosječne $\text{p}K_a$ vrijednosti hidroksilnih skupina katehola 9,2 i 13, što je određivano potencijometrijskom titracijom, dok su $\text{p}K_a$ vrijednosti hidroksilnih skupina 4-NC 6,7 i 10,8 (Cornard *et al.*, 2005).

1.2.2. Humusna kiselina

Humusne tvari (HT) su kompleksne komponente prirodne organske tvari prisutne u tlu, vodenom okolišu i sedimentu. Čine glavnu komponentu organske tvari u morskoj vodi (Gillam i Riley, 1982) a njihovu točnu koncentraciju u prirodnim vodama teško je odrediti zbog raznolikosti sastava i strukture (Laglera *et al.*, 2007). HT nastaju razgradnjom organske tvari, te ispuštanjem izvanstaničnih metabolita fitoplanktona (Magi, 1997; Raspor *et al.* 1984). HT čine značajnu frakciju koloidne frakcije kromoforne otopljene organske tvari (engl. *colored dissolved organic matter* - CDOM) koja utječe na obojanost morske vode. Boja humusne kiseline potječe od mnoštva konjugiranih dvostrukih veza u kompleksnim aromatskim strukturama (Reddy, 2018). Sastoje se od tri osnovne komponente: fulvinske kiseline (FK), humusne kiseline (HK) i humina koje se dijele na temelju topljivosti u vodi. FK je topljiva u kiselom i lužnatom mediju, humusna samo u lužnatom, dok je humin netopljiv. HK je makromolekula građena od aromatskih prstenova i alifatskih lanaca na koje su vezane brojne funkcijske skupine (slika 6.). O funkcijskim skupinama (karboksilne, fenolne, hidroksilne, karbonilne i amino) ovisi reaktivnost HK s metalima ili drugim organskim spojevima, a njihova količina ovisi o starosti, podrijetlu, klimi i biološkim uvjetima okoliša (De Melo *et al.*, 2016). Molekulska masa HK varira od 100 do 1000 Da (Leenheer, 2003). HK imaju važnu ulogu u topljivosti, mobilnosti, prijenosu, vezanju metala u tragovima u morskom okolišu, odnosno biodostupnosti (Hirata, 1981). Metali se većinom vežu na karboksilne i fenolne skupine koje lako reagiraju s kationima i stvaraju stabilne komplekse (Jackson *et al.*, 1978).



Slika 6. Model strukture humusne kiseline-preuzeto s https://en.wikipedia.org/wiki/Humic_substance (pristup 16.9.2022, 11:10)

Zasupljenost HK u prirodnim vodama od velikog je značaja za procjenu stanja okoliša.

Rashid ukazuje 1971. godine na snažne interakcije između dvovalentnog kobalta i HK (Rashid, 1971). Mantoura je u radu iz 1978. istražio je kompleksiranje Ca, Mg, Mn, Co, Ni, Cu, Zn, Cd i Hg s HK u prirodnim vodama pri pH 8,0 korištenjem gel filtracijske kromatografije. Pokazano je kako je u morskoj vodi više od 99% HT kompleksirano s kalcijem i magnezijem. Stabilnost kompleksa slijedi niz: $Mg < Ca < Cd \approx Mn < Co < Zn \approx Ni < Cu < Hg$ (Mantoura *et al.*, 1978). Pandey u radu iz 2000. koristi metodu ionske izmjene za određivanje konstanti stabilnosti kompleksa Co(II) i HK. Mjerenja su provedena pri pH = 3,5 kad je HK u potpunosti u protoniranom obliku. Koncentracije HK varirane od 0 do $15 \times 10^{-5} \text{ mol dm}^{-3}$ nisu utjecale na vrijednost konstante stabilnosti koja je određena za kompleks stehiometrije 1:2 i iznosi $\log K = 2,81$ (Pandey *et al.*, 2000). Yang i Van den Berg u članku iz 2009. odredili su konstantu stabilnosti kompleksa HA s Cu, Zn, Al, Co i Fe pri pH vrijednosti morske vode. Konstanta je određena metodom voltometrije katodnog otapanja (engl. *cathodic stripping voltammetry-CSV*). Metoda je bazirana na kompeticiji metala s Fe, za koji je prethodno određena konstanta stabilnosti s humusnom kiselinom na temelju kompeticije s EDTA. Određena konstanta stabilnosti s kobaltom je iznosila $\log K = 8,73$. Stabilnosti kompleksa s drugim metalima su se smanjivale redom: $Cu > Zn > Co > Fe > Al$ (Yang i Van Den Berg, 2009). Laglera *et al.* u članku iz 2009. prikazuje novu metodu temeljenu na direktnom određivanju Fe-HK kompleksa voltametrijom katodnog otapanja. Određen je kapacitet kompleksiranja i iznosi $32 \pm 2,2 \text{ nmol Fe}$ za 1 mg humusne kiseline. Određena je i konstanta stabilnosti kompleksa koja iznosi $\log K'_{Fe-HA} = 11,1$ (Laglera i Van Den Berg, 2009). Whitby u članku iz 2014. godine istražio je kompleksiranje Cu(II) s HK korištenjem CSV metode pri pH 8,2 u estuariju. Određene su konstante stabilnosti kompleksa $\log K'_{Cu-HA} = 12,08$ i kapacitet vezanja od 18 nmol Cu za 1 mg humusne kiseline (Whitby i Van den Berg, 2015).

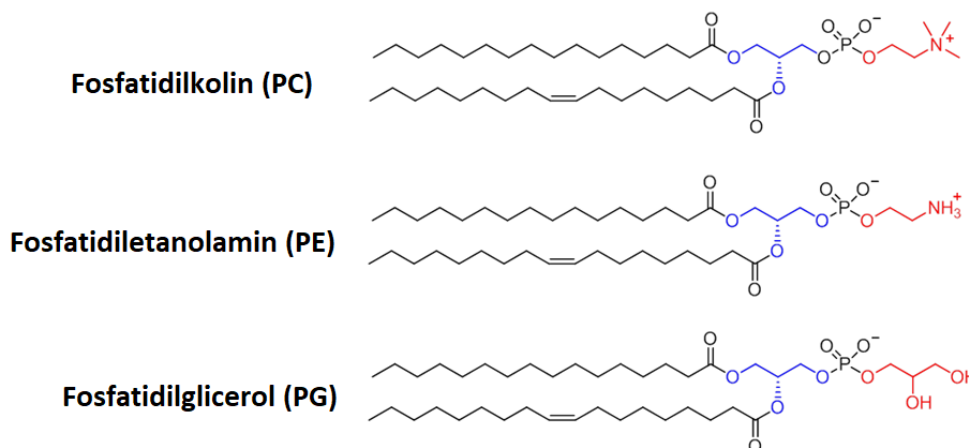
1.2.3. Fosfolipidi

Lipidi su spojevi topljivi u organskim otapalima, a slabo topljivi u vodi za razliku od ostalih klasa prirodnih biomolekula kao što su proteini, aminokiseline, ugljikohidrati te monosaharidi, od kojih su i biološki otporniji. Bogati su ugljikom i služe kao molekule za pohranu energije, te kao takvi čine bitnu komponentu staničnog metabolizma organizama, posebno u oceanima (Wakeham *et al.*, 1997; Triesch *et al.*, 2021). Raspodijeljeni su u morskom okolišu u otopljenom i čestičnom obliku i sudjeluju u brojnim biološkim procesima (Arts *et al.*, 2001; Frka *et al.*, 2011). Čine oko 4-30,5 % otopljene organske tvari u različitim područjima oceana i taj se sadržaj ne mijenja bitno s udaljenošću od obale ili dubinom vodenog stupca (Derieux *et al.*, 1998; Penezić *et al.*, 2010), te 10-25 % čestične organske tvari u površinskim vodama (Duursma i Dawson, 2015). Alge su poznati izvori lipida, bogate nutritivne vrijednosti i značajnih bioaktivnih svojstva u moru (Rosário Domingues i Calado, 2022; Vrana *et al.*, 2022).

Globalne klimatske promjene imaju značajan utjecaj na sastav lipida, a samim time i na integritet i fiziologiju stanice (Vrana Špoljarić *et al.*, 2021). Stanične membrane određuju izdržljivost stanice, štite unutrašnjost od vanjskih faktora i definiraju izgled stanice (Petelska i Naumowicz, 2017a). Okolišne promjene utječu na potrebu fitoplanktona da se prilagodi i mijenja sastav lipida pod utjecajem planktonske zajednice i okolišnih uvjeta (pH, ionska jakost). Interakcija između membranskih lipida i iona ima bitnu ulogu u mnogim biološkim procesima kao što je membranska fuzija, regulacija enzima te prijenos signala. Primarna funkcija membranskih lipida je permeabilnost stanice za male polarne i nabijene molekule, ione, organske molekule i makromolekule (Chao *et al.*, 1994; Dowhan i Bogdanov, 2002; Micelli *et al.*, 2002; Niles *et al.*, 1996; Ravoo *et al.*, 1999; Sovago *et al.*, 2007). Pojam „membranski lipidi“ odnosi se na fosfolipide. Fosfolipidi su amfipatski lipidi izgrađeni od alkohola glicerola ili sfingozina na koji se vežu masne kisline i fosfatna skupina (Pine, 1994).

Fosfolipidi sudjeluju u procesu permeabilnosti iona i molekula kroz staničnu membranu i organele, u raznim katalitičkim procesima, sintezi makromolekula kao građevni elementi i u procesima/reakcijama na membrani (Bogdanov i Dowhan, 1998; Dowhan, 1997). Fosfolipide (slika 7.) fosfatidilkolin (PC) i fosfatidiletanolamin (PE), nalazimo u ekstracelularnim membranama stanice dok je fosfatidilglicerol (PG) smješten u tilakoidnim membranama gdje sudjeluje u fotosintetskom transportu elektrona, stvaranju kloroplasta i toleranciji na niže

temperature (Wada i Murata, 2007). PC i PE su najzastupljeniji membranski lipidi i čine ~ 68–80% ukupnih fosfolipida (Van Meer *et al.*, 2008).



Slika 7. Kemijske strukture fosfolipida (Geraldo *et al.*, 2013)

PC i PE su zwitterion fosfolipidi te slabije i rjeđe vežu ione metale, dok je PG anionski fosfolipid (Sule *et al.*, 2020). Divalentni ioni metala vežu se na negativno nabijenu fosfatnu skupinu zwitteriona. Vezanje iona i lipida izaziva konformacijske modifikacije unutar skupine i mijenja hidrataciju lipida (Kewalramani *et al.*, 2010).

1996. godine Fullington i Hendrickson su pokazali mogućnost kompleksiranja fosfolipida i metala. Potvrdili su svoj model kompleksiranja fosfatidilserina i trifosfoinozitida s Ca(II), Mg(II) i Ni(II) u prirodnom lipoproteinskom sustavu. Također, miješani kompleksi su određeni u modelnim sustavima koji sadrže fosfolipide, metalne ione i koligande kao što su polilizin, poli-L-asparaginska kiselina i etilendiamin. Analize su pokazale formiranje stabilnih kompleksa fosfolipid-metal-koligand (Fullington i Hendrickson, 1966; Mlakar *et al.*, 2018). Postoje mnoga istraživanja o interakcijama metala s monoslojem fosfolipida koja su važna za proučavanje mehanizama biofizičkih i biokemijskih pojava u živim stanicama (Petelska i Naumowicz, 2017a). Takva istraživanja nam omogućuju razumijevanje procesa koji se odvijaju kako u samom monosloju tako i na njegovoj površini (Petelska i Naumowicz, 2017b). Sule, 2020. je istraživao afinitet vezanja divalentnih Co(II), Ni(II) i Mn(II) na membranske fosfolipide. Rezultati ukazuju na mjesta s visokim afinitetom vezanja navedenih metala. Afiniteti vezanja su određeni korištenjem ³¹P NMR-a, elektoroforeze i mjerenjem zeta potencijala. Istraživanja su provedena s tri fosfolipida PC, PG i PS, te je pokazan snažan afinitet vezanja metala s PS, a najslabije s PC vrlo vjerojatno zbog afiniteta vezanja na negativno

nabijene fosfolipide. Praćen je i utjecaj metala na fluidnost i permeabilnost membrane te je pokazano kako Co(II) i Ni(II) značajno utječu na rigidnost posebice na gušće pakiranje molekula (Sule *et al.*, 2020).

1.3. Analitičke tehnike i metode za određivanje kompleksa kobalta s organskim ligandima

1.3.1. Voltometrija

Voltametrijom se naziva skup elektroanalitičkih tehnika koje se koriste za kvalitativnu i kvantitativnu analizu uzorka kojima je električni napon signal pobude, a električna struja signal odziva članka.

Metoda se provodi na statičnim mikroelektodama stalne površine (živina kap, živin film, zlatna žica itd.) u otopini koja miruje. Iznimno je osjetljiva pa se može primjenjivati za analize vrlo niskih koncentracija analita (do 10^{-12} mol dm⁻³), kao što su metali u morskom okolišu. Oblik i vremenska ovisnost amplitude signala pobude mogu biti različiti pa tako postoji linearna promjena potencijala, impulsna, pravokutnovalna i izmjenična promjena potencijala (Piljac, 1995). U ovoj doktorskoj disertaciji za analizu kompleksa s kobaltom korištena je živina kap kao radna elektroda, dok je kao referentna korištena Ag/AgCl elektroda, prema kojoj su određeni potencijali redukcije. Za nastajanje i analizu kompleksa korištene su: pravokutnovalna voltometrija, ciklička voltometrija i voltometrija izmjenične struje.

1.3.1.1. Pravokutno-valna voltometrija

Kod pravokutno-valne voltetrije (PVV) pobuda se sastoji od naizmjeničnih katodnih i anodnih pulseva velike amplitude ($100/n$ mV) koji se pojavljuju nekom frekvencijom f . Budući da sliče pravokutnoj sinusoidi, metoda je dobila ime voltetrija s pravokutnim valovima potencijala (Piljac, 1995). Poslije svakog ciklusa dodaje se srednjem potencijalu određeno povećanje potencijala ΔE . Prividna brzina promjene srednjeg potencijala jednaka je umnošku ΔE i f . Trajanje svakog pulsa obrnuto je proporcionalno frekvenciji pobude: $\tau = (2f)^{-1}$. Struja se uzorkuje pri kraju svakog pulsa, a bilježi se razlika struja uzorkovana na dva uzastopna pulsa kao funkcija srednjeg potencijala tog ciklusa: $\Delta i = i_1 - i_2$. Odziv je zvonolika krivulja strujana-
napon određena maksimalnom strujom (Δi_{\max}) i njenim potencijalom (E_{\max}). Maksimalna struja zvona Δi_{\max} proporcionalna je koncentraciji reaktanta u masi otopine (Mirceski *et al.*, 2013).

Ako je redoks reakcija reverzibilna, onda se za vrijeme katodnih pulseva reaktant reducira, a za vrijeme anodnih pulseva nastali se produkt reoksidira. Tako su struje i_1 i i_2 obrnutog predznaka pa je njihova razlika Δi veća po apsolutnoj vrijednosti od svake od njih pojedinačno. Moguće je bilježiti i pojedinačne struje i_1 i i_2 i tako dobiti informacije slične onima koje se dobivaju cikličkom voltametrijom ili dvostrukom kronoamperometrijom. Ako je redoks reakcija totalno ireverzibilna, onda će tijekom katodnih pulseva dolaziti do redukcije reaktanata, ali tijekom anodnih pulseva neće dolaziti do reoksidacije produkta nego će se samo reaktant slabije reducirati. Tako su obje struje i_1 i i_2 istog predznaka pa je njihova razlika Δi manja od katodne struje i_1 (Piljac, 1995). PVV pobudi prethodi po volji dugotrajan period t_o tijekom kojeg je elektroda nabijena na konstantni potencijal $E_{poč}$. Ovaj se period može iskoristiti za akumulaciju tragova analita na površinu elektrode. Akumulirana se količina zatim oksidacijom ili redukcijom ukloni s elektrode pomoću PVV pobude i mjeri se strujni odziv. Pravokutno valna voltometrija je metoda koja daje mogućnost određivanja mnogih karakteristika redoks procesa kao što su kinetika procesa, konstante brzine redoks reakcije, reverzibilnost, koeficijent prijenosa, itd. (Mirčeski *et al.*, 2013).

Metoda PVV pokazala se jednostavnom i prikladnom za istraživanje kompleksa metala s organskim ligandima u moru. Razvijene su metode s mnogim metalima, a najviše istraživani su željezo, bakar, cink, kadmij, kobalt i nikal. Najveći broj istraživanja kompleksiranja željeza je sa sideroforima, organskim ligandima koje proizvode morske bakterije (Vukosav *et al.*, 2012a). PVV je korištena kako bi se istražilo kompleksiranje kobalta s vitaminom B₁₂ i koenzimom (Bruland *et al.*, 1991; Zhang *et al.*, 1990). Bakar pokazuje izraženi afinitet za spojeve s tiolnim skupinama zbog čega su istraživana kompleksiranja bakra s aminokiselinama koje u svojoj strukturi sadrže sumpor (Dupont *et al.*, 2004). Kadmij i cink također pokazuju afinitet prema ligandima manje molekulske mase s tiolnim skupinama (Dupont i Ahner, 2005).

1.3.1.2. Ciklička voltametrija

Kod cikličke voltametrije potencijal snimanja (pomak potencijala) na radnoj elektrodi mijenja smjer kod odabrane vrijednosti. Promjena potencijala (pomak) u oba smjera, anodom i katodnom, je linearna (Piljac, 1995). Cikličkom pobudnom signalu prethodi period t_0 (isti se može i izostaviti) tijekom kojeg je elektroda nabijena na odabrani početni potencijal, što se može iskoristiti za akumulaciju elektroaktivne vrste na površini elektrode ukoliko je adsorbabilna ili stvara amalgam, čime se povećava osjetljivost. Kod polazne promjene potencijala (prema negativnijim vrijednostima) struja odziva je katodna struja redukcije koja ima „klasičan“ oblik voltametrijskog vala. Na početku promjene smjera potencijala proces redukcije O u R i dalje traje jer je potencijal elektrode još uvijek znatno negativniji od E° . Daljnjim pomakom potencijala u pozitivnom smjeru potencijal elektrode se približava E° te se odvija proces oksidacije reduciranog oblika (R) uz površinu elektrode (nastalog procesom redukcije), a struja radne elektrodne je anodna. Kod određenog potencijala anodna struja postiže maksimalnu vrijednost, a zatim zbog iscrpljivanja reducirane vrste u otopini uz površinu elektrode, postupno opada. Signal odziva cikličke voltametrije tako ima karakterističan oblik vala s katodnim i anodnim vrhom (Osteryoung i Osteryoung, 1985; Piljac, 1995). Ciklička voltametrija je jedna od najčešće korištenih tehnika za prikupljanje kvalitativnih informacija o elektrokemijskim reakcijama jer pruža mogućnost brzog određivanja podatka koji govore o termodinamici redoks procesa, kinetici heterogenih procesa prijenosa elektrona ili adsorpcijskim procesima (Lovrić, 2007). U cikličkoj voltametriji određeni su prosudbeni kriteriji koji nam daju informaciju o mehanizmu reakcije. Tako je reverzibilni elektrodni proces karakteriziran sljedećim eksperimentalnim parametrima:

$E_{p,k}$ i $E_{p,a}$ – ne ovise o brzini promjene potencijala (v)

$E_{p,k} - E_{p,a}$ je 57/z mV, kod 25 ° C, ne ovisi o v

$i_{p,k} / i_{p,a}$ – je 1, ne ovisi o v

$i_p / v^{1/2}$ - konstantan, ne ovisi o v

Elektrokemijska reverzibilnost reakcije ovisi i o brzini promjene potencijala radne elektrode. Stoga reverzibilnost pokazuju oni elektrodni procesi čija je standardna konstanta brzine elektrodne reakcije $k^0 > 0,1 \text{ cm s}^{-1}$ (Piljac, 1995).

1.3.1.3. Voltometrija izmjenične struje

Kod voltetrije izmjenične struje (engl. *alternating current voltammetry* - ACV) signal pobude je potencijal koji se linearno mijenja, a na koji je superponiran sinusoidalni izmjenični potencijal male amplitude. Signal odziva je izmjenična komponenta ukupne struje, prikazana u ovisnosti o primjenjenom, linearno promjenjivom potencijalu (Piljac, 1995).

Kako faradejska i kapacitetna struja imaju različite fazne kuteve u odnosu na primjenjeni izmjenični sinusoidalni potencijal, fazno osjetljiva mjerenja, ako se izvode izvan faze, odnosno pod faznim kutem $\varphi = 90^\circ$, omogućuju mjerenje samo kapacitetne komponente ukupne struje. AC voltetrijom može se pratiti promjena kapaciteta dvosloja prilikom adsorpcije organskih molekula na hidrofobnu živinu površinu, a kapacitetna struja ovisi o vremenu nabijanja električnog dvosloja, odnosno o brzini promjene potencijala elektroda (Zoski, 2007). Priroda sila ovisi o prirodi interakcija između molekula koje se adsorbiraju i elektrode koje su najčešće elektrostatske prirode. Istraživanje adsorpcije površinski aktivnih molekula na živinu površinu naziva se tenzometrijom (Rashid, 2015).

Procese na elektrodi hidrofobnih aromatskih spojeva (polinuklearnih aromatskih ugljikovodika, polikloriranih bifenila, neurotoksičnih pesticida i fenotiazina) su pratili (Nelson *et al.*, 1990). Nelson i Benton u radu iz 1986. prikazuju adsorpciju fosfolipida na površini živine elektrode. Promjenom potencijala u negativnom smjeru dolazi do reorijentacije molekula fosfolipida. Nakon reorijentacije dolazi do stvaranja pora u samom sloju, pri čemu sloj postaje propustan za ione prisutne u otopini (Nelson i Benton, 1986). Fosfolipidi, npr. dioleil fosfatidilkolin (DOPC), stvaraju fluidne nepropusne monoslojeve na živinoj elektrodi. Iz tog razloga kako bi se istraživala propusnost membrane potrebno je modificirati monosloj aktivnim komponentama. Na takav način moguće je pratiti interakcije s proteinima, interakcije između membrane i antibiotika, funkcije ionskih kanala i interakcije membrane i peptida, s iznimnom osjetljivošću, točnošću i ponovljivošću (Zhang *et al.*, 2011). Sve membranske aktivne molekule mogu potiskivati kapacitivni val (zbog inhibicije prodiranja elektrolita), smanjiti kapacitet monosloja (zbog ugradnje u pore/praznine između skupina lipidnih glava koje povećavaju debljinu monosloja) i/ili pomaknuti kapacitetni signal prema negativnijim potencijalima. Mlakar *et al.* u radu iz 2018. prikazuju adsorpciju fosfatidilglicerola na površinu živine elektrode, te interakciju s fenantrolinom i bakrom stvarajući na taj način miješani kompleks na površini elektrode (Mlakar *et al.*, 2018). AC voltetrija je korištena za praćenje kinetike redoks reakcija na elektroaktivnim monoslojevima, interakcija monoslojeva na

površinu žive i adsorpciju monoslojeva na živu (Moncelli *et al.*, 1996). Ovom metodom moguće je proučavati funkcioniranje ionskih kanala, prijenos iona kroz fosfolipidni monosloj, te interakcije fosfolipida i hidrofobnih organskih molekula.

1.3.1.4. Adsorpcijska voltometrija katodnog otapanja

S obzirom na niske koncentracije metala u realnim uzorcima za njihovo određivanje često su potrebne metode s pretkoncentracijskim korakom. Elektrokemijske metode s pretkoncentriranjem su: voltometrija anodnog otapanja (engl. *anodic stripping voltammetry* - ASV), adsorpcijska voltometrija katodnog otapanja (engl. *adsorptive cathodic stripping voltammetry* - AdCSV), potenciometrijska stripping analiza (engl. *potentiometric stripping analysis* - PSA) (Fogg, 1999).

Metodom ASV mogu se analizirati samo metali topivi u živi, koji stvaraju amalgam (npr. Zn, Cd, Pb, Cu, itd.), dok se za određivanje koncentracija metala koji sa živom ne stvaraju amalgam (Co, Ni, Sb, Fe, itd.) koristi adsorpcijska voltometrija katodnog otapanja, AdCSV.

Metoda AdCSV je najčešće korištena za specijaciju metala netopivih u živi, određivanje koncentracije liganda vezanog za metal i određivanje uvjetnih konstanti stabilnosti kompleksa u morskoj vodi. Metoda je iznimno osjetljiva, a razlog tome je pretkoncentriranje analita od interesa. Korak pretkoncentriranja je prvi korak koji se odvija akumulacijom određivane tvari (adsorbensa) na elektrodi procesima *elektrolize* ili *adsorpcije*, dok se drugi korak, korak otapanja s elektrode provodi promjenom potencijala radne elektrode, provođenjem kontrolirane struje kroz radnu elektrodu ili pak heterogenom kemijskom reakcijom. Otapanje adsorbensa s površine elektrode naziva se engl. *stripping*, pa te metode nazivamo "*stripping*" metodama. Metal s organskom tvari stvara kompleks koji se adsorbira na visećoj živinoj kapi pri potencijalu pozitivnijem od standardnog potencijala tog kompleksa. Promjenom potencijala prema negativnijim vrijednostima dolazi do redukcije adsorbiranog kompleksa te se mjeri odziv struje. Metoda se odvija u dva koraka. Akumulacija kompleksa (jednadžba 1 i 2) odvija se uz miješanje otopine elektrolita i ovisi o koncentraciji određivanog iona u otopini. Nakon pretkoncentracijem odvija se proces otapanja promjenom potencijala radne elektrode prema negativnijim potencijalima. Adsorbirani kompleks na elektrodi se reducira (jednadžbe 3-5) i mjeri se struja tijekom promjene potencijala. Proces se može prikazati nizom kemijskih i elektrokemijskih reakcija (Zhang *et al.*, 1990; Ellwood *et al.*, 2005):

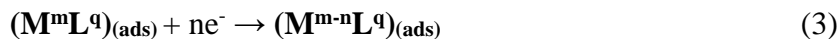
- vodena otopina:



- adsorpcija na površinu elektrode:



- elektrokemijska redukcija:



Gdje su: $\mathbf{M}^m_{(aq)}$ otopljeni kationi, $\mathbf{L}^q_{(aq)}$ slobodni ligandi - uključuje labilan ligand i kompleksiran s H, Ca, Mg, Na, $(\mathbf{M}^m\mathbf{L}^q)_{(aq)}$ otopljeni metalni kompleks u otopini, $(\mathbf{M}^m\mathbf{L}^q)_{(ads)}$ adsorbirani metalni kompleks na živinoj kapi, \mathbf{M}^{m-n} reducirani metalni ioni, \mathbf{L}^{q-n} reducirani ligand. Do redukcije adsorbiranog kompleksa može doći redukcijom metalnog iona (jednadžba 4) ili redukcijom liganda iz adsorbiranog kompleksa (jednadžba 5).

Zbog slabe topljivosti u živi i niske koncentracije u morskim uzorcima za određivanje i specijaciju kobalta u uzorcima morske vode AdCSV metoda je najčešće korištena (Batley *et al.* 2004). Osjetljivost metode ovisi o potencijalu akumulacije i vremenu akumulacije te brzini adsorpcije. Granica osjetljivosti ovisi i o veličini adsorpcijske površine tj. radne elektrode živina kap. Glavna prednost CSV metode je činjenica da nije ograničena samo na slobodne metale, već je moguće mjeriti različite vrste. Metoda je selektivna jer svaka kemijska vrsta daje odziv na različitim vrijednostima potencijala.

1.3.1.5. Određivanje uvjetnih konstanti stabilnosti metodom kompeticije

Važan parametar za karakterizaciju kompleksa metala s organskim ligandima je uvjetna konstanta stabilnosti kompleksa koja je određena pri određenim eksperimentalnim uvjetima i jedinstvena je za određeni kompleks, a ovisi o kemijskim svojstvima (vrsti donora atoma, broju doniranih atoma i dr.) organskih liganada i metalnog kompleksa. Konstante stabilnosti organskih kompleksa s mikronutrijentima mogu se odrediti tzv. metodom kompeticije s dva liganda gdje je jedan elektroinaktivan poznate konstante i jedan nepoznate/trazene koji kompleksira s metalom (Saito i Moffett, 2001) (slika 8.). Metoda kompetitivne izmjene liganda je dobra metoda za korištenje ukoliko postoji kompleks metala s nekim modelnim ligandom prisutnim u bazi podataka.

Uvjetna konstanta stabilnosti kompleksa može se prikazati sljedećom jednadžbom:

$$K'_{ML} = [ML] / [M^{n+}][L'] \quad (6)$$

Gdje su:

[L']- koncentracija slobodnog liganda

[Mⁿ⁺] – koncentracija slobodnog metala

[ML] – koncentracija nastalog kompleksa

A ukupnu koncentracija liganda čine slobodni i kompleksirani ligand $c_L = [L'] + [ML]$, dok ukupnu koncentraciju metala čine slobodni i kompleksirani metal $c_{M^{n+}} = [M^{n+}] + [ML]$.

Ukupna koncentracija metala npr. Co²⁺, uz EDTA kao kompeticijski ligand, može se prikazati sljedećim izrazom:

$$c_{Co} = [Co'] + [CoL_y] + [CoEDTA] \quad (7)$$

gdje je [Co'] označava zbroj koncentracija svih anorganskih vrsta kobalta (npr. Co(OH)⁻, Co(OH)₂), [CoL_y] predstavlja koncentraciju kompleksa kobalta s ligandom čiju konstantu stabilnosti određujemo (y je obično 1, 2 ili 3), a [CoEDTA] je zbroj koncentracija svih vrsta kompleksa Co-EDTA prisutnih u otopini kod određenog pH.

Vršna struja, i_p , povezana je s [CoL_y] faktorom proporcionalnosti, S:

$$i_p = S[CoL_y] \quad (8)$$

U otopini koja sadrži samo ligand koji stvara kompleks čiju konstantu određujemo, vršna strujala redukcije kompleksa se može opisati izrazima:

$$i_{p,0} = S[\text{CoL}_y] = S(c_{\text{Co}} - [\text{Co}']) \quad (9)$$

Pod uvjetom da se $[\text{Co}']$ ne mijenja, $i_{p,0}$ se može opisati kao:

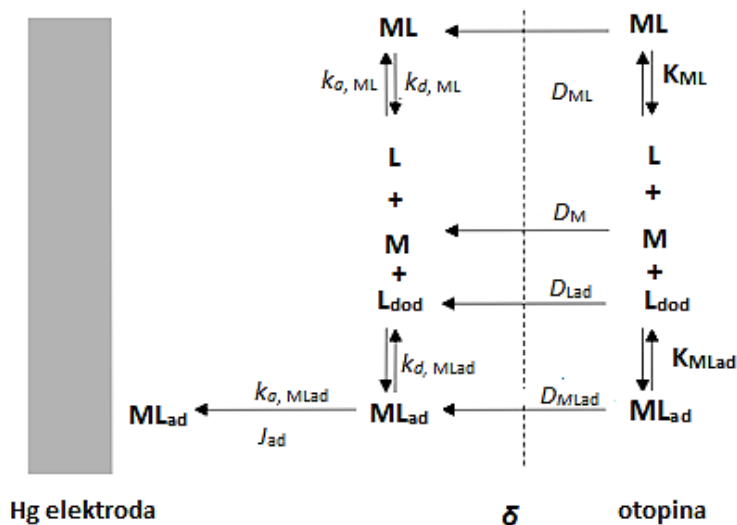
$$i_{p,0} = S(c_{\text{Co}} - [\text{Co}']) = S([\text{CoL}_y] + [\text{CoEDTA}]) \quad (10)$$

Omjer (X) vršnih struja vala redukcije kompleksa u prisustvu ($i_{p,i}$) kompeticijskog liganda i bez ($i_{p,0}$) kompeticijskog liganda, može se zapisati kao:

$$X = i_{p,i}/i_{p,0} = [\text{CoL}_y]/([\text{CoL}_y] + [\text{CoEDTA}]) \quad (11)$$

Metoda kompeticije se koristi od 1982. godine kada je prvi puta proveden eksperiment s EDTA (etilendiamintetraoctena kiselina) (Hirose, 2022).

Za određivanje konstanti stabilnosti kompleksa s kobaltom često je korištena metoda kompeticije (engl. *competing ligand - adsorptive cathodic stripping voltammetry* - CLE-AdCSV) shematski prikazana na slici 8. U otopini se nalazi metalni ion M i ligand L koji grade kompleks ML, te dodani ligand L_{dod} koji s metalom M stvara površinski aktivni kompleks ML_{ad} . Parametri koji određuju udio ML akumuliran tijekom adsorpcijskog koraka su: konstanta stabilnosti K kompleksa ML i ML_{ad} , koeficijent difuzije D za M, ML, L_{ad} i ML_{ad} , konstanta brzine asocijacije (k_a) i disocijacije (k_d) ML i ML_{ad} , adsorpcijski protok (J_{ad}) i debljina difuzijskog sloja δ (Jansen, 2004). Kao poznati modelni ligandi najčešće se koriste etilendiamintetraoctena kiselina (EDTA) i nitrilotrioctena kiselina (NTA). CLE-AdCSV metoda omogućuje određivanje kompleksa različitih oksidacijskih stanja metala i različite stehiometrije (Vega i Van den Berg, 1997; Zhang *et al.*, 1990).



Slika 8. Shematski prikaz vrsta i interakcija u blizini površine Hg elektrode u CLE-AdSV eksperimentu. Modificirano prema Jansen, 2004

Bown *et al.* je 2012 godine istraživao specijaciju kobalta s organskim ligandima metodom CLE-AdCSV korištenjem nioxima kao kompetitivnog liganda. Određene uvjetne konstante stabilnosti $\log K_{\text{CoL}}$ su iznosile između 17.9 – 20.1. Rad pokazuje i da je 60 - 99 % otopljenog kobalta kompleksirano s organskim ligandima dok su određene koncentracije slobodnog (ionskog) kobalta bile od 10^{-17} do 10^{-19} mol dm⁻³ (Bown *et al.*, 2012a). Ellwood i van den Berg 2001. istražuju specijaciju otopljenog kobalta s organskim ligandima CLE-AdCSV metodom pri pH ~ 9,1 (korištenjem amonijeva pufera), titracijom s nioksimom i nitritom kao oksidansom. Dobiveni logaritmi uvjetnih konstanti stabilnosti kompleksa Co s organskim ligandima su u rasponu od 15,6 do 16,1 (Ellwood i Van den Berg, 2001). Saito & Moffet u radu iz 2001. godine ukazuju na iznimnu stabilnost kompleksa s dimetilglioksimom (DMG) koji karakteriziran prisustvom oksimne skupine C=N-OH ima značajan porast signala redukcije Co u usporedbi s redukcijom Co(II) kationa ili drugih kompleksa kobalta s organskim ligandima. U radu iz 2001 Saito i Moffet su odredili $K_{\text{CoHMDG2}} = 11.5 \pm 0.3$ pri pH 8, dok je K_{CoL} bila 16.3 ± 0.9 (Saito i Moffett, 2001).

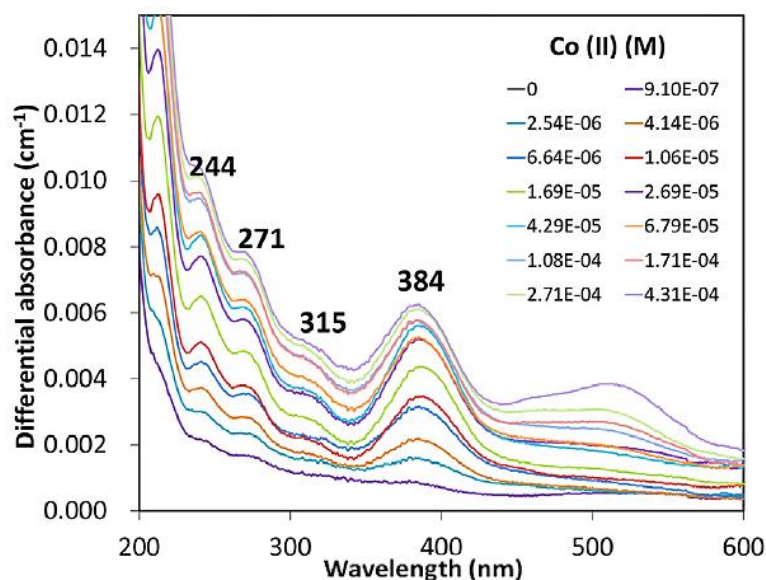
Uvjetne konstante stabilnosti posebno su određivane za komplekse željeza s organskim ligandima, najviše sideroforima, kako modelnim tako i realnim (Bonnet *et al.*, 2013; Bruland, 1995; Luther *et al.*, 2001; Reid *et al.* 1993; Rue i Bruland 1995, Witter *et al.* 2000). Macrellis *et al.* (2001) su identificirali hidroksiamate i kateholate, dvije željezo(III) kompleksirajuće skupine, te su identificirali siderofore izolirane iz mora i odredili konstante stabilnosti sa željezom koje su bile u rasponu $\log K_{\text{FeL}}$ od 11.5 do 11.9 (Macrellis *et al.*, 2001). Dobivene vrijednosti su bile usporedive s vrijednostima određenim sa sideroforima pročišćenim i proizvedenim uzgojom i drugim istraživanjima Fe(III)-ligand kompleksa u moru (Hutchins *et al.*, 1999; Lewis *et al.*, 1995; Rue i Bruland, 1995; Witter *et al.*, 2000).

1.3.2. UV/Vis spektrofotometrija

UV/Vis spektrofotometrija je analitička metoda koja proučava međudjelovanje tvari i elektromagnetskog zračenja u ultraljubičastom ($\lambda = 400\text{-}200\text{ nm}$) ili vidljivom ($\lambda = 800\text{-}400\text{ nm}$) dijelu spektra. Osim za identifikaciju i analizu brojnih organskih i anorganskih vrsta tvari (kvalitativna analiza), UV/Vis spektroskopija se primjenjuje i za kvantitativnu analizu (Yu *et al.*, 2018). Najjednostavnija i najčešća primjena je za mjerenja na određenoj valnoj duljini za koju je prethodno utvrđeno da predstavlja maksimum apsorpcije za željenu komponentu, a ako otopina sadrži više apsorbirajućih komponenti valna duljina se bira u području u kojem ostale vrste ne apsorbiraju ili apsorbiraju minimalno. Količina apsorbiranog zračenja na danoj valnoj duljini ovisi o veličini apsorbirajućeg sustava i vjerojatnosti elektronskog prijelaza. Ovo svojstvo molekule se naziva molarni apsorpcijski koeficijent ili molarni koeficijent ekstinkcije (ϵ). Molarni apsorpcijski koeficijent je konstanta svojstvena svakoj molekulskoj vrsti i ovisna o valnoj duljini svjetlosti. Osim toga, apsorpcija raste s porastom broja molekula koje apsorbiraju svjetlost odgovarajuće energije (Skoog i Holler, 1998). Mjerenja se također mogu provoditi na višestrukim valnim duljinama što često daje uvid u pojedinosti o sastavu ili čistoći uzorka. Međutim, kod kompleksnijih istraživanja snima se spektar svih valnih duljina. Iz snimljenog UV/Vis spektra možemo očitati na kojoj valnoj duljini ispitivana molekula najbolje apsorpira, dok uz pomoć Beer-Lambertova zakona možemo odrediti koncentraciju ispitivane molekule poznatog apsorpcijskog koeficijenta iz izmjerene apsorpcije. Organske molekule mogu apsorbirati elektromagnetsko zračenje jer sadrže valentne elektrone koje je moguće pobuditi u više energetska stanja. Dio molekule u kojem razlika između dva energetska nivoa odgovara energiji UV i vidljivog dijela spektra naziva se kromofor (grč. *chroma* – boja, *phorein* – nositi). Apsorbancijska spektrometrija se često koristi u svrhu karakterizacije organske tvari, a sve više i za interakcije metala s organskim ligandima (Vukosav *et al.*, 2012b; Yan *et al.*, 2013). Odnosi se na kromofornu komponentu organske tvari tzv. chromophoric DOM koja predstavlja frakciju DOM-a koja apsorpira svjetlost. Određivanje metala kolorimetrijskim metodama temelji se na nastajanju njihovih obojenih kompleksa. Određivanje metala u složenoj matrici spektrofotometrijskim metodama ima mnogo ograničenja zbog brojnih smetnji. Uklanjanje smetnji je važno u ovim metodama.

Hosseini *et al.* u radu iz 2012. prikazali su spektrofotometrijsko istraživanje kompleksa Co(II), Mn(II), Cd(II), Pb(II) i Zn(II) s jodokinolom u organskim otapalima dimetilformaldehydu/metanolu pri ionskoj jakosti otopine od 0,1 (25 °C). Određene su konstante stabilnosti kompleksa, a stabilnost kompleksa je slijedila niz: Zn(II) > Co(II) > Pb(II)

$>Mn(II) >Cd(II)$. Stabilnost kompleksa značajno ovisi o prirodi otapala (medija) u kojem kompleksi nastaju. Solvatacija liganda i metala ovisi o afinitetu otpuštanja elektrona, dielektričnoj konstanti otapala, obliku i veličini molekula otapala. Općenito, očekuje se da konstante stabilnosti kompleksa opadaju zbog kompeticije liganda i otapala za metal. Tijekom kompleksiranja ligand može zamjeniti molekule otapala u solvatacijskoj ljusci ili metal može zamjeniti molekule otapala s ligandima (Hosseini *et al.*, 2017). Yan *et al.* u radu iz 2013 prikazao je interakciju Cu(II), Cd(II) i Al(III) sa frakcijama DOM-a te ih usporedio s interakcijama između metala i standardnih uzoraka humusne i fulvinske kiseline (Yan *et al.*, 2013). Zhang *et al.* u radu iz 2021 prikazuje interakciju metalnih iona Cu(II), Ni(II), Co(II), Fe(III), Cr(III), Al(III), Zn(II), Ca(II), Mg(II) i Pb(II) s eskuletinom (Zhang *et al.*, 2021). Nastajanje kompleksa s kobaltom prikazano na slici 9.



Slika 9. UV/Vis spektri kompleksa kobalta(II) s eskuletinom snimljeni pri različitim koncentracijama kobalta (Zhang *et al.*, 2021)

Ali *et al.* u radu iz 2022. prikazuje spektrofotometrijsku analizu kompleksa kadmija, kobalta, bakra, željeza i olova. Metoda je temeljena na stvaranju kompleksa s fenolom, natrijevim nitritom i streptomycinom u kiselom mediju. Sniman je cijeli spektar s variranjem koncentracija metala i pH vrijednosti (Ali *et al.*, 2022). Prednost metode UV/Vis je što ne zahtijeva pretkoncentriranje uzorka, lako se koristi, te daje strukturne informacije o specifičnim ligandima. Međutim, UV/Vis spektrofotometrija nije selektivna i često je nedovoljno osjetljiva za koncentracije analita prisutnih u morskoj vodi. Stoga se najčešće koristi u istraživanjima s modelnim ligandima i metalima u koncentracijama višim od 10^{-6} mol dm⁻³. Ravnotežne

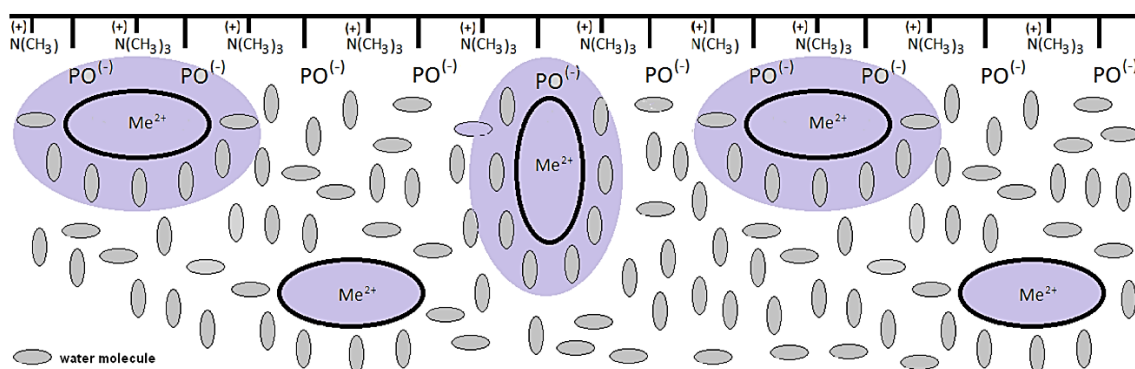
konstante stabilnosti kompleksa nastalih u otopini mogu se odrediti programom *Specfit* koji se temelji na multivarijantnom, nelinearnom korištenju regresijske analize najmanjih kvadrata (Cornard *et al.*, 2005; Gampp *et al.*, 1985; Maeder, 1990).

1.3.3. Langmuir-Blodgettova metoda

Langmuir-Blodgettova metoda prati promjene na Langmuirovim monoslojevima površinski aktivnih tvari na granici faza zrak/voda (Giner-Casares *et al.*, 2014).

Temelji se na stvaranju monoslojeva pod utjecajem tlaka (Crawford i Leblanc, 2014). Proučavanjem monoslojeva možemo dobiti informacije o njihovoj interakciji i o sastojcima subfaze, o interakcijama unutar njih, o svojstvima lipidinih membrana, orijentaciji molekula te gustoći slaganja molekula. Metoda se provodi u Langmuirovoj kadi (Velázquez *et al.*, 2016). Površinski tlak (π) i srednja molekulska površina (A) se kontinuirano mjere tijekom kompresije. U kadu se na površinu medija, tzv. subfaze mikro injekcijom nanosi uzorak otopljen/pomiješan s hlapivim nepolarnim otapalom (Crawford i Leblanc, 2014; Dynarowicz-Łątka *et al.*, 2001). Uzorak se raširi brzo i prekrije dostupnu površinu. Kad otapalo ispari nanese površinski aktivne tvari kao amfifilne molekule orijentiraju se na njima prirodan način na granici faza voda-zrak. Hidrofilne glave bit će uronjene u vodeni medij dok će hidrofobni ugljikovodični lanci stajati u zraku. Kompresijom monosloja dolazi do promjene površinskog tlaka (π), a ugradnjom komponenti iz subfaze dolazi i do promjene u molekulskom pakiranju što će se odraziti na razliku u π - A izotermama (Dynarowicz-Latka *et al.* 2001). Mjerenja se provode pretežno pod pseudo ravnotežnim uvjetima, kontinuiranom kompresijom monosloja dok se mjeri površinski tlak. Smanjenjem površine dolazi do stvaranja odbojnih sila između molekula. Kompresijom monoslojeva površinski tlak se mijenja, a molekule se slažu u uređena stanja koja se mogu usporediti stanjima trodimenzionalnih plinova, tekućina i krutina (Laszuk, 2020). Plinovito stanje je početno stanje koje zauzimaju molekule prilikom nanošenja na subfazu. U takvom stanju interakcije između molekula su slabe i a molekule zauzimaju njima najpovoljnije, najčešće najudaljenije položaje. Komprimiranjem monoslojeva molekule oni se međusobno približavaju pa dolazi do faznog prijelaza iz plinovitog u tekuće ekspanzirano stanje, što je vidljivo kao plato na π - A izoterma. Daljnjom kompresijom dolazi do stvaranja tekućeg ekspaniranog dijela. U tekućem ekspaniranom stanju hidrofobni su dijelovi molekula uronjeni u tekućinu dok se hidrofobni lanci uzdižu iznad površine, a njihova orijentacija je slobodna. Daljnjom kompresijom dolazi do sve većeg zblizavanja molekula te prelaska u tzv.

tekuće kondenzirano stanje. U tom stanju polarne glave su još gušće složene te dolazi do stvaranja međumolekulskih sila između molekula. Nastavkom kompresije postiže se kondenzirana faza. U toj fazi molekule su vrlo gusto i pravilno složene. Daljna kompresija dovodi do tlaka kolapsa, nakon čega daljnim povećavanjem tlaka dolazi do raspada sloja u izolirane nakupine ili višeslojne strukture nastale istiskivanjem molekula iz monosloja (Laszuk *et al.*, 2020). Monoslojevi nastali na kadi pokazali su se kao dobar model stanične membrane od 1917. godine kada je Irving Langmuir predstavio LB-metodu. (Laszuk *et al.*, 2020). Interes za korištenjem metode značajno raste u periodu između 1990 i 1999. razvojem novih metoda koje je moguće bilo povezati s metodom LB kao što su UV/Vis, IR refleksijska spektroskopija, mikroskopija pod Brewsterovim kutom, difrakcija i refleksijska X-zraka (Giner-Casares *et al.*, 2014). Metoda je izuzetno prikladna za određivanje mnogih fizikalno-kemijskih parametara pri karakterizaciji membrana. Amfifilni monoslojevi su korisni za istraživanja mehanizama biofizikalnih i biokemijskih fenomena u živim stanicama, te nam mogu dati informacije o sastavu amfifilnih molekula (npr. sastav masnih kiselina, lipida, proteina i miješanih filmova). Metoda LB se može koristiti za istraživanje interakcija fosfolipida s metalnim ionima i organskim molekulama u vodenom mediju. Metodom LB može se pratiti utjecaj metala na fosfolipidne molekule. Negativno nabijen fosfolipid privlači pozitivan metalni kation (slika 10.).



Slika 10. Shematski prikaz monosloja L- α -fosfatidilkolina s divalentnim ionima (LMe^{2+} i L_2Me^{2+} kompleksi) (Petelska i Naumowicz, 2017a)

Utjecaj metala jako ovisi o veličini kationa koji djeluje na gustoću površinskog naboja. Petelska *et al.* u svojim radovima iz 2013. i 2017. prikazuju interakciju Ca^{2+} , $Mg(II)$, $Na(II)$, $Zn(II)$, $Sr(II)$, $Cd(II)$, $Ba(II)$, $Pb(II)$ s monoslojem L- α -fosfatidilkolina. Dodatkom iona u subfazu dolazi do stabilizacije monoslojeva i porasta limitirajuće površine (slika 10.). Izračunate konstante stabilnosti kompleksa su pokazale najmanju stabilnost s ionima Mg a

najveću s ionima Pb za komplekse stehiometrije 1:1 (LM^{2+}) i 2:1 (L_2M^{2+}). Tijekom formiranja miješanog dvokomponentnog monosloja na elektrolitnoj površini, individualne komponentne (L^- , M^{2+} i LM^+) mogu stvarati komplekse (Petelska i Figaszewski, 2013; Petelska i Naumowicz, 2017a). Ravnoteža takvog sustava je opisana reakcijama kompleksiranja. Ako pretpostavimo da na granici faza nastaju miješani kompleksi 1:1 i 2:1 reakcije kojima se mogu opisati su:



Konstante kojima se reakcije mogu opisati su:

$$K_1 = \frac{a_{LM^+}}{a_{L^-} \cdot a_{M^{2+}}} \quad (14)$$

$$K_2 = \frac{a_{L_2M}}{a_{LM^+} \cdot a_{L^-}} \quad (15)$$

$$a_{L^-} A_{L^-} + a_{LM^+} A_{LM^+} + a_{L_2M} A_{L_2M} = 1 \quad (16)$$

$$a_{L^-} + a_{LM^+} + a_{L_2M} = C \quad (17)$$

gdje su:

a_{L^-} , a_{LM^+} i a_{L_2M} [mol/m²] površinske koncentracije komponenti L^- , LM^+ i L_2M ;

A_{L^-} , A_{LM^+} , A_{L_2M} [mol/m²] površine koje zauzima 1 mol komponente L^- , LM^+ i L_2M ;

K_1 i K_2 [mol/m²] su konstante stabilnosti kompleksa LM^+ i L_2M ;

C [mol/m²] je ukupna površinska koncentracija.

Eliminacijom a_{L^-} , a_{LM^+} i a_{L_2M} parametara iz 14-17 jednadžbe može se postaviti pojednostavljena jednadžba:

$$y = m_1 x_1 + m_2 x_2 + m_3 x_3 + m_4 x_4 + m_5 x_5 + b \quad (18)$$

Gdje je:

$$y = C^2 a_{M^{2+}} \quad (19)$$

$$m_1 = K_1 K_2^{-1} A_{L_2M}^{-2} A_{LM^+} (2 A_{LM^+} - A_{L_2M}) \quad (20)$$

$$x_1 = C a_{M^{2+}}^2 \quad (21)$$

$$m_2 = K_2^{-1} A_{L_2M}^{-2} [4 K_2 A_{L_2M} + A_{L^-} (2 A_{LM^+} - A_{L_2M}) + A_{LM^+} (2 A_{L^-} - A_{L_2M})] \quad (22)$$

$$x_2 = C a_{M^{2+}} \quad (23)$$

$$m_3 = K_1^{-1} K_2^{-1} A_{L2M}^{-2} A_{L-} (2A_{L-} - A_{L2M}) \quad (24)$$

$$x_3 = C \quad (25)$$

$$m_4 = -K_1 K_2^{-1} A_{L2M}^{-2} (2A_{LM+} - A_{L2M}) \quad (26)$$

$$x_4 = a_{M2+}^2 \quad (27)$$

$$m_5 = -K_2^{-1} A_{L2M}^{-2} [4 K_2 + (2A_{L-} - A_{L2M}) + (2A_{LM+} - A_{L2M})] \quad (28)$$

$$x_5 = a_{M2+} \quad (29)$$

$$b = -K_1^{-1} K_2^{-1} A_{L2M}^{-2} (2A_{L-} - A_{L2M}) \quad (30)$$

gdje su K_1 , K_2 , A_{L-} , A_{LM+} i A_{L2M} parametri izračunati iz jednadžbi:

$$A_{L-} = (-m_3)/b \quad (31)$$

$$A_{LM+} = (-m_1)/m_4 \quad (32)$$

$$A_{L2M} = 2 (A_{L-} * m_4 - A_{LM+} * b)/(m_4 - b) \quad (33)$$

$$K_1 = -\frac{2 * A_{LM+} - A_{L2M}}{m_4 * K_2 * A_{L2M}^2} \quad (34)$$

$$K_2 = -\frac{(A_{L-} - A_{L2M}) + (2A_{LM+} - A_{L2M})}{4 + m_5 * A_{L2M}^2} \quad (35)$$

Langmuirovi monoslojevi na granici faza dobar su model za istraživanje interakcija biološki važnih molekula (npr. DNA) i fosfolipida. Takvi eksperimenti često uključuju prisutnost divalentnih iona koji su potrebni za bolju adsorciju molekula na monosloj. Istraživanje Petelske *et al.* iz 2017. godine pokazuje snažnu elektrostatsku interakciju između DNA i monosloja fosfatidilkolina u prisustvu Ca^{2+} iona (Petelska i Naumowicz, 2017a).

1.3.4. Mikroskopija atomskih sila

Mikroskopija atomskih sila (engl. *Atomic force microscopy* - AFM) (Binnig *et al.*, 1986) pripada novoj generaciji mikroskopa s pretražnom probom (engl. *scanning probe microscopy*, SPM). AFM omogućuje stvarne topografske prikaze površina u tri dimenzije. Površina uzorka se pretražuje pomoću oštrog šiljka. AFM se, osim u oslikavajućem načinu rada, koristi i za ispitivanje mehaničkih svojstava u neoslikavajućem načinu rada. Neinvazivna je tehnika visoke rezolucije koja služi za karakterizaciju površine. Najveća prednost tehnike je mogućnost provođenja kvantitativnih morfoloških mjerenja, mogućnost istraživanja u realnom vremenu i s visokom rezolucijom površinske nanostrukture biomolekula, direktno mjerenje fizikalnih svojstava na nanoskali, provođenje mjerenja u različitim okolišima i proučavanje promjena na biološkim površinama u kontroliranim uvjetima (konstantni tlak, temperatura) (Dufrêne *et al.*, 2017; Farré, 2012). AFM je korišten za snimanje molekula iz vodenog okoliša, uključujući humusne tvari, polisaharide i razne koloide (Al-Rekabi i Contera, 2018). Mjerenja je trebalo prilagoditi fizikalnim i kemijskim svojstvima uzorka. Prilikom pripreme uzorka treba paziti na koncentraciju uzorka koja može dovesti do stvaranja agregata, na pH i ionsku jakost. To je važno kad se mjerenja izvode na zraku jer proces sušenja može stvoriti strukturne artefakte ili restrukturirati čestice AFM substrata (Balnois i Wilkinson, 2002). Uvođenje tzv. „tapping mode“ AFM-a omogućilo je istraživanje iznimno mekih i osjetljivih uzoraka što je povećalo interes za istraživanjem lipida (Pompeo *et al.*, 2005). AFM omogućava istraživanje lipidnih dvoslojeva u vodenom okolišu na nanometarskoj rezoluciji, omogućavajući vizualizaciju lipidnih faza (Maté *et al.*, 2014). Bitna prednost AFM-a je da može proučavati biološki važne spojeve kao što je DNA, do proteina i makromolekula. AFM je pružio novi pogled na mehanizam membrana na nanometarskoj skali. Istraživanje interakcije membrana s ionima je od značajnog interesa zbog naboja membrane i nabijenih iona.

Al Rekabi i Contera u radu iz 2017. prikazuju utjecaj kolesterola na dvosloj fosfatidilkolina u koncentraciji od 0 do 60%. AFM slike pokazale su kako pri nižim koncentracijama kolesterola lipidni dvosloj pokazuje jasno fazno razdvajanje i elastičnost dok pri višim koncentracijama dvosloj postaje homogeniji te pokazuje i elastična i viskozna svojstva (Al-Rekabi i Contera, 2018). Nordin *et al.* u radu iz 2012. AFM-om prate interakcije izvanstaničnog proteina fibronektina sa jednostavnim lipidnim dvoslojem koji omogućava dobro definirano fazno razdvajanje. AFM-om su vidljiva fazna razdvajanja različitih visina faza, dok je dodatak fibronektina stvorio prstenaste strukture i povećao rezoluciju slike u istom mjernom području (Nordin *et al.*, 2012). U radu Rinia *et al.* iz 1999. godine snimane su strukture

fosfolipida. U strukturama su vidljivi defekti, ali su ti oblici različiti, tako fosfatidiletanolamin ima pravilnije, okrugle dok su kod fosfatidilkolina nepravilnih oblika. Oblik ukazuje na energetski povoljnije strukture fosfatidiletanolamina - jaču interakciju između fosfolipidnih molekula. Dodatkom metala u uzorak dolazi do stvaranja različitih uzvisina, mogućih artefakata koje bi se mogle objasniti različitom površinskom gustoćom. Povišenjem ionske jakosti uzorka dolazi do smanjenja visine nabijenih površina što se može objasniti time da je prisutnost dvovalentnih kationa dovela do nestanka uzvišenja (Rinia *et al.*, 1999). Na temelju navedenih literaturnih navoda metoda atomske mikroskopije pokazala se pogodnom za istraživanje interakcija između fosfolipida, organskih molekula i dvovalentnih metalnih iona.

2. CILJ I HIPOTEZA RADA

Cilj ovog doktorskog rada je bio istražiti komplekse kobalta i prirodno prisutnih organskih liganada iz morskog okoliša, koji su od posebnog značaja za praćenje biogeokemije kobalta u moru, njegov utjecaj na morske organizme, kao i posljedično praćenje utjecaja klimatskih promjena, npr. variranjem fizikalno-kemijskih parametara (pH). U tu svrhu korištene su prirodno prisutne organske molekule topljive u morskoj vodi (4-nitrokatehol, humusna kiselina), kao i amfifilne (lipidi) koje su teško topljive

Postavljena hipoteza je da kobalt(II) u moru stvara komplekse s organskim ligandima, posebno onim koji sadrže nitro funkcijske skupine, te na takav način utječe na svoju biodostupnost. Kako bi se istražila biodostupnost kobalta za morske organizme ispitivana je interakcija kobalta s fosfolipidnim molekulama. Pretpostavljeno je da bi prolazak iona kobalta kroz lipidnu staničnu membranu bio moguć samo formiranjem intermedijernog kompleksa topljivog u morskoj vodi, koji neutralizira naboj kobaltovog iona. Na taj bi način neutralni miješani kompleks interagirao s membranskim fosfolipidima i otvarao membranske stanične pore, te omogućio prolaz kobalta u unutarstanični prostor. Takvi kompleksi bi ujedno mogli i povećati biodostupnost kobalta kao esencijalnog mikronutrijenta.

Predloženo istraživanje pružit će uvid u procese koji kontroliraju biogeokemijski ciklus kobalta u moru, njegovu biodostupnost, mobilnost i stabilnost koje su od velike važnosti za razumijevanje specijacije kobalta u moru. Prikazat će se potencijal stvaranja kompleksa s lipidima koji su u morskom okolišu biomarkeri za okolišni stres (klimatske promjene).

3. PUBLIKACIJE NA KOJIMA SE TEMELJI DOKTORSKI RAD

3.1. Publikacija I

Naslov: Electrochemical characterization of Cobalt(II)-Complexes involved in marine biogeochemical processes. I. Co(II)-4-nitrocatechol and Co(II)-Humate

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Electrochemical characterization of Cobalt(II)-Complexes involved in marine biogeochemical processes. I. Co(II)-4-nitrocatechol and Co(II)-Humate



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ABSTRACT

Cobalt (II) as an important micronutrient for numerous biological processes in marine environment, has been tested in model aqueous solutions with naturally occurring ligands 4-nitrocatechol (4NC) and humic acid (HA) by voltammetry and spectrophotometry. Irreversible, two-electron reduction processes of Co(II)-4NC and Co(II)-HA complexes adsorbed on the mercury drop surface, depending on accumulation time and composition of the solution (c_{Co} , c_{4NC} , c_{HA} and pH), were detected. Complexes Co:L = 1:1 stability constants formed only in the solution, were determined by spectrophotometry and amount to $\log K_{Co(II)4NC} = 3.98$ (pH = 8.2) and $\log K_{Co(II)4NC} = 5.76$ (pH = 6.5), and $\log K_{Co(II)HA} = 3.80$ (pH = 8.2). Co:L = 1:2 complexes Co(II)-(4NC)₂ at pH 6.5 and 8.2 and Co(II)-(HA)₂ at pH 8.2, formed at the mercury drop electrode surface as hydrophobic species, were detected only by voltammetric measurements. Conditional stability constants were calculated by the CLE/ACSV method: $\log K_{Co(II)(4NC)_2} = 21.86$ (pH = 8.2), $\log K_{Co(II)(4NC)_2} = 21.11$ (pH = 6.5) and $\log K_{Co(II)(HA)_2} = 11.32$ (pH = 8.2).

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1. Introduction

Global climate changes involve complex processes in marine ecosystems, especially in the surface layers. Using a multidisciplinary, holistic approach, it is requisite to study the atmospheric influence on biogeochemical responses in the marine environment. A gradual approach and specific objectives for the assessment of concentrations, sources and fate of atmospheric components, the impact on marine biogeochemistry of macro- and micronutrients and inevitable effects in the biological organization of marine environment, are of great importance [1].

The sea surface micro-layer represents one of the largest natural phase boundaries on Earth, as an interfacial area it is crucial in gases, particles and energy exchange processes between sea and atmosphere [2–6]. The atmosphere impact is a significant external source of macro- and micronutrients.

Trace elements in oceans are present in very low concentrations $<10^{-9}$ mol dm⁻³ [7]. Their bioavailability in surface waters

regulates phytoplankton growth. As phytoplankton consumes atmospheric carbon dioxide and enters it into the seawater column, the key components that regulate the carbon cycle are bioavailable trace metals linked to the organic matter. In addition to iron, other bioactive micronutrients such as cobalt, copper, nickel and zinc play an important role in phytoplankton adaptation and growth regulation of, vital for the carbon biological pump [8,9].

Cobalt is an important micronutrient involved in vitamin B12 (cobalamin) biosynthesis and can replace zinc as a metal co-factor [10–13]. Cobalt speciation in oceans is a dynamic process as it is a redox-active metal. Part of the dissolved cobalt under the euphotic zone remains unbound or weakly bound to the labile complexes [14]. The cobalt distribution in seawater often shows a maximum within the upper boundary of the thermocline as a result of atmospheric intake. In deep ocean layers it is exhausted due to the removal from seawater column by sedimentation processes [15]. Improvements in cobalt biogeochemistry understanding should give an insight into other current hypotheses, such as the Sunda Scenario [16], C–Co–Zn colimitation [12], as well as speciation influence on phytoplankton abundance.

In order to make cobalt speciation measurement at seawater, it is necessary to consider the redox state of cobalt. Co existing in +2 and +3 oxidation state under typical environmental E_H and pH

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conditions and is a redox active element that tends to be strongly bound to organic complexes in upper water column [17].

Cobalt (II) complexes formation with naturally occurring ligands at the hydrophobic mercury drop surface as a model for hydrophobic cell membranes in NaCl solution is studied, mimicking seawater conditions. Electrochemical characterization and speciation of cobalt (II) complexes with catecholate and humic acid type ligands was examined, as essential in the marine environment. Catecholates, *ortho*-dihydroxybenzene and 4-nitro-dihydroxybenzene, as simple organic ligands from aerosols are present as a structural part of complex marine ligands (humic acid, siderophores, etc.). Humic substances as heterogeneous biomolecules are a characteristic component of the dissolved organic carbon (DOC) pool in marine systems and important for phytoplankton communities [18–21].

2. Experimental

2.1. Equipment and measurements procedure

2.1.1. Electrochemical measurements

Electrochemical experiments were performed using a μ -AUTO-LAB multi mode potentiostat (ECO Chemie, Utrecht, The Netherlands) equipped with a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland). The instrument was computer-controlled using GPES 4.9 control software. The working electrode was a static mercury drop electrode (SMDE, size 2, i.e. 0.40 mm²), the counter electrode was a glassy carbon rod and the reference electrode was Ag/AgCl (sat. NaCl) (+0.197 V vs. SHE). Measurements were performed in electroanalytical quartz cell at 25 ± 1 °C in NaCl of $I = 0.55 \text{ mol dm}^{-3}$ (I = ionic strength), maintaining pH by borate buffer (0.1 mol dm⁻³), as HEPES and phosphate buffers showed noticeable impact on the Co–4NC and Co–HA complexes voltammograms. Applied electrochemical techniques were square-wave voltammetry (SWV) with the pulse amplitude, $a = 25 \text{ mV}$; frequency, $f = 50 \text{ s}^{-1}$; potential step increment, $E_{\text{inc}} = 2 \text{ mV}$, and cyclic voltammetry (CV) with scan rate, $v = 0.05 \text{ V s}^{-1}$. Scan direction in all techniques was cathodic, i.e. voltammograms were recorded by scanning from positive toward negative potentials. Prior to electrochemical measurements, solutions were deaerated by bubbling with extra pure nitrogen for about 20 min. The solution was stirred during deaeration and accumulation (3000 rpm). Nitrogen circulated above the solution during measurements. The pH in solutions was measured using a combined glass-Ag/AgCl electrode connected to an ATI Orion PerpHecTMeter, model 320 (Cambridge, USA). pH electrode was immersed into the electroanalytical cell throughout majority of voltammetric measurements. of the Glass electrode calibration was performed using standard buffer solutions.

2.1.2. UV/vis spectrophotometric measurements

The UV–visible absorption spectra were recorded on a PerkinElmer spectrophotometer Lambda 45 in an optical quartz cell 1 cm path length, using software Winlab 2.85. Continuous spectra were measured within range 220–650 nm. Measurements were performed in 0.5 mol dm⁻³ NaCl at pH 8.2 maintained by borate buffer ($C_{\text{H}_3\text{BO}_3} = 0.05 \text{ mol dm}^{-3}$), ionic strength ($I = 0.55$). Measurements with 4NC were provided at pH 6.5 and 8.2, while with HA at 8.2.

UV–Vis spectra were analyzed through the entire range of measured wavelengths (400–900 nm) by multivariate non-linear least squares regression analysis using Specfit program [22–24].

2.2. Chemicals and solutions

Standard solution of cobalt (II) nitrate ($1.69 \times 10^{-2} \text{ mol dm}^{-3}$)

(Fluka Chemie GmbH, Buchs, Switzerland) was used as source of cobalt (II) in all experiments. The aqueous stock solution of 4NC (*p.a.* Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland) and HA (1000 mg dm⁻³, 600–1000 Da, Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland) was prepared by solid dissolving in deionized water from a Mili-pore Mili-Q system (Bedford, USA). Sodium chloride (*s.p.* NaCl Merck, Damstadt, Germany) was also dissolved in deionized water to obtain stock aqueous solution (0.5 mol dm⁻³). Solutions' pH was adjusted by adding diluted *s.p.* HCl or NaOH (Merck, Damstadt, Germany).

3. Results and discussion

3.1. Voltammetric characterization of Co(II)–4NC and Co(II)–HA complexes

Measurements were performed by square-wave (SW) and cyclic voltammetry (CV). Standard potential (E°) for the two-electron reduction of Co(II)_(aq) ion in aqueous solutions is -0.28 V .

Catechol (1,2-dihydroxybenzene) and nitrocatechol are weak diprotic acids, present in a wide scale of environmental compounds as chelating ligands [25]. Nitro substituent leads to an increase hydroxyl groups acidity in phenol derivatives [26]. Several estimates of acidic dissociation constant in aqueous medium and average values of pK_a of two nitrocatecholate hydroxyls, have been reported [27,28]. These were obtained by potentiometric titrations, amount to 6.7 and 10.8 at $I < 0.55 \text{ mol dm}^{-3}$.

Humic acid (HA) has a typical chemical formula $C_{187}H_{186}O_{89}N_9S_1$ [29]. Measured HA pK_a values depend on their components and it is a mixture of dibasic acids with pK_1 value about 4 for the carboxyl groups, and about 8 for phenolate groups protonation, being completely dissociated at $pH > 8$. It is an electroinactive molecule under experimental conditions used [30].

Co(II)-complexes voltammograms were measured using SMDE in the pH range 2–11, with $c_{\text{Co}} = 1\text{--}5 \times 10^{-5} \text{ mol dm}^{-3}$, $\gamma_{\text{HA}} = 0.1\text{--}0.9 \text{ mg dm}^{-3}$, $c_{\text{Cat}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, $c_{4\text{NA}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, accumulation time $t_{\text{acc}} 0\text{--}300 \text{ s}$ at $E_{\text{acc}} = 0.0 \text{ V}$. Without accumulation Co(II)- complexes were not registered.

The Co(II)-catechol reduction process was measured within pH range 5–12, however, the complex reduction response was not observed. Cobalt (II) reduction peak at -1.3 V appeared corresponding to the Co(OH)₂ complex at pH above 11.

The Co(II)–4NC reduction response was registered within pH range 5.5–10.5 (Fig. 1) at -1.05 V , with the reduction current maximum at $pH = 6.5$. At pH range above 6.5, Co(II)–4NC reduction peak decreased and shifted towards more negative values. At higher pH values poorly soluble Co(II)-hydroxide formed progressively, and presumably, due to adsorption at the electrode surface, inhibited Co(II)–4NC reduction process. At pH values above 9.5 reduction peak at -1.36 V appeared, corresponding to reduction of Co(OH)₂ (Fig. 1, curve 6).

Co(II)–4NC reduction peak current at -1.05 V ($pH = 8.2$) increased exponentially, depending on $c_{4\text{NC}}$ within range $1\text{--}5 \times 10^{-5} \text{ mol dm}^{-3}$, when reached the plateau due to mercury drop surface saturation with complex molecules. At the same time Co(II)4NC complex reduction peak potential shifted towards negative values for $\approx 50 \text{ mV}$.

Co(II)–4NC reduction current depending on cobalt (II) concentration with constant $c_{4\text{NC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$ ($pH = 8.2$, $t_{\text{acc}} = 180 \text{ s}$), increased linearly with the slope $12.75 \pm 0.21 \text{ nA mol}^{-1} \text{ dm}^3$, while the reduction peak potential remained constant.

By adding HA to the Co(II) solution, Co(II)–HA complex was formed and the reduction response was registered in pH range

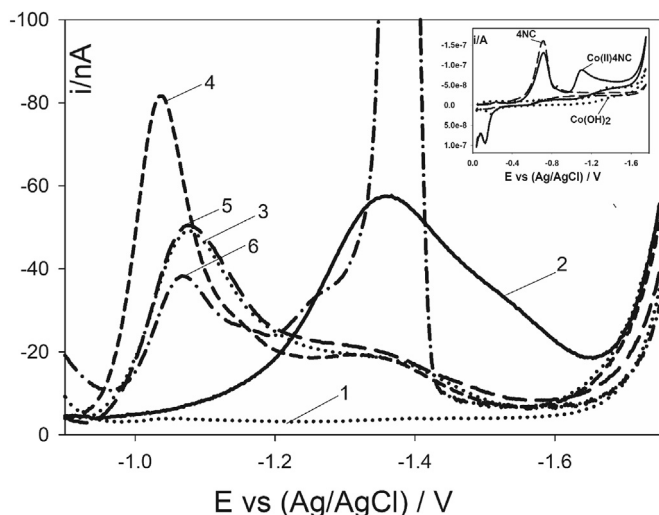


Fig. 1. SW voltammograms of Co(II)–4NC complexes in 0.55 mol dm^{-3} NaCl with $c_{\text{Co}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$; $c_{4\text{NC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$ at different pH: (1) 4NC pH = 8.2; (2) Co(OH)_2 pH = 8.2; (3) 5.5, (4) 6.5, (5) 7.7, (6) 8.2, (6) 9.5. SW parameters: $f_{\text{SW}} = 50 \text{ s}^{-1}$, $a_{\text{SW}} = 75 \text{ mV}$, $E_{\text{inc}} = 2 \text{ mV}$, $t_{\text{eq}} = 5 \text{ s}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{acc}} = 0.0 \text{ V}$. Inset: CV of Co(II) (OH)₂, 4NC, Co(II)–4NC complex (pH = 8.2).

4.4–10.8 (Fig. 2). The reduction current reached maximum at pH 6.5 and with further increase in pH dropped to zero at pH ≈ 10 . The complex reduction current increased and peak potential moved towards positive values for about 50 mV in the pH range 4.4–8.2. At higher pH values reduction current decreased, whereas the peak potential remained constant.

Only weakly soluble Co(OH)_2 reduction peak was registered at -1.3 V without accumulation, while Co(II)-HA complex reduction peak at -1.05 V was registered (pH = 8.2) with accumulation times from 60 to 300 s. The reduction current increased until $t_{\text{acc}} = 300 \text{ s}$ reaching maximum, and the reduction potential shifted towards more positive values for about 30 mV.

Co(II)-HA complex reduction current (pH = 8.2, $t_{\text{acc}} = 180$) depending on γ_{HA} (0.1 – 0.9 mg dm^{-3}) increased linearly, with the slope of $143.38 \pm 0.14 \text{ nA mg}^{-1} \text{ dm}^3$ until $\gamma_{\text{HA}} = 0.55 \text{ mg dm}^{-3}$.

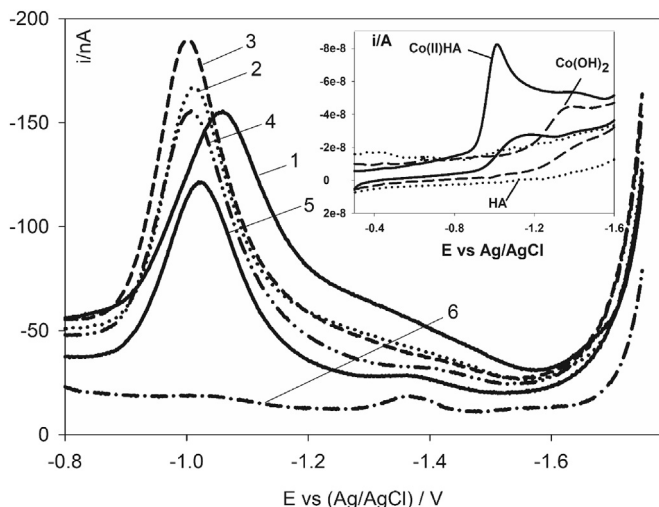


Fig. 2. SW voltammograms of Co(II)-HA complexes in 0.55 mol dm^{-3} NaCl with $c_{\text{Co}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$; $\gamma_{\text{HA}} = 0.9 \text{ mg dm}^{-3}$ at different pH: (1) 4.4, (2) 5.9, (3) 6.5, (4) 8.2, (5) 9.5, (6) 10.8. SW parameters: $f_{\text{SW}} = 50 \text{ s}^{-1}$, $a_{\text{SW}} = 75 \text{ mV}$, $E_{\text{inc}} = 2 \text{ mV}$, $t_{\text{eq}} = 5 \text{ s}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{acc}} = 0.0 \text{ V}$. Inset: CV of Co(II) (OH)₂, HA, Co(II)-HA complex (pH = 8.2).

Reduction current dependence reached plateau with HA concentration above 0.9 mg dm^{-3} as the electrode surface was saturated with Co(II)-HA complex molecules. Depending on HA concentration Co(II)-HA reduction potential shifted towards positive values for about 30 mV.

Co(II)-HA complex SW reduction current examined in dependence of cobalt (II) concentration within range 1 – $5 \times 10^{-5} \text{ mol dm}^{-3}$, with constant HA concentration of 0.4 mg dm^{-3} ($t_{\text{acc}} = 180 \text{ s}$), increased linearly with the slope of $11.51 \pm 0.08 \text{ nA mol}^{-1} \text{ dm}^3$ in the entire examined concentration range, while the peak potential remained constant.

3.2. Estimation of the redox process mechanisms

Reduction process mechanisms and electrochemical characteristics of Co (II) complexes described above, were examined by SWV and CV. Their reduction peak current and potential on SW frequency and SW amplitude and CV scan rate dependencies, were measured [31,32]. Voltammograms were measured with $\gamma_{\text{HA}} = 0.9 \text{ mg dm}^{-3}$, $c_{4\text{NC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$ and $c_{\text{Co}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$.

As SWV technique discriminate faraday and capacitive current, and provide an insight into both half-electrode reactions, it is particularly suitable for studying the mechanisms of electrode processes of investigated Co (II) complexes. The SWV response separation into forward current, measured before the “down” pulse and backward, reverse current measured at the “down” pulse of staircase, showed their reduction characteristics [33]. SW forward-backward (f-b) scans of both, Co(II)–4NC and Co(II)-HA complex reduction at about -1.05 V showed that processes are totally irreversible. CV voltammograms showing total irreversibility of both complexes, as on the anodic side oxidation peaks were not registered are presented in insets of Figs. 1 and 2.

SW peak current and peak potential (E_p) variation with f , provide an important tool for distinguishing electrochemical mechanisms [36,37]. The Co(II)–4NC reduction peak current of at pH 6.5 ($E_p \approx -1.05 \text{ V}$, $t_{\text{acc}} = 180 \text{ s}$) depends on SW frequency linearly within range (8 – 100) s^{-1} , with the slope of $-4.64 \pm 0.11 \text{ nAs}$. The Co(II)–4NC complex peak potential dependence at pH 8.2, $t_{\text{acc}} = 180 \text{ s}$, on $\log f$ was linear, with the slope of 10.4 mV/d.u [35,36].

Co(II)-HA complex reduction process is irreversible as well. Namely, the reduction peak current at pH 8.2, $t_{\text{acc}} = 180 \text{ s}$ depend on SW frequency within range 8 – 300 s^{-1} linearly, with the slope of $0.67 \pm 0.05 \text{ nAs}$. Additionally, the Co(II)-HA complex reduction peak potential dependence on $\log f$ was linear (slope -20.3 mV/d.u.) confirming the assumption of the Co(II)-HA complex reduction irreversibility.

Linear dependence of the reduction current on the scan rate (CV) with the shift of the reduction potential toward negative values and the linear dependence of E_p on $\log f$, are characteristic to completely irreversible reduction processes with the reactant adsorption [34–37]. The Co(II)–4NC complex reduction current increased linearly with the scan rate enhancement, with the slope of $0.075 \pm 0.02 \text{ nA/V}$ [34], while the Co(II)-HA complex reduction current increased linearly with the slope of $0.1 \pm 0.05 \text{ nA/V}$. Both complexes reduction currents increased exponentially with $v^{1/2}$. Complex peak potentials moved toward negative potentials with the scan rate increase. In addition, no oxidation peak was observed on the anodic side, suggesting a completely irreversible reduction process followed by the complex dissociation. Reactant adsorption influence on totally irreversible redox SW voltammograms analysis, showed that the reduction peak width at half-height, satisfied the relationship $\Delta E_{p/2} \text{ (mV)} = (63.5 \pm 0.5)/\alpha n$, where n is the number of simultaneously transferred electrons and α is the average transfer

coefficient [38,39]. The obtained peak width at half-height of Co(II)–4NC complex was $E_{p/2} = 60$ mV and relates to $\alpha = 0.67 \pm 0.03$, while for Co(II)-HA with $E_{p/2} = 45$ mV to $\alpha = 0.35 \pm 0.01$, taking into account that the reduction of both complexes are two-electron processes.

Furthermore, all reduction reactions depended on SW amplitude [34,39–41]. Co(II)–4NC complex reduction peak current dependence on SW amplitude was linear with the slope of $((\Delta i_p / \Delta a)_{a \leq 25}) = 1.1059 \times 10^{-3} \text{ A V}^{-1}$, while for Co(II)-HA complex $((\Delta i_p / \Delta a)_{a \leq 25}) = 1.623 \text{ A V}^{-1}$. According to equation (1):

$$X = \frac{K_{\text{Co(4NC/HA)}_n}^{\text{cond}} [4\text{NC} / \text{HA}']_n}{(K_{\text{Co(4NC/HA)}_n}^{\text{cond}} [4\text{NC} / \text{HA}']_n + K_{\text{CoOHNTA/EDTA}} [\text{NTA} / \text{EDTA}'])} \quad (4)$$

$$i_p = (5 \pm 1) \times 10^2 q a n^2 F a \Delta E \Gamma \quad (1)$$

where the amount of the adsorbed reactant (Γ) was calculated from the slope i_p/a using values $\alpha_{\text{Co(II)-4NC}} = 0.67$ and $\alpha_{\text{Co(II)-HA}} = 0.35$, $n = 2$ (number of electrons involved in the reduction process) and $q = 0.004 \text{ cm}^2$ (the surface of the mercury drop), $F = 96485 \text{ s A/mol}$ (Faraday constant) and ΔE (square-wave scan increment). Calculated maximum concentration of adsorbed complexes for Co(II)–4NC complex was $\Gamma = 1.70 \times 10^{-12} \text{ mol cm}^{-2}$, while for Co(II)-HA complex $\Gamma = 5.11 \times 10^{-12} \text{ mol cm}^{-2}$.

The peak width at half height remained constant with SW amplitude change, which is characteristic of completely irreversible reduction processes with the reactant adsorption [37,40–42].

3.3. Determination of conditional stability constant of Co-ligands complex

Co (II) complexes with organic ligands stability constants described in the literature, are $\log K > 16.8$ and are significantly higher than those for other transition metals, such as iron ($\log K > 13.1$) [17]. Conditional stability constants and apparent stoichiometry of described Co(II) complexes was determined by CLE/ACSV method [43]. Competition between investigated ligands and well characterized complexes with EDTA and NTA, was applied. The method was described as competitive ligand equilibrium (CLE) followed by adsorptive cathode stripping measurements (ACSV). The process consists of two stages. First one was equilibrium completion between known quantity of competitive ligand (EDTA, NTA) and investigated ligand. The second phase is voltammetric (ACSV) determination of the Co (II) -complex concentration left after competitive equilibrium. Although, obtained stability constants are conditional and model derived, this characterization provide a good assessment of the complexation degree.

Nitrilotriacetic acid (NTA) was used as competing ligand for the Co(II)–4NC complex (reduced at -1.10 V) at pH 8.2, while etilendiamintetraacetic acid (EDTA) for Co(II)-HA reduced at $E_p = -1.05$ V. The mass balance for Co (II) can be described as follows [37]:

$$c_{\text{Co}} = [\text{Co}'] + [\text{Co(4NC / HA)}_n] + [\text{Co NTA / EDTA}] \quad (1a)$$

where $[\text{Co}']$ is the concentration sum of all inorganic species (Co(OH)^+ and Co(OH)_2) and Co(4NC/HA)_n (complex of Co^{2+} with n molecules of ligand; n can be 1 or 2), $[\text{Co(II)NTA/EDTA}]$ represents the concentration quantity of all present Co(II) species with NTA/EDTA at given pH. The peak current was directly related to $[\text{Co(4NC/HA)}_n]$ complexes through the proportionally factor S :

$$i_p = S[\text{Co(4NC/HA)}_n] \quad (2)$$

The ratio, X , of cobalt peak current in presence of NTA/EDTA ($i_{p,i}$) to one without it, ($i_{p,o}$) can be written as:

$$X = \frac{i_{p,i}}{i_{p,o}} = \frac{[\text{Co(4NC/HA)}_n]}{[\text{Co(4NC/HA)}_n] + [\text{CoNTA / EDTA}^-]} \quad (3)$$

Using expressions for $[\text{Co(4NC/HA)}_n]$:

Instead of free, $[\text{HA}']$, total HA concentration is used, as its concentration was much higher than that of cobalt, while the free EDTA concentration, $[\text{EDTA}']$, was computed from expression:

$$[\text{NTA} / \text{EDTA}'] = C_{\text{NTA/EDTA}} - (1 - X)C_{\text{Co}} \quad (5)$$

$c_{\text{Co}} = 4 \times 10^{-5} \text{ mol dm}^{-3}$, $c_{4\text{NC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$ solutions at pH 6.5 and 8.2 were titrated with NTA within concentration range $0.25\text{--}3 \times 10^{-5} \text{ mol dm}^{-3}$. Co(II)–4NC complex reduction peaks were measured after titration and X was calculated for each titration point.

It was assumed that complex formed at the electrode surface was Co:4NC with 1:2 ratio, as calculated curve agreed well with our experimental data (Fig. 3A). Conditional stability constant was calculated from the mean value of experimentally obtained points: $\log K_{\text{Co(II)4NC2}} = 21.86$ (pH = 8.2), $\log K_{\text{Co(II)4NC2}} = 21.11$ (pH = 6.5).

3B. Conditional stability constant of Co(II)-HA complex determination using CLE-voltammetric method (pH = 8.2): (●) experimental values; (–) calculated data; (...) confidence interval; (–) prediction interval.

Co(II)-HA stability constants for distribution calculations were taken from the literature, $\log K_{\text{CoHA}} = 5.03$, $\log K_{\text{Co2(HA)}} = 2.8$ (pH = 3.5) [36]. Taking into account these constants, Co_2HA complex was predominant species within pH range 2–5, and Co(II)-HA is present from 5 to 9.5, while Co(II) hydrolysis takes place at pH values about 9.5. Within pH range between 10 and 12 Co(OH)_2 is predominant species, while Co(OH)_3 in the pH range 12–14 [43].

The experiment competing reaction with Co(II)-HA at $E_p = 1.05$ V was performed with EDTA. Titration was performed with $\gamma_{\text{HA}} = 0.9 \text{ mg dm}^{-3}$, $c_{\text{Co}} = 4 \times 10^{-5} \text{ mol dm}^{-3}$ in the EDTA concentration range from 4×10^{-6} to $3 \times 10^{-5} \text{ mol dm}^{-3}$. It was assumed that complex formed at the electrode surface was Co:humate with ratio 1:2, as calculated curve agreed well with our experimental data (Fig. 3B). The conditional stability constant calculated from the mean value of experimentally obtained points amount to $\log K_{\text{Co(II)HA2}} = 11.32$.

3.4. UV–vis spectrophotometric characterization of Co(II)–4NC and Co(II)-HA complexes

The Co(II) complexes formation and stoichiometry was also examined by UV–Vis spectrophotometry. UV–Vis spectrophotometric ligands (4NC or HA) with Co(II) titrations, were performed in order to determine discrete Co(II) complexes stability constants and stoichiometry in model water solutions.

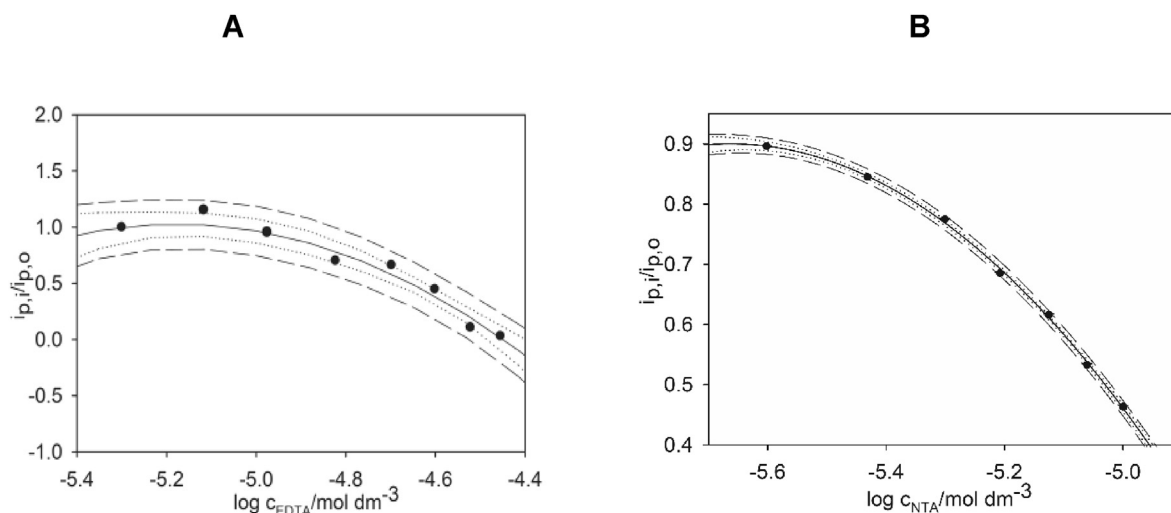


Fig. 3. A. Conditional stability constant of Co(II)–4NC complex determination using CLE-voltammetric method (pH 8.2): (●) experimental values; (–) calculated data; (···) confidence interval; (– –) prediction interval.

Catechol (1,2-dihydroxybenzene) is a weak diprotic acid, where the acidity of hydroxyl groups is enhanced due to nitro substituent. pKa value of 6.7 for 4NC was determined by means of the pH spectrophotometric UV–Vis titration, which was in good agreement with previously determined pK values [44,45].

4NC titration with cobalt (II) was followed by UV–Vis technique at pH = 6.5 and pH = 8.2 in order to test complexation with protonated and deprotonated 4NC form in solution. HA titration with cobalt (II) was performed only at pH = 8.2, at which HA was completely deprotonated. Set of 4NC solutions was prepared (10 solutions at pH 6.5; 9 solutions at pH 8.2). 4NC concentration was the same in all solutions ($1 \times 10^{-5} \text{ mol dm}^{-3}$ at pH 6.5; $2 \times 10^{-5} \text{ mol dm}^{-3}$ at pH 8.2), while cobalt (II) concentration varied ($2 \times 10^{-6} \text{ mol dm}^{-3}$ – $1 \times 10^{-4} \text{ mol dm}^{-3}$). Similarly, set of 15 HA solutions were prepared; HA concentration was constant ($1.5 \times 10^{-6} \text{ mol dm}^{-3}$), while cobalt (II) concentration varied in the range from $5 \times 10^{-8} \text{ mol dm}^{-3}$ to $5 \times 10^{-4} \text{ mol dm}^{-3}$. Constant pH was controlled by borate buffer and solutions were equilibrated overnight. UV–Vis spectra were analyzed by multivariate, non-

linear, least squares regression analysis using Specfit program [23–25].

Significant bathochromic shift (>80 nm at pH 6.5, >60 nm at pH 8.2) occurred as a result of Co(II)–4NC complex formation. Specfit calculations confirmed the Co (II) –4NC complex formation. As a result of analysis, for both acidic and alkalic conditions, the best fitting model describing the experimental data was formation of one complex form, with stoichiometry 1:1 [Co(II)–4NC]. The 1:2 complex content was too low to fit the data (Fig. 4, Fig. 5). Stability constant $\log K_{\text{Co(II)4NC}} = 5.76 \pm 0.40$ was calculated for [Co(II)–4NC] complex at pH 6.5. Calculated Co(II)-complex and ligand electronic spectra and corresponding distribution diagram are presented in Fig. 4B.

Stability constant $\log K = 3.98 \pm 0.17$ was obtained at pH 8.2, indicating a lower affinity of 4NC for Co(II) in weakly alkalic conditions (Fig. 5A). Calculated Co(II)-complex electronic spectra and corresponding distribution diagram are presented in Fig. 5B.

As a result of Co(II)–HA complexation, new absorption band occurred at 300 nm. As previously described, Specfit calculations

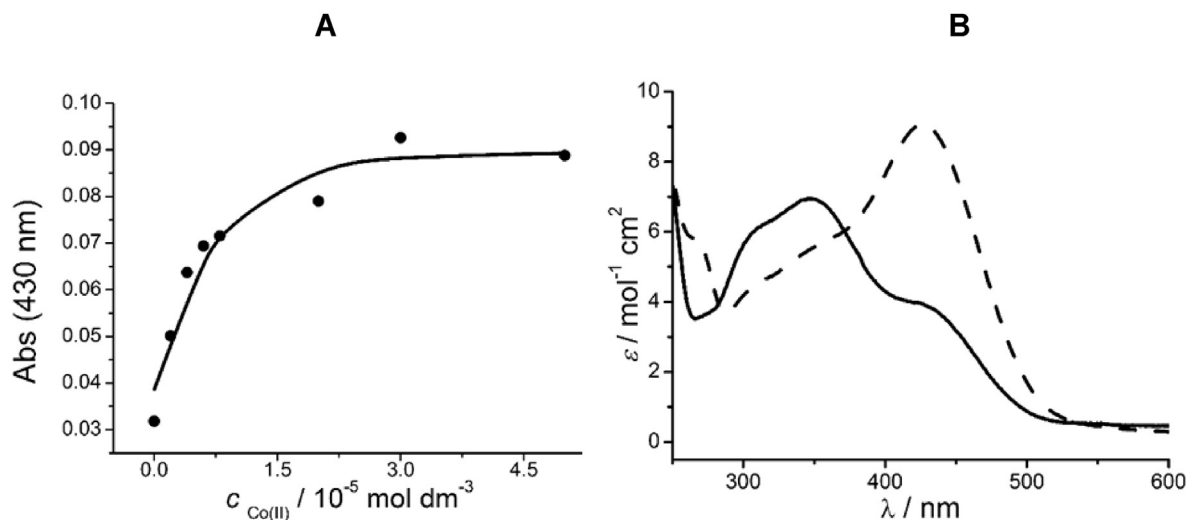


Fig. 4. A) Experimental (●) and calculated (–) UV–Vis spectra at $\lambda = 430 \text{ nm}$ of 4NC recorded as a function of Co(II) concentration. B) Calculated electronic spectra of 4NC (–) and [Co(II)–4NC] complex (– –). $c_{4\text{NC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, pH 6.5 ± 0.05 , $I = 0.55 \text{ mol dm}^{-3}$ (NaCl).

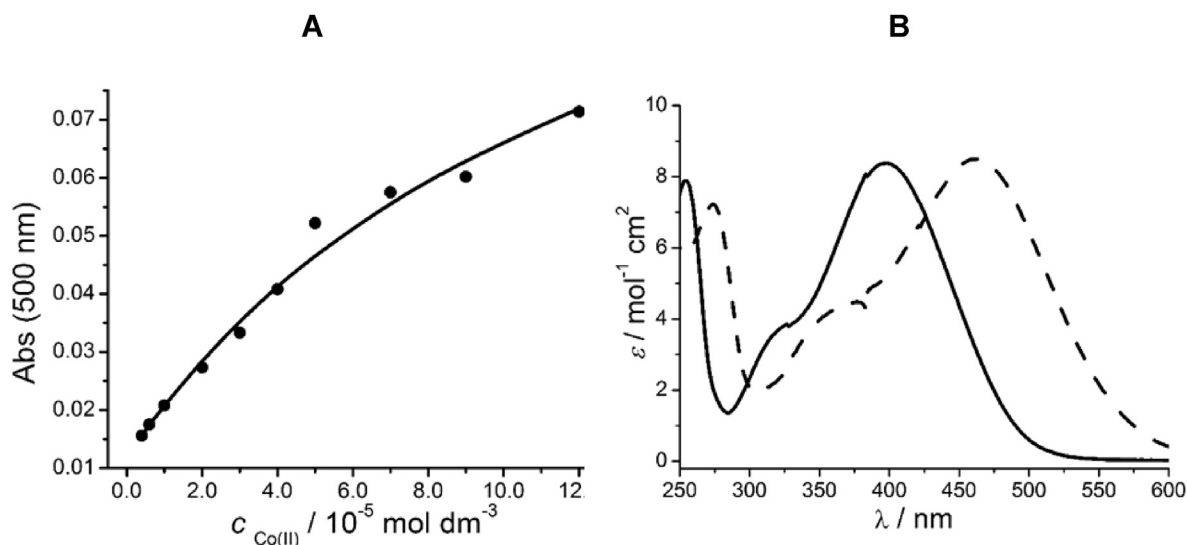


Fig. 5. A) Experimental (●) and calculated (–) UV–Vis spectra at $\lambda = 500 \text{ nm}$ of 4NC recorded as a function of Co(II) concentration. B) Calculated electronic spectra of 4NC (–) and [Co(II)–4NC] complex (– –). $c_{4\text{NC}} = 2 \times 10^{-5} \text{ mol dm}^{-3}$, $\text{pH} = 8.20 \pm 0.05$, $I = 0.55 \text{ mol dm}^{-3}$ (NaCl).

approved formation of [Co(II)-HA] complex registered in voltammetric measurements, (Fig. 6A and B). The best-fitting model described formation of one type 1:1 complex, with $\log K = 3.80 \pm 0.29$ stability constant.

4. Conclusion

Cobalt speciation understanding is critical for its bioavailability evaluation in dynamic marine environment, involving living cells, aquatic and sediment compartments. Co(II) complexes have an important role in numerous biological processes, and therefore, a clear description of their chemistry is of substantial relevance.

Cobalt (II)–4NC and cobalt (II)–HA complexes characterization, using square-wave and cyclic voltammetry, in combination with UV–Vis spectrophotometry, were performed. Voltammetric measurements in model aqueous solutions of cobalt (II) with 4NC and HA as ligands, revealed two-electron adsorption controlled

irreversible reduction processes, followed by dissociation (EC process). Well defined reduction peaks were detected within pH range 4.5–9.5. Regarding Co(II) complexes with 4NC and HA by analysing their voltammetric characteristics, it was assumed that the crucial atom for the Co(II) complexes formation in both ligands is nitrogen, since Co (II) with catechol did not give voltammetric signal.

Micronutrient complexes stability constants determination and their speciation are crucial to presume their behavior in the marine ecosystem. Dissolved Co(II) complexes stability constants were determined spectrophotometrically with stoichiometry 1:1 ($\log K_{\text{Co(II)4NC}} = 3.98$ (pH = 8.2), $\log K_{\text{Co(II)4NC}} = 5.76$ (pH = 6.5) and $\log K_{\text{Co(II)HA}} = 3.80$ (pH = 8.2)). High stability constants of Co(II)–4NC₂ and Co(II)–HA₂ complexes formed only at the mercury drop electrode surface, ($\log K_{\text{Co(II) (4NC)}_2} = 21.86$ (pH = 8.2), $\log K_{\text{Co(II) (4NC)}_2} = 21.11$ (pH = 6.5), $\log K_{\text{Co(II) (HA)}_2} = 11.32$ (pH = 8.2)), were calculated using CLE/ACSV method. As hydrophobic species with stoichiometry Co:L = 1:2, they enter into biological structures

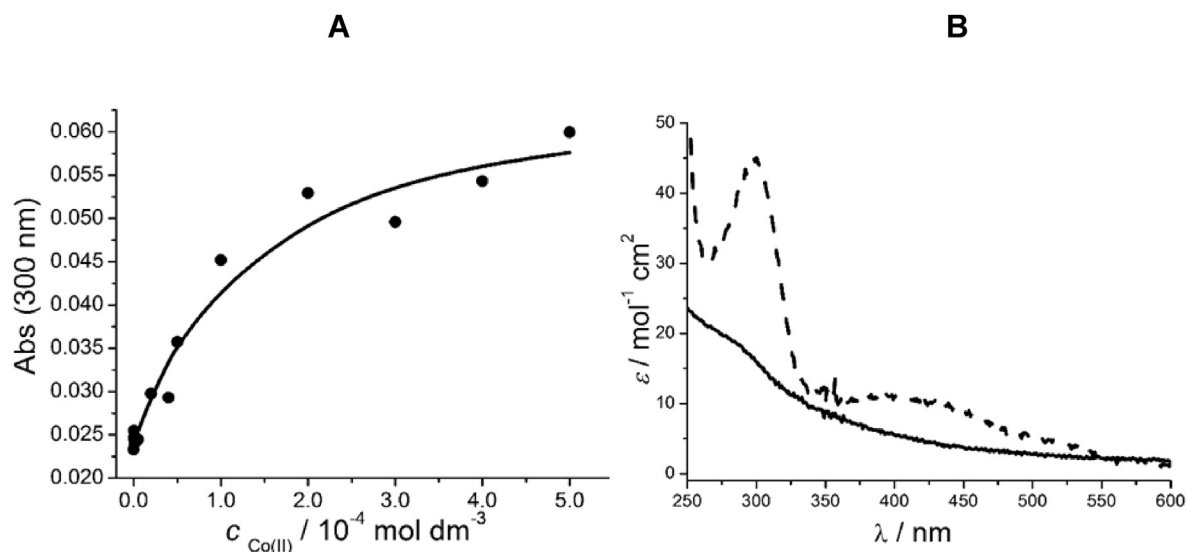


Fig. 6. A) Experimental (●) and calculated (–) UV–Vis spectra at $\lambda = 300 \text{ nm}$ of HA recorded as a function of Co(II) concentration. B) Calculated electronic spectra of HA (–) and [Co(II)-HA] complex (– –). $c_{\text{HA}} = 1.5 \times 10^{-6} \text{ mol dm}^{-3}$, $\text{pH} = 8.20 \pm 0.05$, $I = 0.55 \text{ mol dm}^{-3}$ (NaCl).

through the hydrophobic phospholipid membrane [46].

CRediT authorship contribution statement

Andela Baćinić: Methodology, Investigation, Validation, Data curation, Writing - original draft. **Lidija-Marija Tumir:** Investigation, Data curation, Writing - original draft. **Marina Mlakar:** Conceptualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

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3.2. Publikacija II

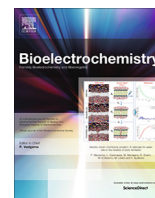
Naslov: A study of cobalt (II) complexes involved in marine biogeochemical processes: Co(II)-1,10-Phenanthroline and Co(II)-1,10-Phenanthroline-L- α -Phosphatidylcholine

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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 $(1.45 \pm 0.12) \times 10^{-10}$ mol. cm^{-2} . Conditional stability constants $\log K_{\text{Co(II)Phen2PC}} = 23.02 \pm 0.26$ and $\log K_{\text{Co(II)Phen2PC2}} = 29.31 \pm 0.17$ ($I_c = 0.55$) 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 $K_1 = 2.4 \times 10^{-2}$ $\text{m}^3 \text{mol}^{-1}$, while for Co(II)-Phen-PC $K_2 = 4.86 \times 10^{10}$ $\text{m}^2 \text{mol}^{-1}$.

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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 ($\text{C}_{12}\text{H}_8\text{N}_2$, 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 kinks into the hydrocarbon chains and make

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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 adsorbed at model hydrophobic interfaces, such as 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, $\geq 99\%$ (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 (C₁₂H₈N₂, 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 (1.69×10^{-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 multi-mode 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 mm²) 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-Feinttechnik*, Germany), equipped with two movable barriers. Surface pressure was performed at 21 °C with a precision ± 0.01 mN m⁻¹ using a Wilhelmy plate (*Whatman* filter paper, No. 1) as pressure sensor. On the initial surface area ($A_{max} = 160$ cm²), the PC monolayer was formed by carefully applying aliquots ($V = 0.02$ cm³) 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 cm² 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 $\pi = 0$ 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 $pK_a \sim 4.9$. 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 (1×10^{-6} mol dm⁻³) with Co(II) in the range from 1×10^{-7} to 1×10^{-6} mol dm⁻³, the reduction current of the complex ($E_p \approx -1.0$ V) increased until $c_{Co} = 8 \times 10^{-7}$ 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

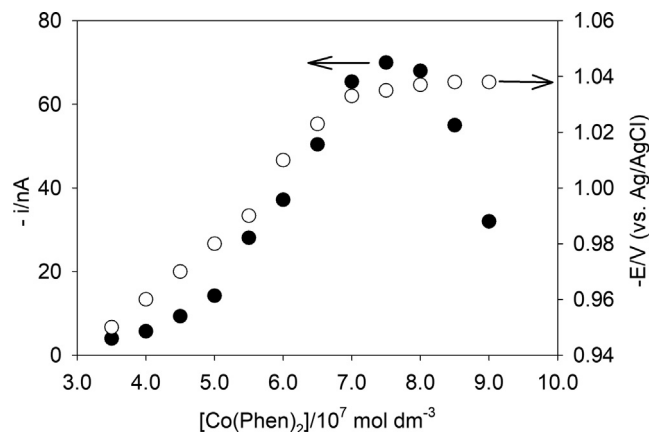


Fig. 1. Dependence of (●) peak current and (○) peak potential of the Co(Phen)₂ complex on c_{Co} in range from 1×10^{-7} to 1×10^{-6} mol dm⁻³; $c_{\text{Phen}} = 1 \times 10^{-6}$ mol dm⁻³, $E_{\text{acc}} = 0$ V, $t_{\text{acc}} = 180$ s, $f = 50$ s⁻¹, $\alpha = 25$ mV.

inhibition. The reduction potential of Co(II)-Phen changed slightly towards negative values (Fig. 1).

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 $E_{\text{acc}} = -1.3$ V for 3 min or more, it was calculated that by SWV measurable Co(II) concentrations would be down to 10^{-10} mol dm⁻³.

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

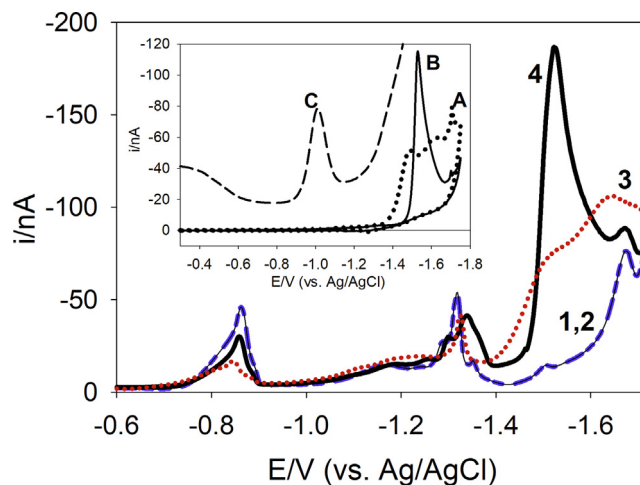


Fig. 2. SW voltammograms of $c_{\text{PC}} = 1 \times 10^{-5}$ mol dm⁻³, $c_{\text{Phen}} = 10^{-6}$ mol dm⁻³, $c_{\text{Co}} = 5 \times 10^{-7}$ mol dm⁻³; 1) PC + Co, 2) PC + Co, 3) Phen + PC, 4) Phen + PC + Co; $E_{\text{acc}} = -0.3$ V, $t_{\text{acc}} = 60$ s, $E_{\text{step}} = 2$ mV, $\alpha = 25$ 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; $c_{\text{Phen}} = 10^{-6}$ mol dm⁻³, $c_{\text{Co}} = 1 \times 10^{-6}$ mol dm⁻³, $E_{\text{acc}} = -0.3$ V, $t_{\text{acc}} = 180$ s, $v = 50$ mV s⁻¹; 0.55 mol dm⁻³ NaCl, pH = 8.2.

(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 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.

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 $c_{\text{Phen}} = 1 \times 10^{-6}$ mol dm⁻³, $c_{\text{Co}} = 1 \times 10^{-6}$ mol dm⁻³ pH = 8.2 for Co(II)-Phen complex. Mixed ligand complex measurements were done with $c_{\text{PC}} = 1 \times 10^{-5}$ mol dm⁻³, $c_{\text{Phen}} = 10^{-6}$ mol dm⁻³, $c_{\text{Co}} = 5 \times 10^{-7}$ mol dm⁻³ at $E_{\text{acc}} = -0.3$ V with $t_{\text{acc}} = 180$ 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. 4A), while of Co(II)-Phen-PC mixed ligand complex completely irreversible (Fig. 4B).

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 nA V⁻¹, while with $A > 50$ mV reduction peak splits in two what is characteristic for reversible reduction processes from adsorbed state [29]. 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 ± 0.02 nA V⁻¹, and shift of the reduction potential towards negative values, is characteristic for reversible reduction processes with the reactant adsorption [16].

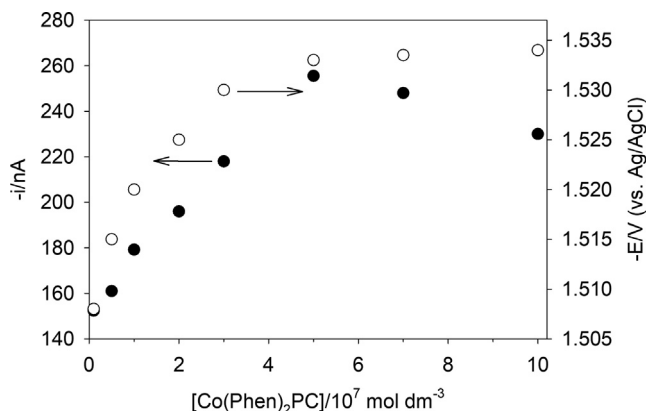


Fig. 3. Dependence of (●) peak current and (○) peak potential of the Co-Phen-PC complex on c_{Co} in range from 1×10^{-9} to 1×10^{-6} mol dm^{-3} . $c_{Phen} = 1 \times 10^{-6}$ mol dm^{-3} , $c_{PC} = 1 \times 10^{-5}$ mol dm^{-3} ; $E_{acc} = -0.3$ V, $t_{acc} = 180$ s, $f = 50$ s $^{-1}$, $a = 25$ mV.

Impact of the SW frequency and amplitude on the net reduction current (i_p) and potential (E_p) of Co(II)-Phen-PC complex was investigated as well. Co(II)-Phen-PC complex reduction current ($E_p \sim -1.5$ 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 A V $^{-1}$.

By reviewing the dependence of the reduction current of the Co(II)-Phen-PC complex by CV technique 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) = $(63.5 \pm 0.5)/\alpha n$, where $n = 2$ (number of simultaneously transferred electrons) and where α is the average transfer coefficient [29,30]. From the experimental data calculated peak width at half-height of Co(II)-Phen was $E_{p/2} = 73$ mV and relates to $\alpha = 0.5 \pm 0.05$, while for Co(II)-Phen-PC complex was $E_{p/2} = 85$ mV and relates to $\alpha = 0.37 \pm 0.02$.

According to equation:

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

from the slope i_p/α using values $\alpha_{Co(II)-Phen-PC} = 0.37$, $n = 2$ and $q = 0.004$ cm 2 (the surface of the mercury drop), $F = 96485$ s A mol $^{-1}$ (Faraday constant) and $\Delta E = 20$ 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.

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

or for the mixed complex:

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

where $[Co']$ is the sum of concentrations of all inorganic species (Co(OH) $^+$ and Co(OH) $_2$ and Co(Phen) $_n$ Co(Phen $_x$ PC $_y$) $_n$ (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 :

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

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

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

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

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

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

$$X = \frac{K_{CoPhen}^{cond} [Phen]n}{K_{CoPhen2}^{cond} [Phen]n + K_{CoOHNTA} [NTA]n} \quad (8)$$

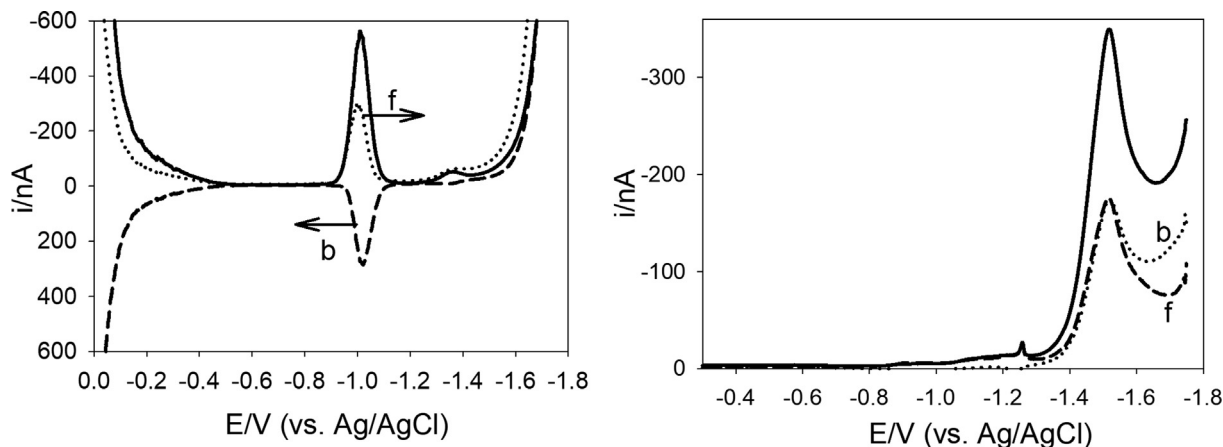


Fig. 4. Backward-forward SW voltammograms of A) Co(II)-Phen; B) Co(II)-Phen-PC complexes; $c_{PC} = 1 \times 10^{-5}$ mol dm^{-3} , $c_{Phen} = 1 \times 10^{-6}$ mol dm^{-3} , $c_{Co} = 10^{-6}$ mol dm^{-3} .

$$X = K_{\text{CoPhenPCy}}^{\text{cond}} [\text{CoPhenPCy}]_n / K_{\text{CoPhenPCy}}^{\text{cond}} [\text{CoPhenPCy}]_n + K_{\text{CoOHPhen}} [\text{Phen}]_n \quad (9)$$

From the mean value of the experimentally obtained values it was calculated $\log K_{\text{Co(II)-1,10-Phen}_2}$ to amount to 22.75 ± 0.48 . Obtained conditional constants of the mixed ligand complex amount to $\log K_{\text{Co(II)Phen}_2\text{PC}} = 23.02 \pm 0.26$ and $\log K_{\text{Co(II)Phen}_2\text{PC}_2} = 29.31 \pm 0.17$. The stoichiometry was determined so that the calculated curve agreed well with our experimental data (Fig. S2 A, B, C).

3.2. Monolayer surface pressure - area measurements

Monolayer studies at the air-water interface were performed to complement the voltammetric conclusions of the mixed ligand complex Co(II)-Phen-PC formation at a model hydrophobic interface. An aqueous solution of 0.55 mol dm^{-3} NaCl (pH = 8.2; 1 mol dm^{-3} borate buffer) was used as pure subphase, while pure subphase with additions of Co(II) ions ($1 \times 10^{-5} \text{ mol dm}^{-3}$), Phen ($1.2 \times 10^{-5} \text{ mol dm}^{-3}$), and/or both Co (II) ions and Phen was further used for penetration experiments. The compression behavior of the PC monolayer on the pure subphase was firstly evaluated (Fig. 4, curve 1). At A_{max} ($>60 \text{ \AA}^2$), PC molecules were in the liquid-expanded state with some degree of cooperative interaction but still not closely packed, as previously well 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 \AA^2 , which is in the range of the previous results. Penetration experiments were further performed during 3 h 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 ($1 \times 10^{-5} \text{ mol dm}^{-3}$) and both Co(II) ions and Phen, are present in subphase (Fig. 5), enables information on the intermolecular interactions within the reference monolayer.

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, $A_{\text{lim}} = (53.0 \pm 0.5) \text{ \AA}^2$, 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 $(64.0 \pm 0.8) \text{ \AA}^2$ 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

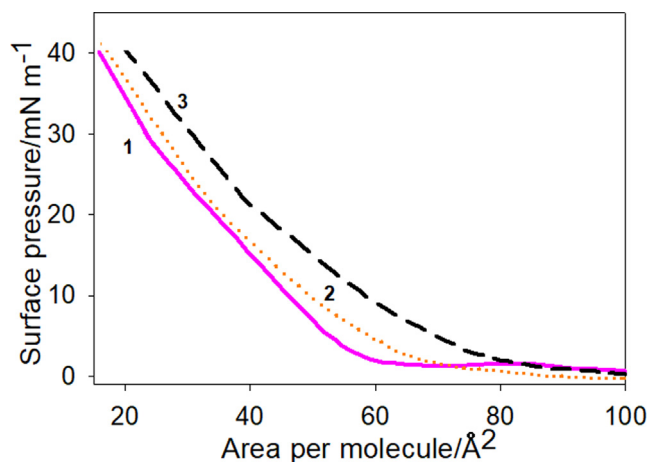
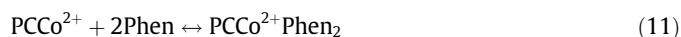


Fig. 5. Surface pressure (π) - area (A) compression isotherms of the PC monolayer on a pure subphase (0.55 mol dm^{-3} NaCl; 1 mmol dm^{-3} borate buffer) (1); pure subphase containing $c_{\text{Co}} 1 \times 10^{-5} \text{ mol dm}^{-3}$ (2); $1 \times 10^{-5} \text{ mol dm}^{-3}$ Co(II) + $1.2 \times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

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

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

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



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

$$K_1 = \frac{c_{\text{PCCo}^{2+}}}{c_{\text{PC}} * c_{\text{Co}^{2+}}} \quad (12)$$

$$K_2 = \frac{c_{\text{PCCo}^{2+}\text{Phen}_2}}{c_{\text{PCCo}^{2+}} * c^2_{\text{Phen}}} \quad (13)$$

$$c_{\text{PC}} * A_{\text{PC}} + c_{\text{PCCo}^{2+}} * A_{\text{PCCo}^{2+}} + c_{\text{PCCo}^{2+}\text{Phen}_2} * A_{\text{PCCo}^{2+}\text{Phen}_2} = 1 \quad (14)$$

$$c_{\text{PC}} + c_{\text{PCCo}^{2+}} + 2c_{\text{PCCo}^{2+}\text{Phen}_2} = C \quad (15)$$

where c_{PC} , $c_{\text{PCCo}^{2+}}$, $c_{\text{PCCo}^{2+}\text{Phen}_2}$ (mol m^{-2}) are the surface concentrations of components PC, PCCo^{2+} , $\text{PCCo}^{2+}\text{Phen}_2$; $c_{\text{Co}^{2+}}$ (mol m^{-3}) is the concentration of Co^{2+} ions; A_{PC} , $A_{\text{PCCo}^{2+}}$, $A_{\text{PCCo}^{2+}\text{Phen}_2}$ ($\text{m}^2 \text{ mol}^{-1}$) are surface areas occupied by 1 mol of components PC, PCCo^{2+} , $\text{PCCo}^{2+}\text{Phen}_2$. K_1 ($\text{m}^3 \text{ mol}^{-1}$) K_2 ($\text{m}^2 \text{ mol}^{-1}$) 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], K_1 , K_2 , A_{PC} , $A_{PCCo^{2+}}$, $A_{PCCo^{2+}Phen_2}$ were calculated. Equations used are shown following instructions from the literature [34]:

$$APC = (-m3)/b \tag{16}$$

$$APCCo = (-m1)/m4 \tag{17}$$

$$APCCoPhen = 2(APC * m4 - APCCo * b)/(m4 - b) \tag{18}$$

$$K_1 = -\frac{2 * APCCo^{2+} - APCCo^{2+}Phen_2}{m_4 * K_2 * A^2 PCCo^{2+}Phen_2} \tag{19}$$

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

The stability constant of the PC-Co system (1:1) was calculated to amount to $K_1 = 2.4 \times 10^{-2} m^3\ mol^{-1}$, while $K_2 = 4.86 \times 10^{10} m^2\ mol^{-1}$ upon the addition of Phen describing Pc-Co-Phen₂ 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 types showed higher stability of the mixed complex than the stability of the Co(II)-Phen or Co(II)-Phen-PC complex.

4. Conclusion

The penetration of cobalt (II) as one of the crucial micronutrient in numerous biological processes, through the cell membrane is important for understanding its fate and role in the marine environment living organisms. Studies of PC adsorbed on hydrophobic surfaces (mercury drop electrode and air–water interface) used as a model for a cell membrane, showed that direct binding between Co(II) and PC is very weak, and not registered by electrochemical measurements. Conditional Stability Constants of the complexes are essential for assessing the ability of ligands to bind micronutrients under actual conditions. The study of the ligand substitution mechanism in the Co(II) coordination sphere enable to predict the micronutrients behavior in biological processes. Co(II) complexation takes place with Phen forming a stable complex with a stoichiometry of 1:2. Co(II)-Phen complex with conditional stability constant $\log K_{Co(II)-1,10-Phen_2} = 22.75 \pm 0.48$. Co(II)-Phen complex as an intermediate interacts with PC molecules by substituting two remained water molecules in its coordination sphere resulting most probably in the stable octahedral configuration. In that way is formed hydrophobic mixed ligand complex Co(II)-Phen-PC. The mixed ligand complex reduction occurs at about $-1.5\ V$ by an irreversible mechanism, followed by dissociation, leaving only PC molecules adsorbed at the electrode surface (EC mechanism). The net stoichiometry of the mixed ligand complexes formed at the mercury drop electrode was calculated to be 1:2:1 and 1:2:2 for Co:Phen:PC. The conditional stability constants of mixed ligand complexes were calculated to amount $\log K_{Co(II)Phen_2PC} = 23.02 \pm 0.26$ and $\log K_{Co(II)Phen_2PC_2} = 29.31 \pm 0.17$ ($I_c = 0.55$). The quantity of

adsorbed reactant (Γ) Co(II)-Phen-PC complex was calculated to be $(1.45 \pm 0.12) \times 10^{-10} mol\ cm^{-2}$. 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 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 $K_1 = 2.4 \times 10^{-2} m^3\ mol^{-1}$. 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 $K_2 = 4.86 \times 10^{10} m^2\ mol^{-1}$. 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioelechem.2021.108009>.

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3.3. Publikacija III

Naslov: Study on the Surface Interactions of Co(II) with Phospholipids from the Marine Environment

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Article

Study on the Surface Interactions of Co(II) with Phospholipids from the Marine Environment

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Abstract: Natural phospholipid (PL)_n extract from cultured green algae *Dunaliella tertiolecta* was used as the main component of cell membranes for studies on the interaction with trace metal Co(II). The phospholipids of the membranes were extracted from other biological components using TiO₂-μSPE cartridges selective for the phosphate group according to a completely new protocol. The interaction of Co(II) with natural and standard phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) are registered only in the presence of the additional chelating ligand 1,10-Phenanthroline (Phen). 1,10-Phenanthroline, as a model of humic substances in the marine environment, formed a neutral complex with Co(II) by the substitution of water molecules of central metal ions. The interaction of hydrophobic mixed-ligand complexes with phospholipids was enabled by the substitution of the remaining water molecules in the coordination shell of Co(II), which was registered by voltammetric measurements. The Co(II)-Phen-PL complex is reduced from the adsorbed state at −1.65 V by the transfer of two electrons, followed by its irreversible dissociation and desorption, indicating an EC mechanism. The interaction between the mixed-ligand complexes Co(II)-Phen-lipids was confirmed by atomic force microscopy (AFM). AFM images of PL, PL with Phen mixture and PL, Phen with Co(II) showed different 3D structures on the mica surface, indicating changes caused by the interaction between cobalt(II), 1,10-Phenanthroline and phospholipids.

Keywords: cobalt(II); 1,10-Phenanthroline; phospholipids; voltammetry; atomic force microscopy



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1. Introduction

Interaction with membrane lipids usually involves divalent metals, micronutrients such as Mg²⁺, Zn²⁺, Fe²⁺, Co²⁺, and Cu²⁺, which are present in trace amounts in seawater but are very important for certain enzymatic reactions. Cobalt is an essential element present in metabolic enzymes necessary for the development of organisms [1]. Vitamin B12, which requires cobalt to function, is involved in many enzymatic reactions [2]. Co(II) tends to bind to electrophilic nitrogen atoms or groups such as amines and imidazoles. As a result, the binding of cobalt (II) to lipids is generally weaker and makes it more difficult to pass through narrow channels in biological membranes [3].

Phytoplankton produce numerous complex biomolecules, including lipids, which are the most important biochemical class of organic matter (OM) in seawater, along with carbohydrates and proteins [4–7]. They are an important cellular component for cell membrane function in physiological processes, energy storage, and trophic interaction in aquatic food webs. Global climate changes have a profound effect on lipids in coastal and estuarine waters and on lipid composition, which in turn affects cell integrity and physiological performance [8]. Lipid content in phytoplankton is highly dependent on environmental conditions such as CO₂ concentration, temperature, salinity, and nutrient availability [9], with CO₂ being the most important.

Numerous studies have shown that phytoplankton can adapt to new environmental conditions by remodeling lipid production [8,10–12]. The most important group of lipids that build cell membranes are phospholipids, which define the membrane bilayers of almost all living organisms. The main function of the major membrane lipids is to form the permeability barrier of the cell [13]. Within this category, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) are among the most important with respect to algal species in the marine environment [4–6]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are predominantly found in the extrachloroplastic membranes of the cell, while phosphatidylglycerol (PG) is the only phospholipid found in the thylakoid membranes and is involved in the photosynthetic transport of electrons.

Therefore, the aim of this study was to investigate the interaction of standard (model) and extracted natural phospholipids with the micronutrient Co(II). Voltammetric results on the interaction of Co(II) mixed-ligand complexes with standard and natural phospholipids were additionally investigated by atomic force microscopy (AFM) imaging.

2. Materials and Methods

2.1. Chemicals and Solutions

The following reagents were used for experiments: methanol (p.a. Kemika d.d., Zagreb, Croatia), n-hexan (Ph. Eur. Carlo Erba, Emmendinger, Germany), 2-propanol (hypergrade for LC-MS, Merck, Darmstadt, Germany), formic acid (98–100%, Kemikad.d. Zagreb, Croatia), ammonia solution (25%, Merck, Germany), 2,5-dihydroxybenzoic acid (puriss. p.a., Merck, Germany), and matrix substance for MALDI-TOF mass spectrometry (MS). Standard solutions of $\text{Co}(\text{NO}_3)_2$ and $\text{Cu}(\text{NO}_3)_2$ (FlukaChemie GmbH, Buchs, Switzerland) were used in all experiments.

The standard lipids used in our experiments were commercially available: lipid standard of L- α -phosphatidylcholine (PC; egg yolk, type XVIIE, $\geq 99\%$ (TLC), lyophilized powder, Sigma-Aldrich, St. Louis, MO, USA), and L- α -phosphatidylglycerol (PG; Sigma-Aldrich).

The original stock solutions of standard phospholipids (PC, PE and PG) dissolved in methanol, $c = 10^{-2} \text{ mol dm}^{-3}$, were kept at -20°C .

Natural lipid extract was obtained from the green algae *Dunaliella tertiolecta* culture, as described in Gašparović et al., 2015 and Gašparović et al., 2017 [14,15].

2.2. Micro-Solid Phase Extraction

The phospholipids were isolated from the lipid extract of *Dunaliella tertiolecta* by micro-solid phase extraction (μSPE) using Agilent AssayMAP Bravo Platform (St. Clara, CA, USA) with TiO_2 micro cartridges (binding $\geq 90\%$ recovery, 80 μg phenyl phosphate load, Resin Pore Size 100 \AA , pH Stability 1–14, Agilent Technologies, St. Clara, CA, USA). TiO_2 - μSPE cartridges are efficient in the preparation of an analyst sample with a phosphoryl group, such as phosphopeptides and phospholipids, using a chelate bidentate bond between the phosphate group and the TiO_2 surface [16]. Standard solutions for solid phase extraction were prepared by dissolving the lipid standards in an equilibration buffer (2-propanol/hexane 8:2 + 1% formic acid) $c = 10^{-2} \text{ mg ml}^{-1}$, since MALDI-TOF MS showed good resolution for the investigated concentration range. Natural samples in triplets were dissolved in a 500 μL equilibration buffer (the calculated concentration was about $10^{-3} \text{ mg mL}^{-1}$). In our experiment, the cartridges were washed and conditioned with 100 μL $> 99.9\%$ MeOH (Sigma-Aldrich), which was used as the initial buffer, with a flow rate of 100 $\mu\text{L min}^{-1}$. After adding the natural sample, the cartridge was equilibrated with 2-propanol/hexane 8:2 + 1% formic acid in a volume of 100 μL and a flow rate of 60 $\mu\text{L min}^{-1}$. Cartridges were washed twice with the equilibration buffer and four times with MQ water using a flow rate of 30 $\mu\text{L min}^{-1}$ to remove all impurities and chlorophyll from the sample. After washing, phospholipids were eluted (flow rate of 30 $\mu\text{L min}^{-1}$) from the cartridge with 10 mol dm^{-3} ammonium in methanol. All fractions were frozen and stored at -20°C for further analysis—Figure 1.

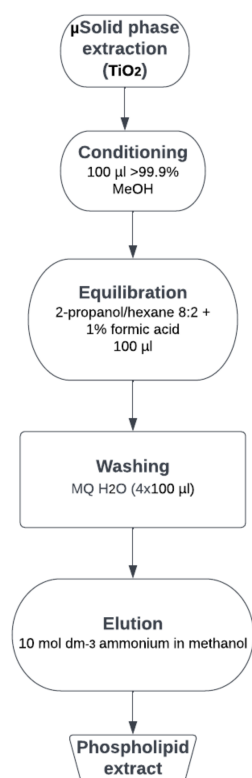


Figure 1. Flowchart of the solid-phase extraction of phospholipids from lipid extract by TiO_2 μ cartridges.

For new analysis, the eluate was lyophilized in the Concentrator 5301 apparatus (Eppendorf, Hamburg, Germany) for 2 h. Dried fractions were diluted in a matrix of 2,5-dihydroxybenzoic (DHB) solution for MALDI measurements (described in following section) and in methanol for other analysis (voltammetry, AFM).

2.3. Mass Spectrometry Analysis

By the MALDI-TOF MS analysis, it was checked whether the process of phospholipids isolation from natural extract was successful. A 4800 MALDI TOF/TOF Analyzer (Applied Biosystems Inc., Foster City, CA, USA) equipped with a 200 Hz, 355 nm Nd: YAG laser was used.

The preparation of MALDI samples is extremely fast and easy to accomplish. Organic solvents are exclusively used, which enhances the homogeneity of the mixture crystallization of the sample with the matrix compound extremely and, thus, the reproducibility of the MALDI-TOF MS measurements is excellent and well-resolved mass spectra were obtained [17]. Imaging was performed in positive ion reflector mode, the laser intensity was fixed at 4000, and the ion mirror was in reflector mode to determine the exact mass with greater precision. The mass range was set from 650 to 900 Da, and the number of shots per spectrum was 1600 and the delay time was 100 ns.

The instrument parameters were set using the 4000 Series Explorer software version (V 3.5.3, Applied Biosystems Inc., Foster City, CA, USA). For calibration of the MALDI-TOF MS, the standard calibration lipids (PG and PE) in the mass range of 300–3000 Da were used.

For the MALDI-TOF MS analysis of the samples, a DHB matrix was used; 5 mg cm^{-3} was prepared by dissolving the matrix in 50:50 acetonitrile (ACN, Sigma Aldrich: milli-Q® water (double-distilled deionized water) (MQ) [18].

The solution was vortexed thoroughly and left to stand at room temperature for several minutes. The dried purified phospholipid samples were diluted in 10 μL of matrix and 1 μL of mixture was spotted onto the MALDI plate to dry. Mass spectra were obtained by averaging 1600 laser shots covering the mass range between m/z 650 and 900. The MALDI spectrum was measured before and after SPE purification.

2.4. Electrochemical Measurements

Voltammetric studies were performed by a μ -AUTOLAB multimode potentiostat (ECO Chemie, Utrecht, The Netherlands) using a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland). The instrument was computer-controlled using GPES 4.9 control software. A static mercury drop electrode (SMDE, size 2, i.e., 0.40 mm²) was the working electrode, and a glassy carbon stick counter electrode and Ag/AgCl (sat. NaCl) (+0.197 V vs. SHE) was used as reference one. Experiments were done in a quartz cell at 25 ± 1 °C in NaCl (Suprapur[®], Merck, Germany) as an electrolyte solution with $I_c = 0.55 \text{ mol dm}^{-3}$ (I_c = ionic strength). Voltammetric methods included alternating current (ACV), out-of-phase mode (tensametry, phase angle 90°), square-wave (SWV), and cyclic voltammetry (CV). Solutions were deaerated by extra pure nitrogen for about 20 min and stirring (3000 rpm). During measurements, nitrogen circulated above the solution. pH was measured by a glass-Ag/AgCl electrode linked to ATI Orion PerpHecT Meter, model 320 (Cambridge, CA, USA). All relevant measurements were repeated at least three times.

For electrochemical experiments, the stock solution of standard phospholipids (PC, PE, PG) and natural phospholipids (PL)_n, $c = 1 \times 10^{-2} \text{ mol dm}^{-3}$, were prepared by dissolution in methanol, as well as PC and PG ($c = 1 \times 10^{-5} \text{ mol dm}^{-3}$). A stock solution of 1,10-Phenanthroline ($c = 1 \times 10^{-2} \text{ mol dm}^{-3}$) was prepared in Milli-Q water.

2.5. Atomic Force Microscopy Imaging

A multimode scanning probe microscope with a Nanoscope IIIa controller (Bruker, Billerica, MA, USA), with a vertical engagement (JV) 125 μm scanner, was used for AFM imaging. The tapping mode using silicon tips (TESP-V2, nominal resonance frequency 320 kHz, nominal spring constant 42 N/m, Bruker) was used in all experiments. To minimize the interaction forces between the tip and the surface during imaging in the tapping mode, the ratio of the set-point amplitude to the free amplitude (A/A_0) was kept at 0.9 (light tapping). The linear scanning rate was optimized between 1.0 and 1.5 Hz with a scan resolution of 512 samples per line. The processing and analysis of images were carried out using NanoScope™ software (Bruker, NanoScope Analysis ver. 2.0). Images are presented as raw data except for the first-order two-dimensional flattening. Measurements were performed in air at room temperature with 30–40% relative humidity to provide a small hydration layer on the sample to enable the conservation of the original molecular structures [19].

For AFM measurements, phospholipides were dissolved in methanol, standard in the concentration of $10^{-5} \text{ mol dm}^{-3}$, while the natural extracted concentration was $10^{-7} \text{ mol dm}^{-3}$. A volume of 5 μL of the phospholipid solution was pipetted directly onto freshly cleaved mica. The thin sheets were placed in closed Petri dishes for 45 min to allow the solvent to evaporate, after which AFM imaging was performed. To obtain AFM images of each lipid with 1,10-Phenanthroline (Phen), dissolved lipid was mixed in a 1:1 ratio with Phen (dissolved in methanol) in a concentration of $10^{-6} \text{ mol dm}^{-3}$.

The same procedure was used in Co(II)-Phen-phospholipid sample preparations, in which the concentration of each component was $10^{-6} \text{ mol dm}^{-3}$ (1:1:1 ratio of Co (II)-Phen-phospholipid).

3. Results and Discussion

3.1. Natural Phospholipids in the Lipid Extract of *Dunaliella Tertiolecta*

Since algae are a well-known source of lipids [20], we extracted the natural phospholipids from the green alga *Dunaliella tertiolecta*. Lipid production is about 30–40% of the *D. tertiolecta* cell biomass [10,12]. To obtain natural phospholipids from extract, first we optimized the micro-solid phase extraction on TiO₂- μSPE . These cartridges were used for lipid isolation from green alga *Dunaliella tertiolecta* for the first time. During the solid phase extraction of natural phospholipids from *D. tertiolecta* extraction described in the Section 2.2, we encountered a problem with the presence of chlorophyll in the eluate, probably because of its amphiphilic properties. Usually, chlorophyll can be removed from the sample with acetone but, in our case, too much of the natural phospholipid was lost. Consequently, by washing the sample with MQ water, the presence of chlorophyll was

reduced to a minimum and a significant yield of the desired compounds was achieved. SPE based on the $\text{TiO}_2/\text{SiO}_2$ composite with an optimized eluent strategy was applied with confirmation of the successful extraction of phospholipids by the MALDI-TOF MS method. The lipid extract spectra were recorded before and after SPE purification (Figure 2). As shown, many signals that correspond to different compounds, as well as many interferences, were recorded in the spectrum of the raw lipid extract (Figure 2a). The complexity of the raw lipid extract can be attributed to the presence of hydrocarbons (HC), fatty acid methyl ester (ME), free fatty acids (FFA), alcohols (ALC), 1,3-diacylglycerols (1,3-DG), 1,2-diacylglycerols (1,2-DG), monoacylglycerols (MG), wax esters (WE), triacylglycerols (TG), and phospholipids (PL), including phosphatidylglycerols (PG), phosphatidylethanolamine (PE), and phosphatidylcholines (PC), as well as glycolipids (GL), which cover sulfoquinovosyldiacylglycerols (SQDG), monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and sterols (ST) [7]. According to the literature, m/z peaks at 676 and 704 were attributed to PE, peaks at 692, 694, 720, 723, 730, 758, 761, and 775 to PC, and 743 and 749 to PG [18,19,21]. After μSPE purification, the spectrum became much cleaner and the non-phospholipid signals were well-reduced (Figure 2b). Results showed that the μSPE cartridge based on a $\text{TiO}_2/\text{SiO}_2$ core-shell composite is very effective in the selective extraction of phospholipids from the natural lipid mixture.

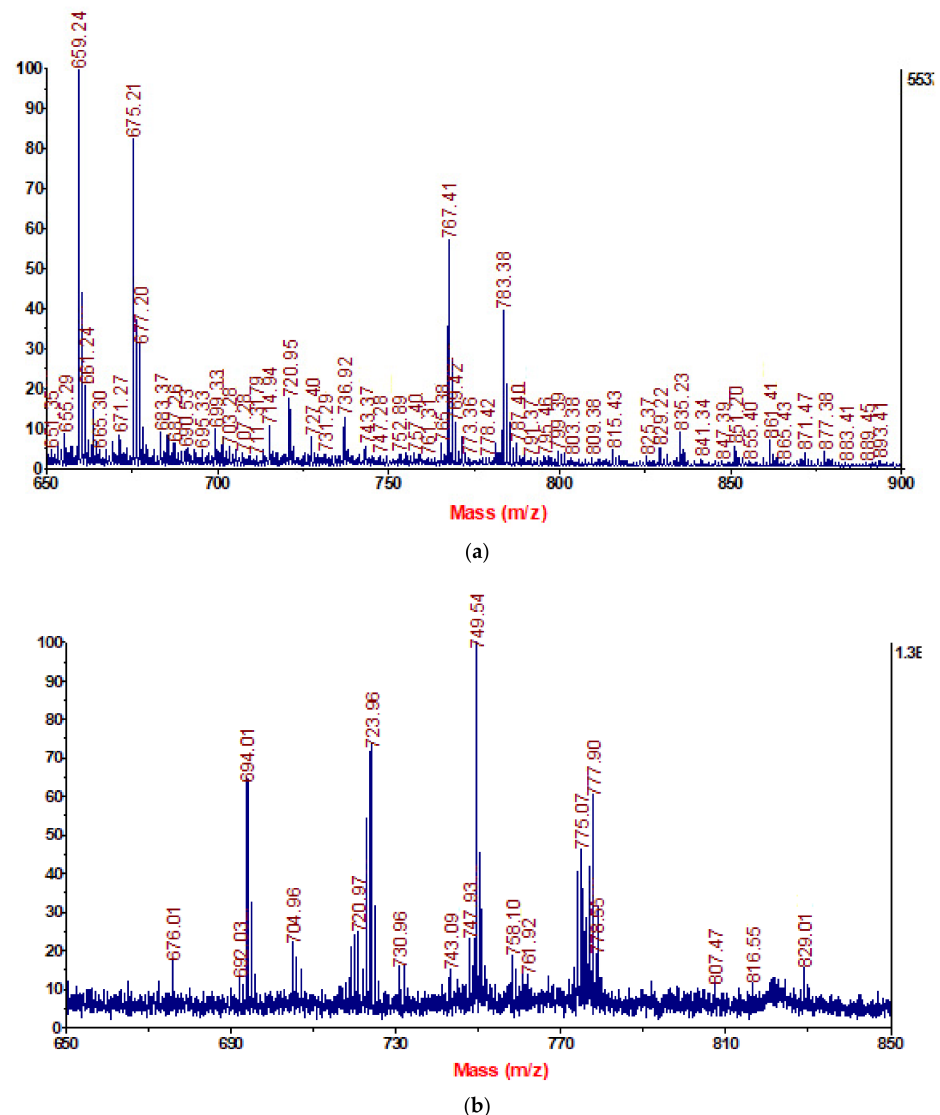


Figure 2. A MALDI-TOF mass spectrum in the positive reflector ion mode (mass range of 600–900 Da) of (a) raw lipid extract from *D. tertiolecta* and (b) phospholipid sample after $\mu\text{SPE-TiO}_2$ protocol.

3.2. Voltammetric Measurements

The interaction of Co(II) with phospholipids was investigated by voltammetric measurements at the mercury drop electrode to mimic interactions at the hydrophobic cell membrane.

Investigations of phospholipid interaction with Co(II) were carried out by square wave (SW), alternating current (AC—tensametry), and cyclic voltammetry (CV). As the SW voltammetric technique discriminates between Faraday and capacitive currents and provides an insight into both half-electrode reactions, it is particularly suitable for studying the mechanisms of complex electrode processes [22].

Before experiments with extracted natural phospholipids, measurements of the interaction of Co(II) with standard phospholipids—phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE)—were carried out. Voltammograms of the standards, a sample of the lipid mixture, and extracted purified phospholipids are presented at Figure 3.

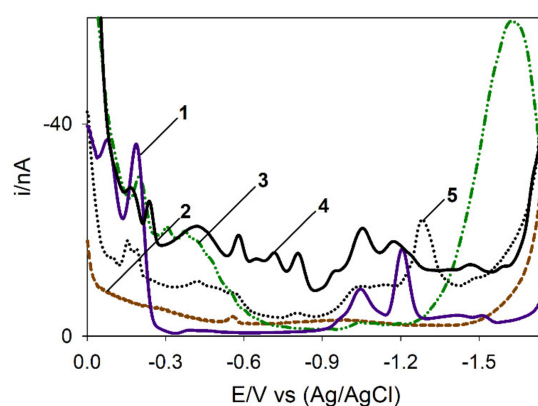


Figure 3. SW voltammograms of (1) PC $c = 10^{-7} \text{ mol dm}^{-3}$, (2) PG $c = 10^{-7} \text{ mol dm}^{-3}$, (3) PE $c = 10^{-7} \text{ mol dm}^{-3}$, (4) mixture of natural lipids, and (5) phospholipids after extraction; $E_{\text{acc}} = 0 \text{ V}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{step}} = 2 \text{ mV}$, $a = 20 \text{ mV}$, $f = 60 \text{ s}^{-1}$, $v = 50 \text{ mV s}^{-1}$; $0.55 \text{ mol dm}^{-3} \text{ NaCl}$, $\text{pH} = 8.2$.

Interaction of Co(II) with 1,10-Phenanthroline and Standard Phospholipids

In the solution of standard phospholipids (either PC, PG and PE), regardless of its concentration, after the addition of Co(II) in the concentration range from 10^{-9} to $10^{-4} \text{ mol dm}^{-3}$, no reduction peak was recorded, indicating that no complex is formed between the metal ions and the mentioned phospholipids. It has been shown that the transport of micronutrients through the lipid membranes of living cells is facilitated by the formation of intermediates. By binding the target ion to the hydrophilic ligand, parts of the water molecules are replaced from the central metal ion and a neutral molecule is formed. In this way, the passage of micronutrient through the lipid membrane is facilitated. In this work, 1,10-Phenanthroline (Phen) was used as a model of natural ligands from the group of humic substances. Phen is good chelating agent for divalent metals. Co(II) forms a complex with Phen that reduces at about $E_p = -1.0 \text{ V}$ [23].

In this work, the interaction of Co(II) with different natural phospholipids was investigated, building on our previous research [24]. Namely, investigations with standard phospholipid showed formation of an irreversible reduction peak of Co(II)-Phen-PC at -1.6 V [24]. As stated for the Co(II)-Phen-PC system in our previous research [24], the order of ligand addition (Phen, PG or PC) to the solution proved to be very important to achieve the desired binding of micronutrients to the lipid surface, that is, the formation of mixed-ligand complexes. Therefore, it was crucial to add both ligands into the electrolyte (Phen and phospholipid). Phen sticks to the phospholipid layer and affects the surface organization of phospholipid molecules. The addition of Co(II) to the solution of both ligands formed a neutral hydrophilic Co(II)-Phen “intermediate” complex in the solution, as a first step, and then, by the accumulation, it interacts with lipid phosphatidyl groups forming a mixed-ligand complex. The reduction signals of the Co(II)-Phen-PC are shown in Figure 4-

inset. The reduction peak of mixed-ligand complex Co(II)-Phen-PG complex was registered at -1.4 V with concentrations of $c_{\text{Phen}} = 5 \times 10^{-7} \text{ mol dm}^{-3}$, $c_{\text{PG}} = 5 \times 10^{-6} \text{ mol dm}^{-3}$, and $c_{\text{Co}} = 2 \times 10^{-6} \text{ mol dm}^{-3}$ (Figure 4-inset.). By titration with Co(II) in the concentration range from 10^{-9} to $10^{-4} \text{ mol dm}^{-3}$, the reduction peak of the complex increased proportionally with the Co(II) addition up to $1 \times 10^{-5} \text{ mol dm}^{-3}$, after which it remained constant because of the saturation of the mercury drop surface. With the variation of the accumulation time from 60–300 s, the reduction current changed significantly, while the reduction potential changed only slightly, as the PG layer was stabilized at the electrode surface.

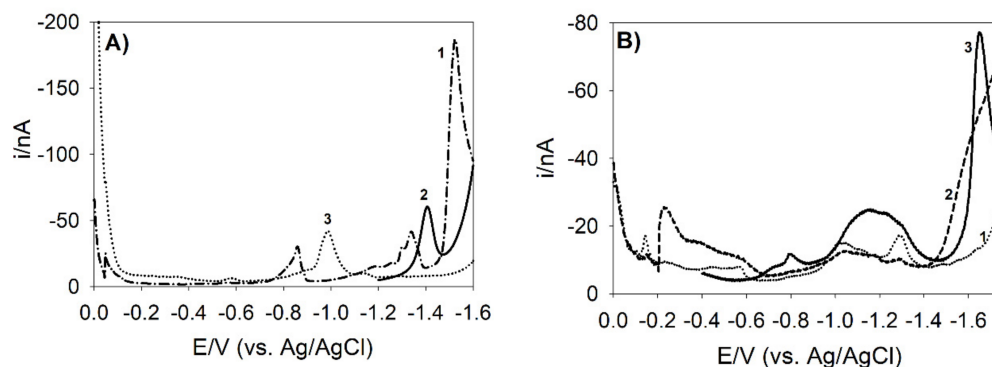


Figure 4. SW voltammograms of standards (A) (1) **Co(II)-Phen-PC** with $c_{\text{PC}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, $c_{\text{Phen}} = 5 \times 10^{-7} \text{ mol dm}^{-3}$, $c_{\text{Co}} = 5 \times 10^{-8} \text{ mol dm}^{-3}$; $E_p = -0.3 \text{ V}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{step}} = 2 \text{ mV}$, $a = 20 \text{ mV}$, $f = 50 \text{ s}^{-1}$; (2) **Co(II)-Phen-PG** with $c_{\text{Co}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, $c_{\text{Phen}} = 5 \times 10^{-7} \text{ mol dm}^{-3}$, $c_{\text{PG}} = 5 \times 10^{-6} \text{ mol dm}^{-3}$; $E_{\text{acc}} = -1.2 \text{ V}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{step}} = 2 \text{ mV}$, $a = 20 \text{ mV}$, $f = 60 \text{ s}^{-1}$, $v = 50 \text{ mV s}^{-1}$; $0.55 \text{ mol dm}^{-3} \text{ NaCl}$, $\text{pH} = 8.2$, (3) **Co(II)-Phen** with $c_{\text{Co}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, $c_{\text{Phen}} = 1 \times 10^{-5} \text{ mol dm}^{-3}$, $E_p = -0 \text{ V}$, $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{step}} = 2 \text{ mV}$, $a = 20 \text{ mV}$, $f = 50 \text{ s}^{-1}$ and natural lipids. (B) (1) **Natural PL+Phen+Co(II)**: $c_{\text{Co}} = 1 \times 10^{-8} \text{ mol dm}^{-3}$, (2) **Natural PL+Phen**: $c_{\text{Phen}} = 1 \times 10^{-8} \text{ mol dm}^{-3}$, (3) **Natural PL extract after SPE** ($c_{\text{PG}} = 4.4 \times 10^{-7} \text{ mol dm}^{-3}$, $c_{\text{PC}} = 2.11 \times 10^{-7} \text{ mol dm}^{-3}$); ($E_{\text{acc}} = -0.4 \text{ V}$); $t_{\text{acc}} = 180 \text{ s}$, $E_{\text{step}} = 2 \text{ mV}$, $a = 20 \text{ mV}$, $f = 60 \text{ s}^{-1}$, $v = 50 \text{ mV s}^{-1}$; $0.55 \text{ mol dm}^{-3} \text{ NaCl}$, $\text{pH} = 8.2$.

The key investigations on Co(II) interaction with extracted natural phospholipide mixture samples by the TiO_2 - μSPE method, described for the first time, were performed. The concentration of natural phospholipids (PL) in the purified mixture were approximately $c_{\text{PG}} = 4.4 \times 10^{-6} \text{ mol dm}^{-3}$ and $c_{\text{PC}} = 2.11 \times 10^{-6} \text{ mol dm}^{-3}$ as determined by thin film chromatography [14]. By the addition of Co(II) ions, ($c_{\text{Co}} = 1 \times 10^{-8} \text{ mol dm}^{-3}$), in the solution of Phen and PL, a peak at about -1.65 V was registered (Figure 4). On the basis of voltammetric results with standard phospholipids, a similar voltammetric response with a natural phospholipid mixture was expected. In the first step of the mentioned process, 1,10-Phenanthroline formed a partially hydrated and hydrophilic uncharged chelate complex with Co(II) ions by substituting part of the water molecules from the Co(II) coordination sphere. PL as a hydrophobic ligand substituted for the remaining water molecules and a mixed-ligand Co(II)-Phen-PL complex was formed and strongly adsorbed at the hydrophobic mercury drop surface. The process was characterized as synergistic adsorption [25,26].

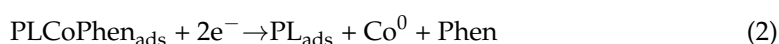
The reduction peak of mixed-ligand complex Co(II)-Phen-PL was registered at -1.65 V as the reduction peak of the Co(II)-Phen-PC (std.) (-1.60 V) [24]. A slight shift of the reduction potential toward more negative values is expected because of the complexity of the natural phospholipid mixture layer. We hypothesized that cobalt will show a stronger binding affinity for phospholipids containing a nitrogen ion (PC).

The dependence of the stripping peak current on the pre-concentration potential was examined in the range from 0 to -1.75 V . A maximum reduction current was obtained with a deposition potential of -0.4 V for Co(II)-Phen-PL. Well-defined reduction signals of the cobalt (II) mixed-ligand complex were registered by the PL molecule stabilization at the

electrode surface, i.e., at the potentials more negative than -0.2 V. By the accumulation time variation from 30 to 300 s, the Co(II)-Phen-PL complex reduction current increased linearly with the slope 18.3 ± 0.07 AV^{-1} , while reduction peak potential changed faintly.

Titration with a Co(II) ion was performed to check the behavior of the mixed-ligand complex reduction at the electrode surface. With the addition of Co(II) in the concentration range from 1×10^{-7} to 1×10^{-4} mol dm^{-3} , the Co(II)-Phen-PL complex reduction current increased up to $c_{\text{Co}} = 1 \times 10^{-5}$ mol dm^{-3} , while with higher concentrations remained constant, as did its reduction potential because of the saturation of the electrode surface with complex molecules.

Therefore, the overall processes for reduction of the Co(II) mixed-ligand complex can be schematically summarized as:



The reduction process of the mixed-ligand complex (2) is totally irreversible, as oxidation does not take place because of its dissociation after Co(II) was reduced to Co^0 .

Furthermore, the reactant adsorption of the irreversible reduction process Co(II)-Phen-PL satisfied the relationship $\Delta E_{\text{p}/2}$ (mV) = $(63.5 \pm 0.5)/\alpha n$, where $n = 2$ (number of simultaneously transferred electrons) and where α is the average transfer coefficient.

From the experimental data, the calculated peak width at the half-height of Co(II)-Phen-PL was $E_{\text{p}/2} = 147.64$ mV and relates to $\alpha = 0.43 \pm 0.05$ [27].

According to Equation (1),

$$i_{\text{p}} = (5 \pm 1) \times 10^2 q \alpha n 2 F \alpha f \Delta E \Gamma \quad (3)$$

from the slope i_{p}/α using values $\alpha\text{Co(II)-Phen-PL} = 0.43$, $n = 2$ and $q = 0.004$ cm^2 (the surface of the mercury drop), $F = 96,485$ s A mol^{-1} (Faraday constant), and $\Delta E = 20$ mV (square-wave scan increment), the amount of the adsorbed reactant (Γ) was calculated. For the Co(II)-Phen-PL complex, the maximum surface concentration adsorbed at the mercury drop electrode was $\Gamma = (1.72 \pm 0.10) \times 10^{-12}$ mol cm^{-2} . If we compare the coverage of the electrode surface of the real sample with the PC standard published in the previous article [24], we can observe that the surface is covered by two orders of magnitude less. That is expected because of the complex composition of the phospholipid mixture sample.

3.3. AFM Characterization of Co(II) Phospholipide Complexes

As previously described, complexes of phospholipids (standard), Phenantrolin, and metals were detected by adsorptive stripping voltammetry at the hydrophobic mercury drop electrode. To confirm the obtained results, a multimode scanning probe microscope with a Nanoscope IIIa controller (Bruker, Billerica, MA, USA) with a vertical engagement (JV) 125 μm scanner was used for AFM imaging. In similar investigations [24], pressure-area (π -A) isotherms at the water-air interface by the Langmuir monolayer technique in a Langmuir-Blodgett trough was applied. However, due to the very small quantities of extracted natural lipid, the Langmuir monolayer technique was not applicable, considering that this technique requires significantly larger amounts of lipids applied to the surface layer of the aqueous solution.

A different structures of lipids (i.e., the formation of vesicles; mono-, bi- and multi-layers, etc.) are usually easily characterized by an atomic force microscope (AFM) because of its exceptional resolution and three-dimensional imaging capability [28–30]. Therefore, AFM imaging was applied to perceive changes in lipid structure that occur because of the addition of Phen and metal, indicating the formation of mixed-ligand complex(es). PC was taken as representative of the standard lipid, while with PG we did not get any significant differences with the addition of ligand and metal in the solution, assuming that this was due to weaker interactions with ligands that do not possess nitrogen in their structure.

The topographic image of PC on the mica surface recorded by AFM is shown in Figure 5a,b. Both amplitude data (Figure 5a) and corresponding height (Figure 5b) images revealed a thick layer of PC, since its concentration in methanol (before methanol evaporation) was very high, i.e., 1 mmol dm^{-3} . PC was spread over almost the entire displayed area of mica, leaving only small holes on its surface. A vertical cross-section shows that the thickness of the PC layers on the mica surface reach about 70 nm in height. By the addition of Phen into the solution of PC (in a 1:1 ratio), AFM imaging revealed a different lipid structure on the mica surface (Figure 5c,d). Due to the electrostatic interaction of Phen and PC, the obtained mixture showed enhanced hydrophobicity and since the mica surface is negatively charged, the PC–Phen mixture formed a vesicle whose size varied from about 40 to 150 nm in height. By the addition of Co(II) into the PC–Phen solution (ratio 1:1:1), a change in the topographic image was registered (Figure 5e,f). In this case, the formation of terraces, as multi-planar ordered surfaces [31], was detected. A vertical cross-section revealed that the height of the terraces varied from the thickness of a single lipid bilayer to the width of several lipid multilayers. A basic comparison of recorded topographic images (in Figure 5a–c) indicates that the Co(II) chemically interacted with a PC–Phen mixture at the mica surface. The influence on hydrophobicity that causes a specific 3D structure of the mixed-ligand complex is evident.

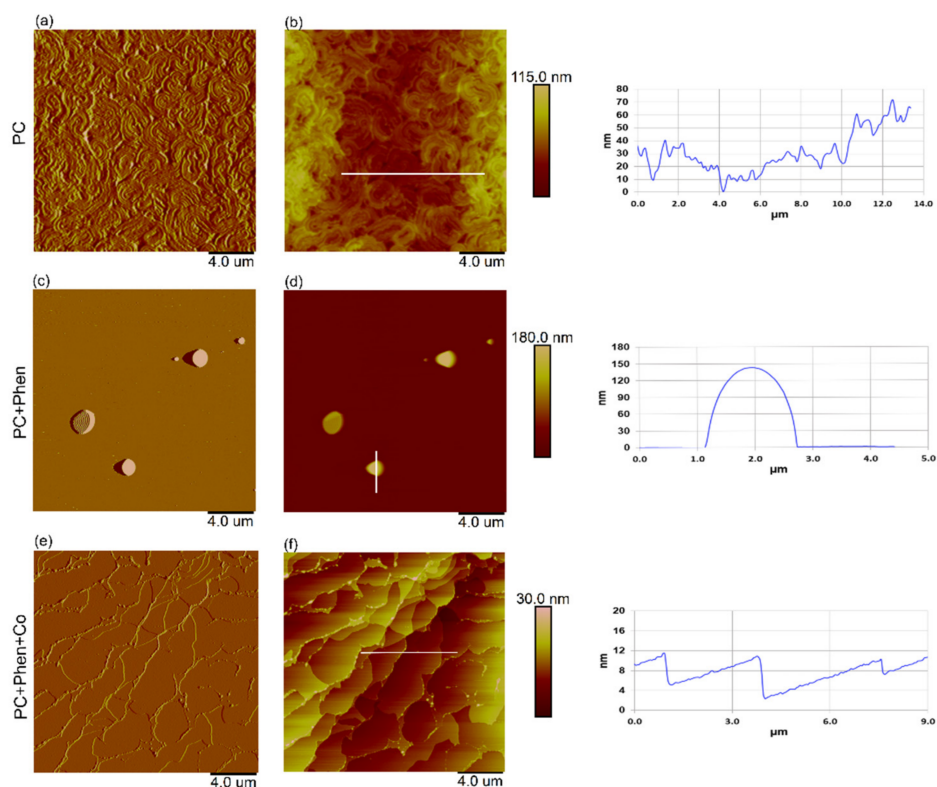


Figure 5. AFM images of PC on mica surface: amplitude data (a) and height (b) of PC alone; amplitude data (c) and height (d) of PC with Phen (1:1); and amplitude data (e) and height (f) of PC, Phen and Co(II) (1:1:1). Heights of cross-sections marked on height images are presented graphically.

Figure 6a,b show topographic images of a natural mixture of phospholipids on the mica surface. Both images show a large area covered by a relatively smooth lipid film in which lipid vesicles were imbedded. The thickness of the smooth film is about 4 nm, which corresponds to the height of the lipid bilayer [32,33], while the vesicle height varied from several nanometers to several tenths of a nanometer. Similar to the topographic images of PC–Phen, in the case of natural lipid and Phen, different-sized vesicles were formed (Figure 6c,d). The addition of Co(II) into the PL–Phen mixture, as in the case of PC,

resulted in detection of different-sized terraces (Figure 6e,f), which confirms the interaction of Co(II)-Phen with PL, presumably forming a mixed-ligand complex.

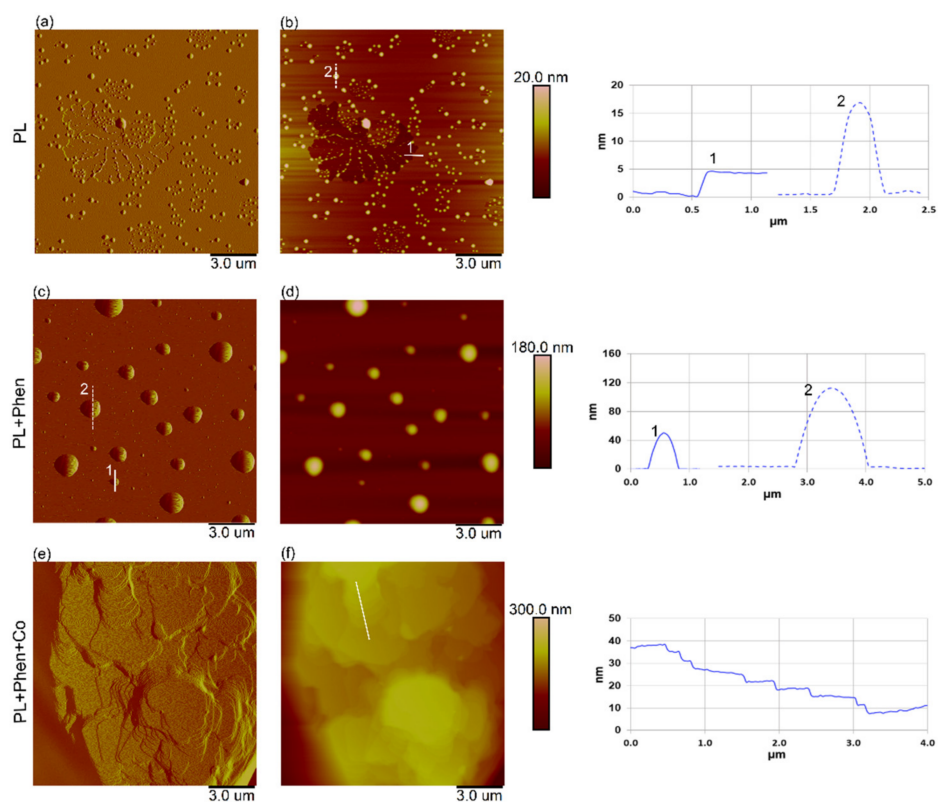


Figure 6. AFM images of natural phospholipid (PL) on the mica surface: amplitude data (a) and height (b) of PC alone; amplitude data (c) and height (d) of PC with Phen (1:1) and amplitude data (e) and height (f) of PC, Phen and Co(II) (1:1:1). Heights of cross-sections marked on height images are presented graphically.

AFM imaging on their basic level showed that interaction of Co(II) with phospholipids and Phenanthroline occurred, confirming results obtained by voltammetric measurements.

4. Conclusions

Phospholipids from the raw lipid extract of the green alga *D. tertiolecta* were separated by the TiO_2 - μSPE method. The results of this study showed that the TiO_2 - μSPE method is suitable and selective for the extraction of phospholipids from the lipid mixtures in real samples. The quality of separation was confirmed by MALDI-TOF analysis. The results showed the presence of the three major membrane phospholipids, PC, PG, and PE. The natural phospholipid mixture/extract and standard phospholipids (PG and PE) were used to study the interactions with the essential trace metal Co (II) by electrochemical measurements. The surface of the mercury electrode mimicked a model for a hydrophobic membrane of living cells. The interaction between Co(II) ions and phospholipids was not demonstrated by the voltammetric techniques used. However, complexes are formed when an intermediate is present in the solution—in our case, a chelating agent: 1,10-Phenanthroline.

When Phen is present in the solution, the formation of mixed-ligand complexes of Co(II) with PG was detected by voltammetric measurements at -1.4 V. When complexes with natural phospholipid samples were examined, a mixed-ligand complex with Co(II) at -1.65 V was detected. It can be assumed that the peak at -1.65 V corresponds to the complex with PC.

AFM images of samples of Co(II)-Phen-PC and Co(II)-Phen-PL systems confirmed that there is an interaction between the metal ions, Phen, and both phospholipids. With

this study, we demonstrated that Co(II) can form mixed-ligand complexes with natural phospholipids with an intermediate to cross the cell membrane. In this way, organisms utilize essential trace metals that penetrate the cell membrane and are involved in numerous important biological processes.

Author Contributions: All authors were responsible for the concept and design of the study, provided critical feedback and helped shape the research. Conceptualization, M.M. and A.B.; methodology, M.M.; software, A.B.; validation, A.B., I.K.; formal analysis, A.B.; investigation, A.B., P.V.; resources, P.V.; data curation, M.M., writing—original draft preparation, A.B.; writing—review and editing, M.M.; visualization, A.B.; supervision, M.M.; project administration, M.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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4. RASPRAVA

Kobalt se u moru osim u obliku slobodnog hidratiziranog iona nalazi adsorbiran na čestice, te kompleksiran s anorganskim (kloridima, karbonatima..) i organskim ligandima (siderofori, kobalofori) što uvelike utječe na dostupnost kobalta morskim organizmima. U najvećoj mjeri kobalt u moru formira komplekse s organskim ligandima što je važno za praćenje njegove kemijske specijacije, posebno iz aspekta utjecaja klimatskih promjena na biogeokemijski ciklus organske tvari u morskom okolišu, uključujući i površinski mikrosloj mora koji je u direktnom kontaktu s atmosferom (Morel i Price, 2003). Nadalje, specijacija kobalta nužna je za razumijevanje njegove raspodjele između morske vode, morskih organizama i sedimenata, kao i njegove kemijske reaktivnosti, biodostupnosti i biogeokemije (Noble *et al.*, 2017).

Ovim doktorskim radom istražen je utjecaj otopljene organske tvari iz različitih izvora na specijaciju kobalta u morskom okolišu, te koliko stabilnost nastalih organskih kompleksa utječe na biodostupnost kobalta kao mikronutrijenta. Istraživali smo kompleksiranje kobalta s 4-NC koji je većinom atmosferskog porijekla, s humusnom kiselinom terigenog podrijetla kao najzastupljenijim organskim ligandom u moru, te lipidima, odnosno fosfolipidima koji su akvagenog podrijetla. Dodatni izvor 4-NC u moru su složene organske molekule koje se razgrađuju ovisno o fizikalno-kemijskim uvjetima. Istraživano je kompleksiranje Co(II) s kateholom i 4-NC. Kompleks s kateholom nije vidljiv, dok je kompleks kobalta (II) s 4-NC identificiran, s obzirom na njegov visok afinitet prema nitro- skupinama (Chen *et al.*, 2014; Ferreira *et al.*, 2018). Kompleks HK s Co(II) identificiran je je elektrokemijskim metodama i spektrofotometrijom. Fosfolipidi izgrađuju staničnu membranu, pa su glavni izvori tih molekula organizmi. Interakcija s fosfolipidima stanične membrane utječe na mobilnost metalnih iona u unutrašnjost stanice (Liang *et al.*, 2007).

Kompleksiranje kobalta s organskim ligandima, s obzirom da kobalt ne stvara amalgam sa živom, moguće je detektirati samo voltametrijom katodnog otapanja (Zhang *et al.*, 1990). Navedena metoda se temelji na stvaranju i adsorpciji kompleksa kobalta na površini živine elektrode, nakon čega se prilikom promjene mjernih parametara inducira redukcija kompleksa te se dobije informacija o mehanizmu elektrokemijske reakcije, stabilnosti kompleksa (Van den Berg, 1998).

Dobiveni rezultati osim elektrokemijski dodatno su potvrđeni korištenjem spektrofotometrije (kompleksi kobalta s 4-NC i HA), LB metodom prikladnom za ispitivanje

biospecifičnih površina, i AFM za praćenje površinskih procesa hidrofobnih miješanih kompleksa kobalta s fosfolipidima. Metoda LB je korištena za praćenje utjecaja iona/molekula na monosloj amfifilnih molekula fosfolipida, dok su AFM metodom oslikane promjene na fosfolipidnom sloju (PC, PG, PE) prilikom interakcije i/ili utjecaja molekula kobalta i 1,10-fenantrolina koji s kobaltom formira intermedijerni kompleks.

4.1. Interakcija kobalta(II) s prirodno prisutnim ligandima u moru

4.1.1. Interakcija kobalta(II) s 4-nitrokateholom i humusnom kiselinom

Atmosfera i riječni donos predstavljaju važan put unosa organskih liganda prirodnog ili antropogenog porijekla u more (Frka *et al.*, 2016). Za naša istraživanja opisana u prvom radu, posebno je bio zanimljiv 4-NC s obzirom na gore spomenuti afinitet kobalta za nitro-skupine (Ferreira *et al.* 2018). Nitro skupine su zastupljene i u složenim organskim molekulama u morskom okolišu kao što su humusne tvari (Chen *et al.*, 2014; Noble *et al.*, 2017), pa se i očekivala interakcija kobalta s takvim organskim ligandima. Kompleksi Co(II) s 4-NC i HA zabilježeni su voltametrijski, praćenjem njihove redukcije. Odzivi struje vidljivi su u području potencijala od -1.0 do -1.1 V u pH uvjetima morske vode (pH 8,2). Porastom pH vrijednosti dolazi do destabilizacije sloja molekula tih kompleksa na površini živine kapi zbog stvaranja kobalt hidroksida, usporedivo s radom Čuljak *et al.*, 1994 u kojem je nastak hidroksida bakra utjecao na stabilnost kompleksa.

Za određivanje mehanizama redukcije tih kompleksa korištene su ovisnosti struje i potencijala redukcije o PVV parametrima (amplituda, a , i frekvencija, f) i onim cikličke voltimetrije (brzina promjene potencijala, v). Također, provedena je i analiza „*backward-forward*“ analiza koja mjeri signal prije i nakon PV pulsa i daje karakteristične valove ovisno o reverzibilnosti redukcijskog procesa (Lovrić i Branica, 1987). Analiza „*backward-forward*“ PV voltamograma oba kompleksa, Co(II)-4-NC i Co(II)-HA ukazuje na ireverzibilnost redukcije. U svrhu detaljne karakterizacije mehanizma redukcije praćena je ovisnost signala struje kompleksa o frekvenciji, i signala potencijala o logaritmu frekvencije. Ovisnost je linearna za komplekse s oba liganda. Linearna ovisnost struje o frekvenciji ukazuje da se radi o adsorpcijskom procesu (Komorsky-Lovrić *et al.*, 1992). Vršni potencijal, E_p , se za oba kompleksa s promjenom frekvencije pomiče prema negativnijim vrijednostima, pri čemu su

ovisnosti E_p vs. $\log f$ linearne što potvrđuje ireverzibilnost tih elektrodnih procesa (Lovrić *et al.*, 1988; Mirceski *et al.*, 2013; Mirčeski i Komorsky-Lovrić, 2007; Mlakar, 1993; Scholz, 2010)

Praćen je i utjecaj PV amplitude (a) na redukcijske procese gore navedenih kompleksa kobalta. Porastom amplitude vršne struje redukcije kompleksa rastu linearno, s tim da je porast struje, i_p redukcijskog procesa s a izraženiji zbog adsorpcije. S obzirom da se u slučaju kompleksa radi o ireverzibilnom elektrodnom procesu s adsorpcijom reaktanta, amplituda nema utjecaja na širinu vala na polovici visine signala struje redukcije.

Ciklički voltamogrami kompleksa također ukazuju na ireverzibilni karakter redukcije s obzirom da na anodnoj strani nije zabilježena oksidacijski proces. Vršna struja redukcije (i_p) proporcionalna je brzini promjene potencijala (v) u CV što je karakteristično za ireverzibilne redukcijske procese s adsorpcijom reaktanta (Bond, 1980; Scholz, 2010). Vršni potencijal se u oba slučaja pri promjeni v pomiče prema negativnijim vrijednostima, pri čemu je ovisnost E_p vs. $\log v$ linearne, što također potvrđuje ireverzibilni karakter redukcije, nakon čega dolazi do disocijacije kompleksa, u tzv. elektrokemijskom-kemijskom (engl. *electrochemical-chemical* - EC) mehanizmu (Bond, 1980; Mirčeski i Lovrić, 2000). Može se zaključiti da su voltametrijske analize redukcijskih procesa kompleksa Co(II)-4-NC i Co(II)-HA pokazale da kompleksi imaju dvoelektronski ireverzibilni karakter uz adsorpciju reaktanta, nakon čega dolazi do disocijacije kompleksa.

UV-Vis spektrofotometrija je korištena kao metoda koja je potvrdila stvaranje kompleksa kobalta s 4-NC i HA u otopini. Nastajanje kompleksa je snimljeno pri pH = 6,5 gdje se 4-NC nalazi u protoniranom obliku i pri pH = 8,2 gdje je deprotoniran (Conrad *et al.*, 2005). Spektar pri pH = 6,5 pokazuje apsorpcijski signal na valnoj duljini $\lambda = \sim 420$ nm, a dodatkom Co u otopinu taj signal opada i nastaje signal kompleksa pri $\lambda = \sim 350$ nm. U deprotoniranom obliku pri pH = 8,2 4-NC pokazuje apsorpcijski signal pri $\lambda = \sim 455$ nm a s Co (II) zabilježen je signal kompleksa pri $\lambda = \sim 375$ nm.

Eksperimenti kompleksa Co (II) s HA provedeni su samo pri pH = 8,2, dakle u području gdje je humusna kiselina potpuno deprotonirana. HA u ispitivanom području valnih duljina ne daje apsorpcijski val, međutim dodatkom kobalta zabilježen je signal kompleksa pri $\lambda = \sim 300$ nm. Ravnotežne konstante stabilnosti određene UV/Vis-om određene su titracijom s kobaltom.

Spektrofotometrijska mjerenja Co(II) s 4-NC i HA do danas nisu istraživana, međutim istraživanja divalentnih metala s kateholom ukazuju na velik afinitet dvovalentnih metala za

kateholnim skupinama (Zhang *et al.* 2021). Vidljivo je stvaranje apsorpcijskih signala kada su u otopini prisutni metali i kateholne molekule (Santana *et al.* 2010) (vidi poglavlje 1.3.2.).

4.1.2. Interakcija kobalta(II) s modelnim i prirodnim fosfolipidima

U drugom i trećem (Bačinić *et al.* 2022a, 2022b) znanstvenom radu istraživano je kompleksiranje kobalta s fosfolipidnim molekulama kao sastavnicama stanične membrane. Korišteni modeli stanične membrane - živina kap i monoslojevi nastali na međufazi zrak-voda pokazali su se dobrima za istraživanja fosfolipida, te interakcija fosfolipida s organskim molekulama i/ili metalima.

Prolaz Co(II) kroz staničnu membranu kao jednog od ključnih mikronutrijenata u brojnim biološkim procesima, važno je za razumijevanje njegove biokemije u morskom okolišu (Liang *et al.*, 2007). Fosfolipidi, kao građevne molekule stanične membrane, hidrofobne su molekule slabo topljive u vodi zbog čega ih je za potrebe eksperimenata bilo potrebno otapati u organskim otapalima (Sule *et al.* 2020). Za potrebe elektrokemijskog istraživanja korišten je metanol čiji odziv ne utječe na dobivene rezultate, dok je za potrebe L-B eksperimenta korišten n-heksan zbog brzog hlapljenja. Eksperimenti su provedeni s tri najzastupljenija fosfolipida u morskim algama, PG, PC i PE. PC i PE su odabrani jer u strukturi hidrofilne glave imaju atom dušika prema kojem kobalt pokazuje afinitet. Ispitivano je kompleksiranje Co(II) s i kako bi se usporedio afinitet vezanja. Svi provedeni modelni eksperimenti s fosfolipidima ponovljeni su s prirodnim uzorcima fosfolipida zelene alge *Dunaliella tertioelecta*. Međutim, navedena istraživanja pokazala su kako je izravno vezanje Co(II) i PC vrlo slabo i nije bilo vidljivo voltametrijskim mjerenjima. Stoga je u eksperiment uveden ligand 1,10-fenantrolin kao dobar kelirajući ligand za dvovalentne metalne ione (Ferreira *et al.*, 2018). Nastali kompleks se reducira na površini živine kapi na potencijalu -1.0 V u pH području od 3,5 do 9,0. Kompleks kobalta i 1,10-fenantrolina zatim reagira s molekulama fosfolipida zamjenjujući preostale molekule vode u koordinacijskoj sferi Co(II). Na takav način dolazi do stvaranja stabilnih miješanih kompleksa između kobalta i fosfolipida. Redukcija kompleksa Co(II)-Phen-PC zabilježena je na potencijalu -1.5 V u pH području od 5 do 9, dok je redukcija kompleksa s PG-om zabilježena na potencijalu -1.4 V.

Kompleks s prirodnim uzorkom smjese fosfolipida na voltamogramu daje odziv na potencijalu od oko -1,6 V što ukazuje na mogućnost stvaranja kompleksa Co(II) s PC iz smjese. Nešto

negativniji potencijal redukcije tog miješanog kompleksa vjerojatno je rezultat složenog prirodnog uzorka od sva tri fosfolipida (omjer PG: PE: PC u smjesi; 55: 12: 21). Linearna ovisnost odziva struje o frekvenciji i potencijala o logaritmu frekvencije ukazuju na ireverzibilnost reakcije. Ovisnost vršne struje o amplitudi miješanih kompleksa također potvrđuje ireverzibilnost elektrodnog procesa. Ciklički voltamogrami svih miješanih kompleksa također ukazuju na ireverzibilni karakter elektrodnih procesa s obzirom da na anodnoj strani nije zabilježena oksidacija. Co(II)-Phen redukcijska struja linearno ovisi o brzini promjene potencijala dok se potencijal pomiče prema negativnijim vrijednostima, što je karakteristično za reverzibilne redukcijske procese uz adsorpciju reaktanta (Bačinić *et al.*, 2022a). Za određivanje signala redukcije miješanog kompleksa Co-Phen-PC, odnosno Co-Phen-PG, vrijeme akumulacije bilo je > 3 min uz $E_{ak} = -1,3$ V, odnosno $-1,2$ V. Kompleks s prirodnom smjesom fosfolipida je trebao jednako minimalno vrijeme akumulacije, a najbolje vidljivi signal zabilježen je uz potencijal akumulacije $E_{ak} = -0,4$ V. PG je potrebno akumulirati pri pozitivnijem potencijalu zbog slabe adsorpcije, dok kod PC vidljiva je snažna adsorpcija na površini živine elektrode i reorijentacija u području potencijala od $-1,0$ do $-1,2$ V.

U drugom radu korištena je LB metoda kako bismo potvrdili pretpostavku nastajanja miješanog kompleksa na živinoj kapi (Bačinić *et al.*, 2022a; Giner-Casares *et al.*, 2014). Za bolju karakterizaciju miješanog kompleksa Co-Phen-PC, analizirani su monoslojevi PC-a te smjese PC, Phen i Co. PC otopljen u heksanu kao lako hlapljivom organskom otapalu, nanesen je na površinu međufaze voda/zrak. U međufazu su dodavani fenantrolin i kobalt te je praćen utjecaj na monosloj PC. Snimljene su π -A izoterme, odnosno izoterme omjera tlaka i površine molekula. Analiza je pokazala značajne razlike između monoslojeva sastavljenih od pojedinačnih molekula PC i miješanog monosloja. Naime, monoslojevi PC porastom tlaka (kompresijom) postižu tekuće kondenzirano stanje. Određena je limitirajuća površina pri najvišem postignutom tlaku $A_{lim} = 58 \text{ \AA}^2$. Utjecaj kobalta i fenantrolina je praćen u vremenskom periodu od 3 sata. Dodatak samog kobalta u međufazu pokazao je interakciju s monoslojem te smanjio limitirajuću površinu na 53 \AA^2 što ukazuje na mogućnost vrlo skromnog povezivanja molekula fosfolipida. Dodatkom fenantrolina dolazi do porasta limitirajuće površine na 64 \AA^2 , odnosno proširenja sloja ugradnjom molekula fenantrolina u monosloj što je u skladu s dobivenim voltametrijskim rezultatima. U provedenom istraživanju dodatak metala u subfazu dovodi do smanjenja površine, što objašnjavamo gušćim povezivanjem molekula nakon dodatka metala, međutim nije u skladu s prethodno objavljenim istraživanjima koja pokazuju povećanje površine dodatkom metala i daljnim dodavanjem organskih molekula u subfazu

(Petelska *et al.*, 2010). Dodatkom fenantrolina u subfazu dolazi do porasta površine zbog inkorporacije molekula u sloj fosfolipida, gdje je vidljiva sličnost s prethodnim publikacijama (Petelska i Figaszewski, 2011).

Trećim znanstvenim radom (Bačinić *et al.*, 2022) povezana su istraživanja na modelnim sustavima fosfolipida s prirodnim uzorkom smjese fosfolipida ekstrahiranim iz zelene alge *Dunaliella tertiolecta*. Morske alge su izabrane s obzirom da su bogate lipidima. Lipidi su razdvojeni od ostalih biomolekula tankoslojnom kromatografijom pokaznom kao dovoljno dobrom za odvajanje lipidnih klasa (Vrana Špoljarić *et al.*, 2021). Ekstrakt lipida razdvajan je ekstrakcijom na čvstoj fazi, i korištene su mikrokolone punjene s titanijevim dioksidom koji selektivno veže fosfatne skupine. Ekstrakt je analiziran Matricom potpomognuta ionizacija laserskom desorpcijom spektrometrija masa s analizatorom masa s vremenom leta (engl. *Matrix Assisted Laser-Mass Spectrometry-Time-of-Flight* - MALDI-TOF MS) metodom kojom je pokazana prisutnost fosfolipida od interesa u uzorku. Dobivena fosfolipidna frakcija je analizirana voltametrijski te su dobiveni signali miješanog kompleksa Co(II), Phen i fosfolipida, u skladu s vrijednostima dobivenim s modelnim PC (vidi poglavlje 4.1.), što je i očekivano zbog prisustva dušika u njegovoj strukturi.

Stvaranje miješanih kompleksa dodatno je ispitano i potvrđeno metodom AFM. Snimljeni su uzorci modelnih fosfolipida (PC, PE i PG) i realnog uzorka fosfolipida. Metoda AFM je korištena za oslikavanje i praćenje mehaničke stabilnosti i morfologije fosfolipida, te utjecaja 1,10-fenantrolina i kobalta na njih (Bačinić *et al.* 2022b). Kao što je vidljivo iz AFM slika PC pokriva cijelu površinu tinjca. Vidljivo je razdvajanje faza u obliku dobro definiranih uzdignutih domena okruženih matriksom. Izmjerena visina presjeka PC pokazala je debljinu PC sloja oko 5 nm, što odgovara visini dvosloja. Za istraživanje utjecaja udjela različitih molekula na organizaciju lipida, PC smo inkubirali s fenantrolinom, a zatim su snimljene uzastopne AFM slike. Kada se 1,10-fenantrolin adsorbira na fosfolipidni sloj, dolazi do značajnog površinskog preuređenja. Lipidne domene (sastavljene od PC) preuređuju se u pravilnije oblike tzv. vezikule za koje se smatra da su posljedica stvaranja stabilnih spojeva zbog smanjenja elektrostatskih odbijanja (Pompeo *et al.*, 2005). Snimanjem samog fosfolipida kao ni snimanjem samog 1,10-fenantrolin nije vidljivo stvaranje ovakvih struktura. Interakcija PC s 1,10-fenantrolinom nije uočena elektrokemijski, ali prethodna istraživanja provedena LB metodom pokazala su mogućnost inkorporacije Phen molekula u lipidnom sloju (Bačinić *et al.* 2022a). Dodatak kobalta u smjesu Phen/PC povezuje molekule i stvara terase. U ovom slučaju terase veličine mikrona se preklapaju kako bi napravile višeplanarnu uređenu površinu.

Snimanjem realnog uzorka vidljiva je i očekivana razlika zbog kompleksnog sastava realnih fosfolipida (PC, PE, PG, PS, itd.) od samog standarda PC. Dodatkom fenantrolina dolazi do stvaranja vezikula kao i kod modelnog uzorka, a dodatkom kobalta u smjesu dolazi do stvaranja “terasa”, zbog pretpostavljenog povezivanja molekula, zbog čega su nam dobiveni rezultati usporedivi, a možemo pretpostaviti i da velik utjecaj ima afinitet kobalta za PC.

4.2. Uvjetne konstante stabilnosti kompleksa

Sukladno navedenim istraživanja za izradu doktorske disertacije birali smo modelne i prirodne ligande prisutne u morskom sustavu, biološki važne i koji u strukturi imaju dušik (Merian *et al.*, 2004). Elektrokemijska metoda kompetitivne izmjene liganda pokazala se kao dobra metoda za istraživanje kompleksa metala s modelnim ligandom čije konstante stabilnosti nalazimo u bazama podataka (Zhang, 1990). Za izračune korištena je National Standard Reference Data Series - NIST baza podataka (Martell *et al.* 2004). Kao kompetitivni ligandi korišteni su EDTA i NTA s obzirom da su njihove konstante stabilnosti poznate. Prilikom kompeticije u prvoj fazi dolazi do ravnoteže između kompetitivnog (EDTA/NTA) i ispitivanog liganda i kobalta nakon čega se u drugoj fazi određuje koncentracija kompleksa nakon postignute kompetitivne ravnoteže. Otopine su titrirane s kompetitivnim ligandom, te je iz omjera struja određenih bez i uz dodatak liganda izračunat parametar X (vidi poglavlje 1.3.1.5). Najbolje slaganje između izračunatih i eksperimentalno dobivenih vrijednosti za kobalt i 4-nitrokatehol dobiveno je uz omjer $\text{Co} : 4\text{-NC} = 1 : 2$, a konstante su određene pri $\text{pH} = 6,9$ na kojem je najbolje vidljiv redukcijski odziv kompleksa, te pri pH vrijednosti morske vode (8,2). Uvjetna konstanta stabilnosti je određena za kompleks s humusnom kiselinom titracijom s EDTA. Najbolje slaganje između izračunatih i eksperimentalno dobivenih rezultata je uz omjer $\text{Co(II)} : \text{HA} = 1 : 2$ ($\text{pH} = 8,2$). Određivanje uvjetnih konstanti stabilnosti s miješanih kompleksa kobalta s fosfolipidima je složenije. Titracija je prvotno provedena s NTA za određivanje uvjetne konstante stabilnosti kompleksa Co(II)-Phen , zatim je Phen korišten kao kompetitivni ligand za određivanje konstante miješanog kompleksa kobalta s fosfolipidom. Određena je uvjetna konstanta stabilnosti s fosfatidilkolinom, dok s fosfatidilglicerolom ju nije bilo moguće odrediti navedenom metodom. Zbog složenosti smjese prirodnih lipida određivanje konstanti stabilnosti nije bilo moguće. Dobivene vrijednosti izračunatih uvjetnih konstanti stabilnosti (tablica 1.) su u skladu s prethodno objavljenim konstantama stabilnosti (Bown *et al.* 2012a,b; Saito *et al.* 2005) (vidi poglavlje 1.3.1.5).

Tablica 1. Uvjetne konstante stabilnosti kompleksa određene voltametrijskom metodom kompetitivnih liganda

Kompleks	pH	Kompetitivni ligand	log K
log $K_{Co(II)-4NC2}$	pH = 8,2	NTA	21,86
log $K_{Co(II)-4NC2}$	pH = 6,9	NTA	21,11
log $K_{Co(II)HA2}$	pH = 8,2	EDTA	11,32
log $K_{Co(II)-1,10-Phen2}$	pH = 8,2	NTA	22,75±0,48
log $K_{Co(II)-1,10-Phen2PC}$	pH = 8,2	Phen	23,02±0,26
log $K_{Co(II)-1,10-Phen2PC2}$	pH = 8,2	Phen	29,31±0,17

Ravnotežne konstante stabilnosti kompleksa u otopini određene su UV/Vis spektrometrijom za komplekse s 4-nitrokateholom i humusnom kiselinom. Za računanje konstanti korišten je Specfit program koji se temelji na multivarijantnom, nelinearnom korištenju regresijske analize najmanjih kvadrata (Gampp *et al.* 1985). Najboljim modelom se pokazao odnos stehiometrije 1 : 1 te su određene ravnotežne konstante stabilnosti $\log K_{Co(II)-4NC} = 5,76 \pm 0,40$ (pH = 6,5), $\log K_{Co(II)-4NC} = 3,98 \pm 0,17$ (pH = 8,2). Za humusnu kiselinu određena je konstanta $\log K_{Co(II)-HA} = 3,80 \pm 0,29$ (pH = 8,2). Kompleksi koji nastaju na površini elektrode živina kap su bitno stabilniji od kompleksa nastalih u otopini. Konstante stabilnosti monosloja, odnosno utjecaj organskih molekula i metalnih iona na fosfolipidni monosloj određivan je Langmuir-Blodgettovom metodom. Statističkom metodom višestruke linearne regresije (objašnjene u poglavlju 1.3.3.) izračunate su konstante stabilnosti nastalog sloja/kompleksa. Ovom metodom je detektirana slaba interakcija između fosfatidilkolina i kobalta u omjeru 1:1, s konstantom stabilnosti $K_1 = 2,4 \times 10^{-2} \text{ m}^3 \text{ mol}^{-1}$. Vrijednost tako dobivene konstante stabilnosti možemo usporediti s elektrokemijskim rezultatima koji nisu pokazali direktnu interakciju kobalta s fosfolipidima, već kompleks kobalta s fosfolipidima nastaje posredno formiranjem intermedijernog kompleksa s 1,10-fenantrolinom, koji omogućava vezanje kobalta s fosfatidilkolinom i formiranje miješanog kompleksa. U slučaju stabilnosti miješanog monosloja na međufazi zrak voda, vrijednost konstante iznosila je $K_2 = 4,86 \times 10^{10} \text{ m}^2 \text{ mol}^{-1}$, što ukazuje na iznimnu stabilnost nastalog kompleksa, koja se također može usporediti s visokim vrijednostima uvjetnih konstanti stabilnosti dobivenih elektrokemijskim mjerenjima adsorpcijskom voltametrijom katodnog otapanja.

4.3. Doprinos istraživanju

- Znanstveni doprinosi doktorskog rada temelje se na:
 - (i) razumijevanju procesa koji kontroliraju biogeokemijski ciklus kobalta(II) u moru, što je od ključnoga značaja za predviđanje njihovog ponašanja, biodostupnosti, mobilnost i stabilnosti s obzirom na promjene vezane uz klimatske ekstreme kojima svjedočimo te,
 - (ii) razvoju i primjeni učinkovitijih metoda za izravnu, brzu i ekonomičnu karakterizaciju kompleksa s hidrofobnim organskim ligandima i fosfolipidima koji su u morskom okolišu pokazatelji stanja stresa fitoplanktona zbog klimatskih promjena (temperatura, salinitet, pH).

- Dobiveni rezultati relevantni su za daljnja ispitivanja esencijalnih mikronutrijenata vezanih za organske ligande i mehanizme kompleksiranja s prirodnim ligandima odgovornim za unošenje mikroelemenata u tragovima u organizme morskog okoliša.

5. ZAKLJUČCI

Iako je značaj kobalta kao mikronutrijenta u morskom okolišu izuzetno važan i ništa manji od ostalih metala u tragovima, broj znanstvenih publikacija je neusporedivo manji ako se uspoređi npr. sa željezom i/ili nekim neesencijalnim metalima. Nedostatak kobalta u morskom okolišu, te nedostupnost može uvelike utjecati na metabolizam morskih organizama.

Istraživanje provedeno u svrhu izrade ove doktorske disertacije, istaknulo je važnost poznavanja specijacije kobalta i razumjevanja biogeokemijskih procesa u kojim sudjeluje u morskom okolišu.

Na temelju voltametrijskih istraživanja modelnih i prirodno prisutnih organskih liganda, standarda i prirodnih uzoraka ekstrahiranih fosfolipida iz morske vode, na elektrodi živina kap, UV/Vis spektrofotometrijskih rezultata, te dobivenih LB i AFM metodom u karakterizaciji kompleksa kobalta(II) doneseni su sljedeći zaključci:

- Kobalt s navedenim ligandima (4-NC, HA, PC) stvara stabilne komplekse visokih uvjetnih konstanti stabilnosti : $\log K_{\text{Co(II)-4NC}_2} = 21,86$ (pH = 8,2), $\log K_{\text{Co(II)-4NC}_2} = 21,11$ (pH = 6,5), $\log K_{\text{Co(II)HA}_2} = 11,32$ (pH = 8,2) stehiometrije 1:2, te $\log K_{\text{Co(II)Phen}_2\text{PC}} = 23,02$ i $\log K_{\text{Co(II)Phen}_2\text{PC}_2} = 29,31$ (pH = 8,2) za komplekse stehiometrije 1:2:1 i 1:2:2, nastale na hidrofobnoj elektrodi živina kap, u modelnoj otopini morske vode (NaCl, pH = 8,2, $I_c = 0,55 \text{ mol dm}^{-3}$)
- Identificiran je mehanizam redukcije istraživanih kompleksa Co(II) metodama CV, PVV analizom „*backward-forward*“ i na temelju ovisnosti o parametrima navedenih tehnika (f vs. I_p , E_p vs $\log f$, $\Delta E_{p1/2} = \text{konst.}$, A vs. I_p – linearno) i zaključeno je da su procesi ireverzibilni dvoelektronski uz adsorpciju reaktanta te da nakon redukcije dolazi do disocijacije kompleksa.
- Optimizirani su uvjeti mjerenja elektrokemijskim metodama PVV i CV (amplituda, frekvencija, brzina promjene potencijala, pH, itd.) te je pokazano da su hidrofobni kompleksi stabilni u uvjetima morske vode (pH, $c(\text{NaCl})$), dok povišenjem pH vrijednosti dolazi do hidrolize kobalta i destabilizacije istraživanih kompleksa kobalta s organskim ligandima.
- Kobalt(II) pokazuje izuzetan afinitet vezanja na organske ligande koji u strukturi sadrže dušik, sukladno tome snažno se veže sa 4-nitrokateholom i na fosfatidilkolinom.

- Istraživani ligandi, modelni i prirodni prisutni u morskoj vodi tvore topljive i/ili hidrofobne Co(II) komplekse.
- Metoda kompetitivnog liganda pokazala se dobrom za određene uvjetnih konstanti stabilnosti za kompleksa s 4-NC, HA, Phen i PC.
- Spektrofotometrijski su određeni kompleksi kobalta topljivi u vodenoj otopini te su izračunate ravnotežne konstante stabilnosti kompleksa za 4-NC i HA: $\log K_{Co(II)-4NC} = 5,76 \pm 0,40$ (pH = 6,5), $\log K_{Co(II)-4NC} = 3,98 \pm 0,17$ (pH = 8,2) i $\log K_{Co(II)-HA} = 3,80 \pm 0,29$ (pH = 8,2).
- Miješani Co(II)-Phen-PC kompleks nastaje samo kad je u otopini uz Co i PC, prisutan i 1,10-fenantrolin. Interakcija kobalta i fosfolipida nije zabilježena elektrokemijskim mjerenjima.
- Metoda LB se pokazala kao optimalna za proučavanje interakcija organskih molekula i metala. Pomak π -A izoterme ukazuje na vezanje 1,10-fenantrolina i kobalta na monosloj PC-a. Dodatkom kobalta dolazi do smanjenja površine što se može objasniti povećanjem gustoće fosfolipidnih molekula zbog koordinacije s kobaltom. Određena konstanta stabilnosti takvog sloja je iznimno niska i iznosi $K_1 = 2,4 \times 10^{-2} \text{ m}^3 \text{ mol}^{-1}$. Dodatkom 1,10-fenantrolina dolazi do povećanja površine monosloja što ukazuje na ugradnju kompleksa kobalta i fenantrolina u fosfolipidni monosloj, dok izračunata konstanta stabilnosti sloja ukazuje na veliku stabilnost sloja i iznosi $K_2 = 4,86 \times 10^{10} \text{ m}^2 \text{ mol}^{-1}$.
- AFM metodom zabilježene su promjene na fosfolipidnim slojevima dodatkom organskih molekula i metalnih iona. Snimanjem realnog uzorka vidljiva je i očekivana razlika zbog kompleksnog sastava realnih fosfolipida od samog standarda. Smjesa fenantrolina i fosfolipida kako standarda tako i realnih pokazuje stvaranja vezikula, a dodatak kobalta u smjesu dovodi do stvaranja “terasa”, zbog pretpostavljenog povezivanja molekula.

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7. ŽIVOTOPIS

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ZNANSTVENE AKTIVNOSTI

3 znanstvena rada, 9 kongresnih priopćenja

8. PRILOZI

I. POPIS ZNANSTVENIH AKTIVNOSTI

Publikacija u CC časopisu; prvi autor

- A. Bačinić, L.M. Tumir, M.Mlakar, Electrochemical Characterization of Cobalt(II)-Complexes Involved in Marine Biogeochemical Processes. I. Co(II)-4-Nitrocatechol and Co(II)-Humate. *Electrochimica Acta*, 2020. 337. IF 5.383, Q1

- A. Bačinić, S. Frka, M. Mlakar, 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. *Bioelectrochemistry* 2022. 108009. IF 5.373, Q2

- A. Bačinić, P. Vukosav, I. Kero, and M. Mlakar, Study on the Surface Interactions of Co(II) with Phospholipids from the Marine Environment, *J. Mar. Sci. Eng.* 2022, 261. IF 2.744, Q2

Usmeno priopćenje na međunarodnom znanstvenom skupu prvi autor

- 1) 25.5.2018 - 8th ISE satellite student regional symposium on electrochemistry; Andela Bačinić, Marina Mlakar, Determination of cobalt-catechol complex by cathodic stripping voltammetry; **oral presentation**, Rudjer Boskovic Institut, Zagreb, Croatia
- 2) 25.2.2019 – Symposium of PhD studies of Natural Science Faculty; Andela Bačinić, Marina Mlakar, Electrochemical characterization of cobalt (II) complexes with 4-nitrocatechol and humic acid; **oral presentation**, University of Zagreb, Zagreb, Croatia
- 3) 2.7.2021- 10th ISE Satellite Student Regional; Andela Bačinić, Marina Mlakar, Formation of Co(II)-Phen-PC mixed ligand complex at the model surface of mercury electrode and Langmuir trough, online, **oral presentation**, Rudjer Boskovic Institut, Zagreb, Croatia
- 4) 1.7.2022 - ISE Satellite Student Regional Symposium on Electrochemistry, Andela Bačinić, Tena Matijević, Ivana Kero, Marina Mlakar, Complexation of membrane phospholipids with cobalt in seawater, **oral presentation**, Zagreb, Croatia
- 5) 15-19.8.2022 - ISE Regional Meeting; Andela Bačinić, Tena Matijević, Ivana Kero, Marina Mlakar, Complexation of membrane phospholipids with metal ions in the marine environment, **oral presentation**, Prague, Czech republic

Poster na međunarodnom znanstvenom skupu; prvi autor

- 1) 10.10.2019 - 42nd Ciesm; Andela Bačinić, Marina Mlakar, Characterization of cobalt(II)-4-nitrocatechol and cobalt(II)-humic acid complexes in seawater model solution; **poster and micro-oral presentation**, Cascais, Portugal
- 2) 30.8 - 4.9.2020 – 71st Annual Meeting of the International Society of Electrochemistry; Andela Bačinić, Marina Mlakar, Voltammetry of cobalt(II) complexes with naturally present organic ligands in seawater; online, **poster**, Belgrade, Serbia

- 3) 25.04.2021. Symposium of PhD studies of Natural Science Faculty; Andela Bačinić, Marina Mlakar, Formation of Co(II)-lipid mixed ligand complex at the model of mercury electrode, online, **poster**, Zagreb, Croatia
- 4) 10-15.7.2022 - [International GEOTRACES Summer School 2022](#), Introducing polar parameters + poster presentation: Andela Bačinić, Marina Mlakar "Electochemical characterisation of Co(II) complexes with organic ligand naturally present in seawater", Bremenhaven, Germany

Poster na domaćem znanstvenom skupu - koautor

- 1) 31.08.-04.09.2020, 71st Annual Meeting of the International Society of Electrochemistry; Sarah Mateša, Andela Bačinić, Claire Durey, Irena Ciglencečki, Electroanalytical characterization of polysulfides (SX₂⁻) in model solution and euxinic seawater conditions; online, poster, Belgrade, Serbia
- 2) 16-18.09.2020, 18th Ružička Days Today science, Sarah Mateša, Andela Bačinić, Claire Durey, Irena Ciglencečki, Elektroanalitička karakterizacija polisulfida (Sx₂⁻) u morskom euksinom okolišu, poster, Vukovar, Croatia

Tečajevi, škole, radionice međunarodne

- 1) 6-10.12.2021 - ISE Satellite Student Regional Symposium on Electrochemistry – 15th PTASchool of Electrochemistry On-line Edition, Sao Paolo, Brazil
- 2) 10-15.7.2022 - [International GEOTRACES Summer School 2022](#), Introducing polar parameters + poster presentation: Andela Bačinić, Marina Mlakar "Electochemical characterisation of Co(II) complexes with organic ligand naturally present in seawater", Bremenhaven, Germany