

Catecholase biomimetic copper(II) complex immobilization via Schiff's base formation onto the cross-linked polymers prepared by drop-casting

Milan Sýs, Michaela Bártová, Aneta Hartmanová,
and Tomáš Mikysek*

*Department of Analytical Chemistry,
The University of Pardubice, CZ–532 10 Pardubice, Czech Republic*

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In this article, a universal protocol is introduced dealing with covalent immobilization of catecholase biomimetic copper(II) complexes with free carbonyl group onto the primary amine-based polymeric substrates (chitosan and collagen, type II) cross-linked with glutar-aldehyde; both being enabled via Schiff's base formation. As a representative of biomimetic catalysts, $[\text{Cu}_2\text{Ac}_2\text{O}(\text{L})\text{ClO}_4]$ consisting of a 2,6-bis(4-methyl piperazin-1-yl-methyl)-4-formyl-phenoxy ligand (L) was selected. The resulting functionalized polymers formed at the surface of a screen-printed carbon electrode were characterized using scanning electron microscopy with energy dispersive X-ray analysis. As a result, the procedure presented herein could find use in the development of artificial biosensors.

Keywords: Biomimetic enzymes; Functionalized polymers; Schiff's base formation; Drop-casting; Scanning electron microscopy.

Introduction

In general, polymers containing the free primary and secondary amino groups (polymer- $\text{CH}_2\text{-NH}_2$ and polymer- NH-R) are able to react with the carbonyl compounds (aldehydes and ketones) to form the corresponding imines and enamines known as Schiff's bases [1]. This reaction is utilized in preparation of functionalized polymer layers, where the most common case represents an attachment of catalysts [2].

* Corresponding author, ✉ tomas.mikysek@upce.cz

They have found wide application in many different areas, namely in environmental remediation [3], development of specific analytical devices (sensors) [4], organic synthesis [5], medicine as innovative drug delivery systems [6], etc. Unfortunately, it is necessary to mention that Schiff's bases are prone to hydrolysis (split after contact with water), giving rise to the respective products of amine and aldehyde or ketone, while the reaction requires acidic medium [7]. Notwithstanding, the Schiff's base formation (coupling) is still used in the preparation of catalytic (enzyme) biosensors and of their completely artificial analogues (biomimetic sensors) [8].

Polymeric layers having the free amino groups can be prepared by several ways including the electropolymerization [9], drop-casting [10], cross-linking [11], light-controlled radical polymerization [12], etc. The combination of drop-casting with the subsequent cross-linking can result in mechanically stable substrate for subsequent immobilisation a catecholase biomimetic copper(II) complex able to catalyse the oxidation of catecholamines (neurotransmitters) [13].

The above-stated is the central theme of this paper and two types of commonly used polymers were selected (chitosan [14] and collagen, type II [15]) and used in our study. Scheme 1 illustrates the functionalized molecular structure of the latter. These layers were characterised using scanning electron microscopy (SEM) with energy dispersive X-ray analysis (an EDX probe). Finally, this work also offers a simple and universal protocol for the preparation of biomimetic sensors for which a long service life is anticipated as the most significant benefit compared to similar devices with rather unstable functional constituents.

Experimental

Chemicals

All the following chemicals and reagents were purchased from Merck KGaA (Darmstadt, Germany), all with purity $\geq 96\%$: Copper(II) acetate hydrate, 4-hydroxybenzaldehyde, paraformaldehyde, *N*-methylpiperazine, methanol (MeOH), sodium perchlorate, collagen (type II) from bovine achilles tendon, glacial acetic acid, chitosan flakes with a molecular weight of 30-80 kDa, glutaraldehyde (GTA), ethanol (EtOH), sodium dihydrogen phosphate dihydrate, and disodium hydrogen phosphate dodecahydrate. An Ultrapure[®] water (with conductivity $\sim 0,055 \mu\text{S cm}^{-1}$ at 25 °C) was prepared by passing of the already deionized water through a Milli-Q[®] purification unit from Merck Millipore (Burlington, MA; USA).

Synthesis of catecholase biomimetic copper(II) complex

According to the protocol reported by Narayanan et al [16], a standard Mannich reaction was used to synthesize the 2,6-bis(4-*N*-methylpiperazine-1-yl-methyl)-4-formylphenol ligand (L), as shown in Scheme 2. First, 4-hydroxybenzaldehyde (3.05 g, 25 mmol) and paraformaldehyde (1.58 g, 50 mmol) were mixed in 100 mL MeOH and heated to reflux for 1 h. Then, *N*-methylpiperazine (5.54 mL, 50 mmol) was added to the resulting reaction mixture and heated to reflux for 24 h. After removal of all volatiles under the reduced pressure, the residue obtained was extracted with 50 mL chloroform. The subsequent evaporation of the solvent led to the ligand as white powder with a yield of 7.5 g (86 %).

A 20 mL aliquot of the ligand solution (1 g, 2.88 mmol) was mixed with 20 mL solution of $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$ (1.15 g, 5.76 mmol); both in MeOH at laboratory temperature. The resulting mixture was refluxed for 7 h. Then, 10 mL of NaClO_4 (0.35 g, 2.88 mol) solution in MeOH was added and refluxed for further one hour. Organic solvent used was evaporated giving rise to a dark-green residue, which was recrystallized from acetonitrile providing $[\text{Cu}_2(\text{L})(\text{OAc})_2]\text{ClO}_4$ complex (see Scheme 3) as a dark-green crystalline material. Yield: 1.28 g (64 %). Anal. Calc. for $\text{C}_{23}\text{H}_{37}\text{N}_4\text{O}_{10}\text{ClCu}_2$: C, 39.9; H, 5.4. Found: C, 39.2; H, 5.2.

Immobilisation of catecholase biomimetic copper(II) complex

Chitosan was dissolved in 1% (v/v) acetic acid to obtain a chitosan solution with a concentration of 1% (w/v). The chitosan-based layer was made by applying 20 μL chitosan solution to the surface of a commercial screen-printed carbon electrode (SPCE, type C110) of 4 mm in diameter from Metrohm DropSens (Asturias, Spain) and the subsequent evaporating the solvent, as shown in Fig. 1. The collagen-based layer was prepared by a similar way when applying 20 μL suspension of collagen, type II (2 mg mL^{-1}) in 0.1 mol L^{-1} phosphate buffer (pH 7).

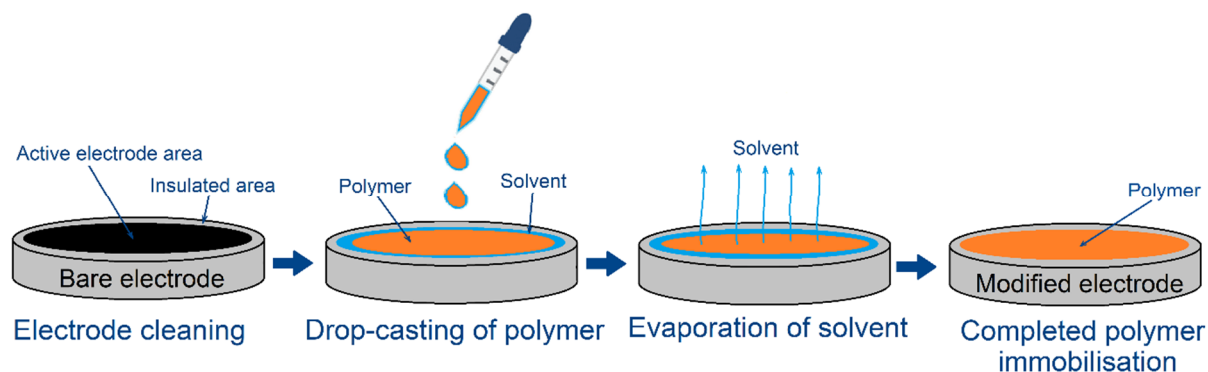
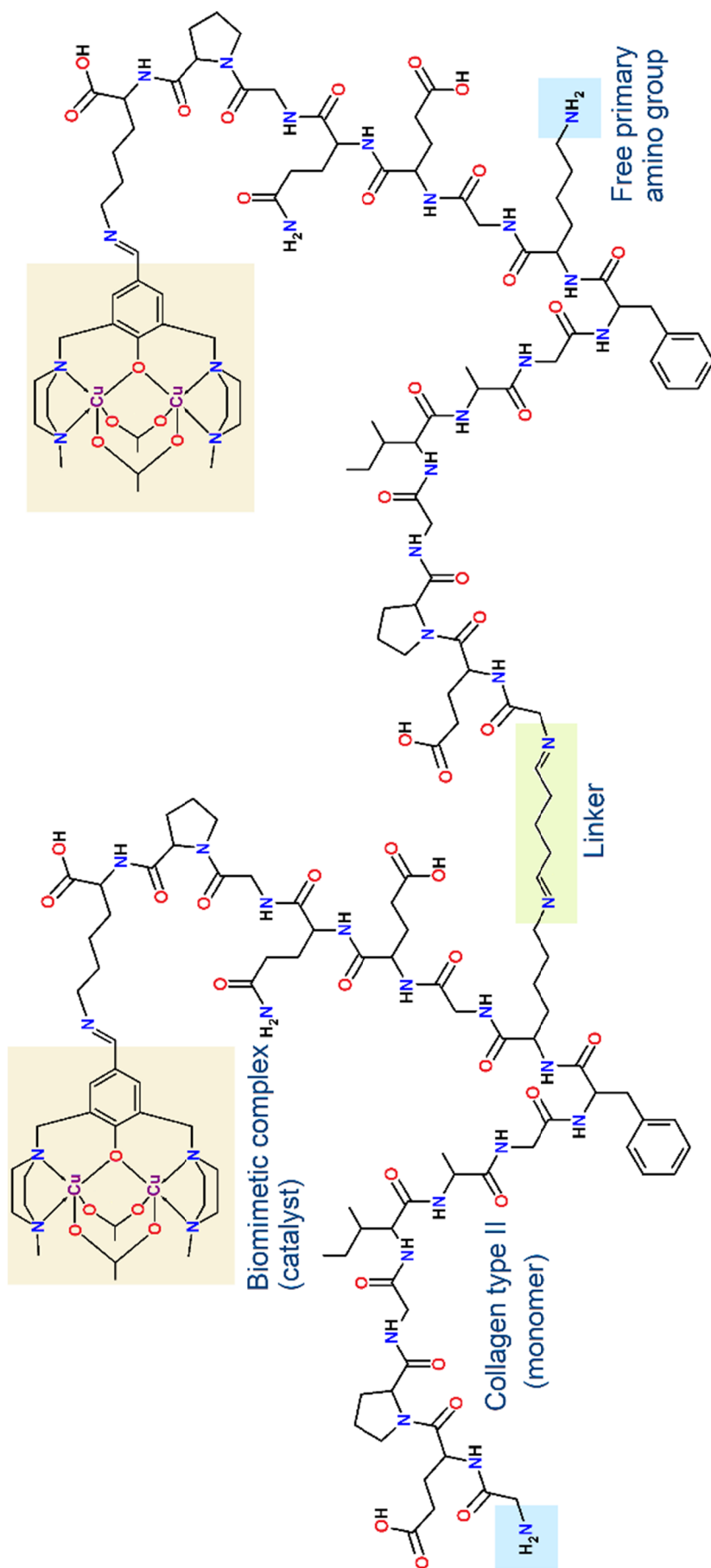
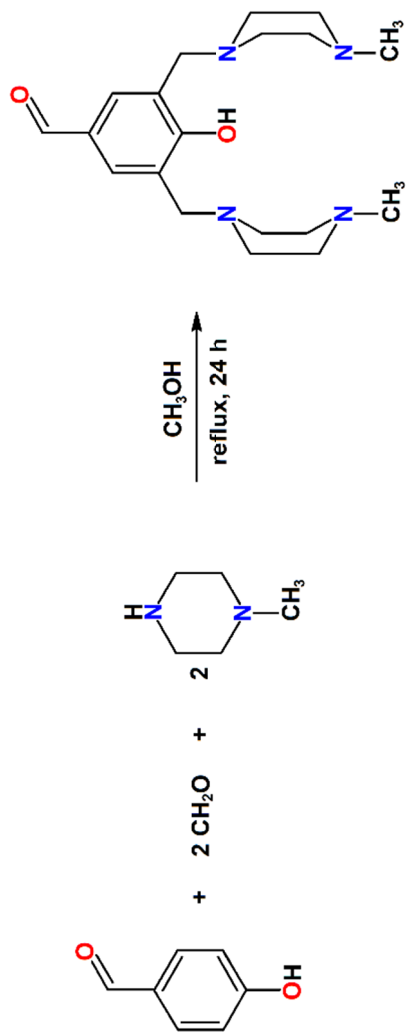


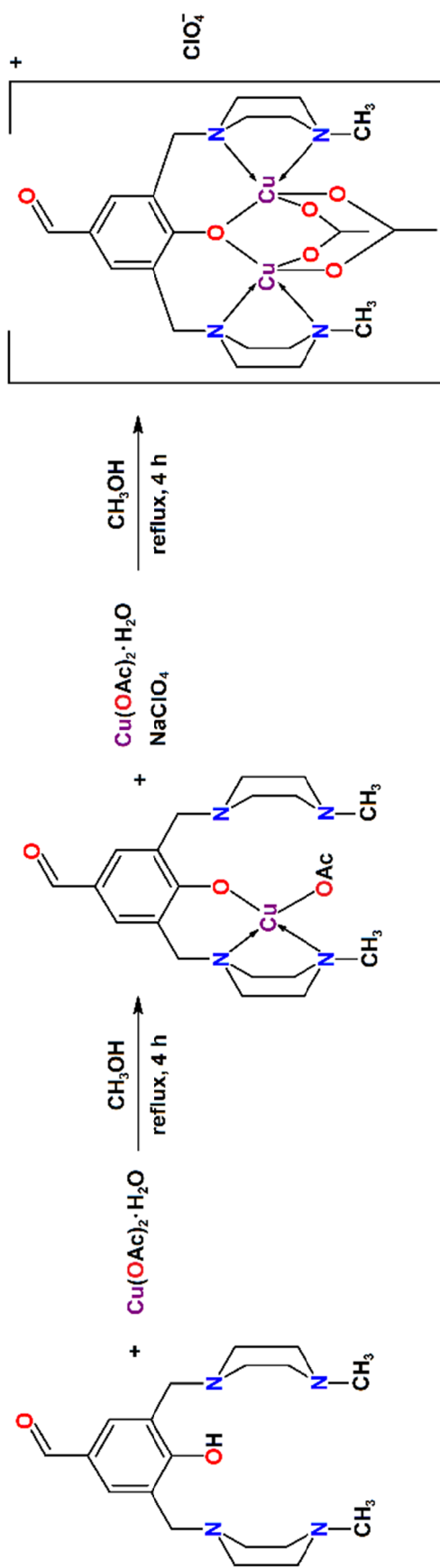
Fig. 1 A schematic showing the drop-casting of polymers onto the electrode



Scheme 1 Probable molecular structure of the cross-linked collagen, type II, with bonded catecholase biomimetic copper(II) complex



Scheme 2 Synthetic reaction of the 2,6-bis(4-N-methylpiperazine-1-yl-methyl)-4-formylphenol ligand (L)



Scheme 3 Synthetic reaction of the representative $[\text{Cu}_2(\text{L})(\text{OAc})_2]\text{ClO}_4$ complex

Before a covalent immobilisation of the catecholase biomimetic complex *via* Schiff's base formation, the absorbed layers of polymers were washed with a stream of deionised water. After that, the individual chains of polymers were cross-linked to mechanically stabilized polymeric layers using 5 μL of 1% GTA (*w/w*) in 50% (*v/v*) EtOH. Self-immobilisation was carried by immersing the SPCE covered by thin layer of cross-linked polymer into methanolic solution of 0.01 mol L⁻¹ copper (II) complex at room temperature for 24 h (see Fig. 2). Then, the resulting biomimetic sensor was treated by deionized water to wash out the non-bonded (directly adsorbed) complex molecules and left to dry at normal conditions.

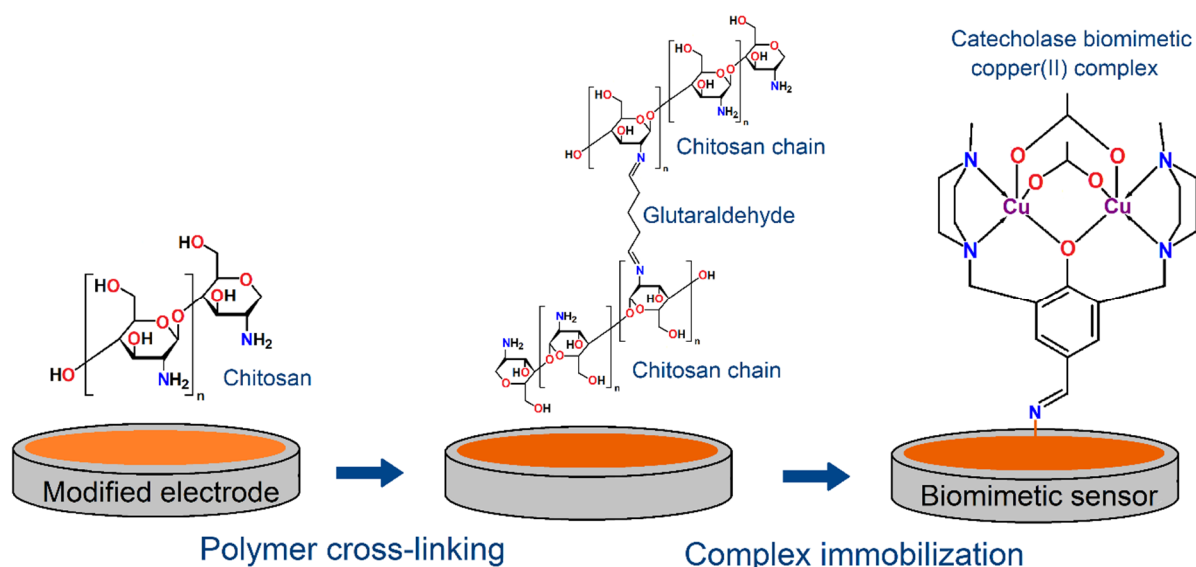


Fig. 2 A scheme showing the covalent immobilisation of catecholase biomimetic copper(II) complex onto polymeric substrate (cross-linked chitosan chains) *via* Schiff's base formation

Instrumentation for characterisation of catalytically active substrates

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) for imaging the morphologies and mapping of the copper(II) complex molecules distribution in the two chosen polymers, respectively, were performed using Vega3 SBU instrument from Tescan Brno (Brno, Czech Republic). For characterisation of the surface microstructure, electron imaging in the secondary mode (SE) was preferred.

Results and discussion

As demonstrated in Fig. 3, SEM imaging of the surface of functionalized polymers has shown a significant difference in their microstructure. The chitosan-based polymer forms a smoother layer than that of collagen and its spongy (porous) texture. An explanation can be found in their different molecular structure with the subsequent crosslinking. Collagen, type II, is a branched molecule compared to a rather straight chain chitosan.

Both polymeric layers were characterized by great mechanical stability because they cannot be removed from the SPCE surface even by manual wiping. In addition, multiple surface rinses did not cause a surface colour change caused by the green coloration of the bonded copper(II) complex molecules. These facts suggest one that the sensors prepared in this way could withstand the conditions of flow injection analysis (FIA), and therefore utilized for the development of sophisticated microfluidic chips. Unfortunately, it is necessary to keep strictly neutral environment because, in an acidic medium, imine hydrolysis (catalyst detachment) and protonation of the nitrogen atoms may cause the release of copper(II) and the consequent inactivation of the catalyst.

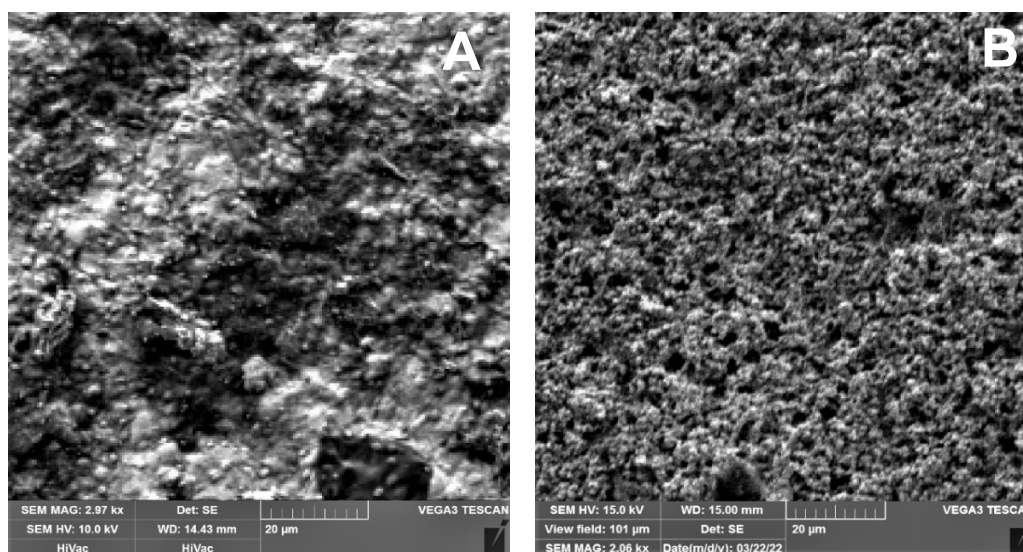


Fig. 3 SEM images of polymeric layers based on the cross-linked chitosan (A) and collagen, type II (B), with covalently immobilized catecholase biomimetic copper(II) complex

Regarding the catalyst distribution (see inset in Fig. 4), it has been found that it is evenly distributed over the whole surface and does not form clusters. This observation confirms the fact that the molecules of the copper(II)-complex were covalently bonded and not adsorbed *via* van-der-vaals interactions. Furthermore, EDX analysis has revealed that chitosan (15.1-17.4 % Cu; *w/w*) is able to bind more catalyst than collagen, type II (3.79-5.9 % Cu; *w/w*), due to the presence of primary amino group in each sugar unit. For demonstration, EDX spectrum of chitosan-based functionalized polymer is illustrated in Fig. 4.

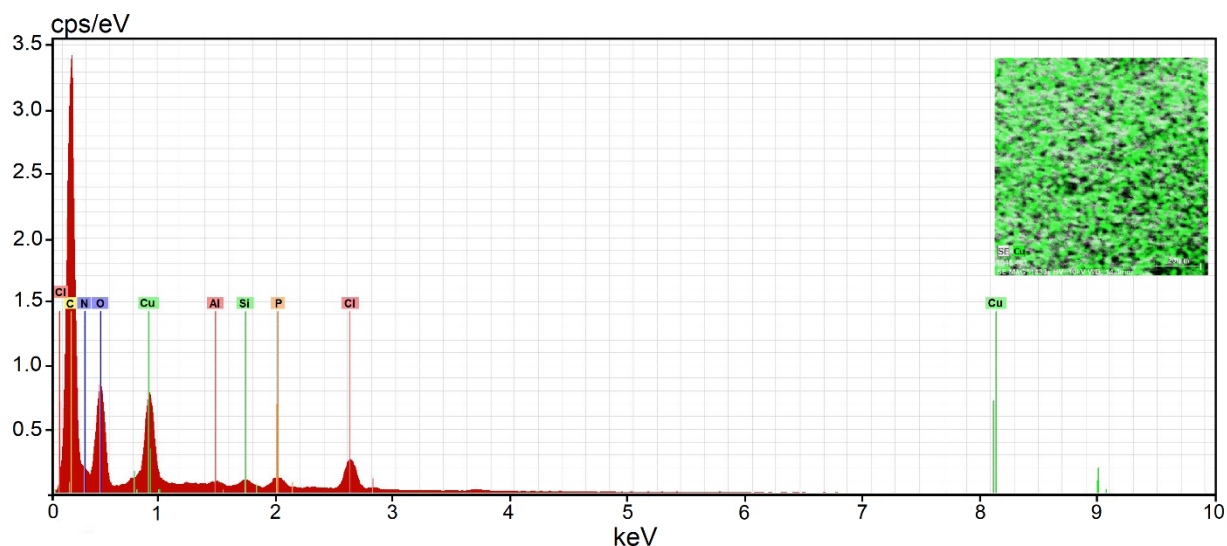


Fig. 4 Energy dispersive X-ray analysis (EDX) spectrum of screen-printed carbon electrode (type C110) covered by thin layer of cross-linked chitosan with the covalently bonded catecholase biomimetic complex

Inset: Mapping of copper distribution in SPCE (type C110) covered by the above-mentioned thin layer

Conclusions

A relatively simple protocol, derived from traditional enzyme immobilization procedure, has been proposed for the covalent bonding of biomimetic catalysts *via* Schiff's base formation to cross-linked polymeric layers prepared by drop-casting onto the solid surfaces (of functionalized polymers). The presented procedure could find its wide application in the development and construction of artificial biosensors.

Acknowledgement

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