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Elektrochemické biosenzory a detektory na bázi pevného stříbrného amalgámu pro analýzu v průtokových systémech

Electrochemical Biosensors and Detectors Based on Silver Solid Amalgam for Analysis in Flow Systems

Disertační práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Contents

1. Abstract

In this Ph.D. thesis new possibilities of using amalgam electrodes are presented. First of all, the tubular detector based on silver solid amalgam (TD-AgSA) was designed for determination of reducible compounds in flow systems. It was tested on model solutions of Cd^{2+}, Zn^{2+} and 4-nitrophenol in amperometric mode under conditions of flow injection analysis. Results have shown that developed tubular detector is simple and low cost device suitable for detection of reducible compounds with good sensitivity, repeatability and long-term stability (at least 2 years) with possibility to work at potentials up to –2 V in aqueous solutions. Afterwards, this newly developed detector was successfully used for the determination of an active ingredient lomustine in pharmaceutical preparation CeeNU® Lomustine by non-stop-flow differential pulse voltammetry based on reduction of present nitroso group.

For measurements in the flow system miniature reference electrodes – saturated calomel electrode, mercury-mercurous sulfate, and mercury-mercuric oxide electrode based on paste silver solid amalgam were fabricated and tested for 14 months. The calomel electrode based on paste silver amalgam proved to be the most resistant to polarization and it was used in all experiments in this thesis.

Next, the electrochemical deposition of 11-mercaptoundecanoic acid monolayer on HMDE and on the electrodes based on solid amalgams – the polished silver solid amalgam electrode (p-AgSAE), the mercury film covered silver solid amalgam electrode (MF-AgSAE), the mercury meniscus covered silver solid amalgam electrode (m-AgSAE), the mercury meniscus covered bismuth-silver solid amalgam electrode (m-BiAgSAE), the mercury meniscus covered copper solid amalgam electrode (m-CuSAE) and the mercury meniscus covered cadmium solid amalgam electrode (m-CdSAE) was studied. Statistical results of repeated preparations of thiol monolayer and its subsequent desorption confirm that amalgam electrodes are a suitable instrument to study the electrochemical properties of thiol films. Moreover, two electrodes (MF-AgSAE and m-AgSAE) were used for preparation of impedimetric biosensors for determination biotin and biotin labeled albumin.

Finally tree types of flow amperometric enzymatic biosensors were designed and fabricated. Two of them are based on the enzymatic reactor and the tubular detector mentioned above. In the first case, the enzymatic reactor is based on *porous* silver solid amalgam. The silver amalgam was modified by thiol 11-mercaptoundecanoic acid. The immobilization of enzyme glucose oxidase at thiol layer was carried out using EDC/NHS chemistry. The biosensor was then successfully used for the determination of glucose in commercial honey.

In the second case, the enzymatic reactor contained *powdered* silver solid amalgam. The amalgam powder was modified by 4-aminothiophenol and enzyme was attached via crosslinking agent glutaraldehyde. Five different biosensors with ascorbate oxidase, glucose oxidase, catalase, tyrosinase, and laccase were prepared for the determination of ascorbic acid, glucose, hydrogen peroxide, catechol, pyrogallol, and dopamine. The biosensor with ascorbate oxidase was used for the determination of ascorbic acid in the vitamin tablets Celascon®.

The last biosensor was constructed using polished silver amalgam electrode which was covered by layer of chitosan. Then, the enzyme sarcosine oxidase was immobilized at the surface of the chitosan via crosslinking agent glutaraldehyde. Thus prepared biosensor was used for determination of sarcosine in model samples.

Abstrakt

Tato disertační práce představuje nové možnosti využití amalgámových elektrod zejména při konstrukci elektrochemických biosenzorů. V první řadě byl navržen a zkonstruován tubulární detektor na bázi stříbrného pevného amalgámu pro stanovení redukujících se analytů v průtokových systémech. Nejdříve byl testován při ampérometrickém stanovení modelových roztoků Cd^{2+}, Zn^{2+} a 4-nitrophenolu pomocí injekční průtokové analýzy. Výsledky ukázaly, že navržený tubulární detektor představuje jednoduché a levné zařízení s dobrou opakovatelností, citlivostí a dlouhodobou stabilitou minimálně 2 roky. Jednou z hlavních jeho výhod je možnost měření při vysokých negativních potenciálech (~ –2 V ve vodném prostředí). Poté byl tubulární detektor úspěšně použit pro stanovení účinné látky lomustinu v chemoterapeutickém léčivu CeeNU® Lomustine metodou průtokové diferenční pulsní voltametrie.

Pro stanovení v průtokových systémech byly taktéž navrženy a 14 měsíců testovány miniaturní referentní elektrody – kalomelová (SCE-AgPA), merkurosulfátová a merkuroxidová na základě stříbrného pastového amalgámu. Všechny referentní elektrody se ukázaly být stabilní po celou dobu zkoumání. Největší odolnost proti polarizaci byla zaregistrována u SCE-AgPA, a proto právě tato referentní elektroda byla použita pro všechny experimenty uvedené v této práci.

Dalším předmětem zkoumání byla elektrochemická depozice monovrstvy thiolu kyseliny undekanthiolové na následujících elektrodách: leštěné pevné stříbrné amalgámové elektrodě (p-AgSAE), stříbrné amalgámové elektrodě pokryté rtuťovým filmem (MF-AgSAE), stříbrné amalgámové elektrodě pokryté rtuťovým meniskem (m-AgSAE), bizmutové stříbrné amalgámové elektrodě pokryté rtuťovým meniskem (m-BiAgSAE), měděné amalgámové elektrodě pokryté rtuťovým meniskem (m-CuSAE), kadmiové amalgámové elektrodě pokryté rtuťovým meniskem (m-CdSAE) a HMDE. Výsledky statistického vyhodnocení přípravy thiolové monovrstvy a její desorpce z povrchu elektrod potvrdily, že dané amalgámové elektrody jsou vhodné pro studium elektrochemických vlastností thiolových vrstev. Dvě elektrody MF-AgSAE a m-AgSAE modifikované undekanthiolovou kyselinou pak byly úspěšně použity pro přípravu impedančních biosenzorů pro stanovení biotinu a biotinem značeného albuminu.

Nakonec byly navrženy a připraveny tři typy ampérometrických enzymatických biosenzorů. První dva se skládají z enzymatického reaktoru a výše zmíněného tubulárního detektoru. V prvním případě je enzymatický reaktor z porézního stříbrného pevného amalgámu modifikovaného kyselinou undekanthiolovou. Enzym glukozoxidáza byl imobilizován na thiolové vrstvě kroslinkingových činidel EDC/NHS. Takto vytvořený biosenzor byl použit ke stanovení glukozy v komerčně vyráběném medu.

Ve druhém případě je enzymatický reaktor plněn práškem z pevného stříbrného amalgámu modifikovaným 4-aminothiophenolem. Enzym byl pak navázán přes kroslinkingové činidlo glutaraldehyd. Pro přípravu pěti různých biosenzorů byly použity následující enzymy: askorbatoxidáza, glukozoxidáza, kataláza, tyrozináza a lakáza pro stanovení kyseliny askorbové, glukozy, peroxidu vodíku, katecholu, pyrogalolu a dopaminu. Glukoza byla dále stanovena i v tabletách Celasconu®. Pro poslední biosenzor byla použita klasická leštěná stříbrná pevná amalgámová elektroda pokryta vrstvou chitosanu s navázaným enzymem sarkozinoxidázou přes glutaraldehyd pro stanovení sarkozinu v modelovém roztoku. Všechny biosenzory vykazovaly dobrou opakovatelnost, citlivost a linearitu koncentračních závislostí.

2. Introduction

Flow analytical techniques as flow injection analysis (FIA), electromigration methods or HPLC makes an important part of instrumental analysis. As in all techniques, detection plays a crucial role in the flow manifold. Depending on the type of analyte, on financial aspects and other criteria, detection methods as UV/VIS, fluorescence spectrometry, mass spectrometry and other are available. However, electrochemical detection comprises a very promising alternative. This type of detection belongs to relatively inexpensive analytical methods and at the same time it is sufficiently sensitive for clinical, pharmaceutical or environmental analysis. Moreover, in comparison to other detection techniques, electrochemical electrodes/detectors are relatively easy to miniaturize and they provide wide possibility of geometric arrangements and chemical modifications.

For measurements in flow systems, there are several electrochemical methods available such as amperometry, voltammetry, potentiometry and others. For this purposes variety types of manifolds, flow cells or flow electrodes of special shapes designed for measurements in flow are available. So in next few pages the overview of the most frequently employed electrochemical techniques, electrodes and their designs used in flow-based systems will be presented.

Undoubtedly, the most frequently investigated, described and applied electrochemical technique in HPLC, FIA and CZE is amperometry where the current is measured as a function of time at optimal constant potential applied to the working electrode. Just the optimization of potential of detection is one of the most important steps because it helps to achieve a good sensitivity and in some cases even reasonable selectivity of analysis. Amperometry has gained its popularity probably because this electrochemical mode (compared to voltammetric techniques) is easily adaptable and simple for using in flow systems. In literature, two basic types of cell design predominate. First, one is wall-jet arrangement where the flow stream jets perpendicularly towards the electrode surface. The second design is thin-layer where flow is heading along the electrode surface. For both of them different types of electrode materials are available. In the first place, there are various types of carbon electrodes. Typical examples are determination of sulfamethizole in pharmaceutical and urine samples on a carbon paste electrode in wall-jet cell by HPLC [1], benzocaine in selected pharmaceuticals on the carbon paste electrode by FIA and HPLC in wall-jet cell [2] or cyanobacierial (blue-green algal) peptide toxins on the glassy carbon in lake water by HPLC [3]. Amperometric detection with glassy carbon was used for fast and simultaneous detection of prominent natural antioxidants including examples of flavonoids and vitamins by capillary electrophoresis [4], selected phenolic compounds on carbon fibers were determined by HPLC [5]. An integrated on-capillary tubular electrochemical detector for capillary electrophoresis systems has been fabricated based on sol-gel technique for determination of dopamine, norepinephrine and catechol. It consists of a sol-gel carbon composite tubular electrode attached permanently onto the outlet of the separation capillary [6]. Properties of electrode surface are also commonly improved by chemical modifications. For example glassy carbon electrodes can be modified by carbon nanotubes [7], bismuth nano-film [8], poly-methylene blue [9] or poly-hematoxylin [10]. Further carbon-based electrodes are boron-doped diamond electrode film electrodes. They have gained popularity in flow systems as well in batch arrangements due to their low and stable background current over a wide potential range, corrosion and fouling resistance, high thermal conductivity, and high

current densities. The varieties in their fabrication, construction, and applications in organic electrochemistry are presented in review of Pecková[11]. The microcrystalline boron-doped diamond electrode in a thin-layer and wall-jet amperometric detection cell was used for determination of 2-, 3-, and 4-aminobiphenyls in model samples of the synthetic colorant tartrazine by HPLC. Results show that higher current densities and higher noise was observed in thin-layer arrangement. On the other hand a wall-jet arrangement provided a slightly wider linear dynamic range of calibration dependencies and lower quantitation limits (in range of 10^{-8} mol dm^{-3}) [12]. Further examples are determination of biomarker of exposition to polycyclic aromatic hydrocarbons (1-hydroxypyrene) in human urine in wall-jet cell by HPLC [13], metoclopramide by FIA [14], catechol estrogens by HPLC [15] or estrogenic compounds by FIA [16]. Above mentioned electrode materials are used mostly for oxidation processes, whereas possibilities of the determinations in cathodic region are significantly limited. Mercury electrodes are the most convenient for analysis of reducible compounds because of their well-known advantages: the high hydrogen overpotential and the easy surface maintenance. Some examples of using mercury electrodes for amperometric measurements are given in papers [17, 18]. However, nowadays they are less frequently used probably due to instability of mercury drop in flow and because of some fear of mercury toxicity. Fortunately the solid and paste amalgam electrodes represent the adequate substitution. They allow cathodic measurements at potentials up to –2 V in aqueous solutions and to construct electrodes/detectors of necessary shapes and sizes. In our laboratory, electrodes of silver solid amalgam have been previously used in a batch arrangement [19], in wall-jet, thin layer or in microcylindrical design for HPLC and FIA [20-24] of electrochemically reducible organic compounds. In this work we presented the tubular detector based on silver solid amalgam for measurements in flow systems (chapter 4.1., and 8. Appendixes/Publication 1-2). It represents a miniature detector with a simple, robust and inexpensive construction which provides sufficient sensitivity and a good repeatability of measurements even at highly negative potentials. Moreover, it was used as a part of enzymatic biosensors (chapter 4.4. and 4.5., 8. Appendixes/Publication 5-6). A good stability at negative detection potentials was also observed at a tubular bismuth film electrode, installed as part of a multisyringe flow injection system. It was used as an amperometric detector to determine the concentration of diclofenac sodium in pharmaceutical formulations [25].

Moreover, electrodes of pure metals are still used. A traditional electrode material is platinum. Platinum electrodes in microcylindrical and tubular arrangements were compared as working electrodes for amperometric detection of 2-aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, and 2-aminonaphthalene by HPLC. Because of factors influencing separation efficiency microcylindrical arrangement are favorable. On the other side, tubular arrangement exhibits higher sensitivities and lower limits of detection [26]. Li and col. described the preparation of a platinum electrode which permits the formation of a platinum tubular electrode inside the capillary, immediately at its outlet and used it in micro liquid chromatography [27]. The suitability of a simple amperometric platinum tubular detector for HPLC analysis of selected phenolic acids is reported by Jirovský [28]. Next example of using platinum tubular detector in conventional and micro-HPLC systems was published by Cvačka [29].

The classical amperometry can be improved by application of a pulse polarization on the working electrode. Then we speak about pulse amperometry. It offers the possibility of cleaning and reactivating the electrode surface effectively after the measuring cycle. In the simplest implementation of pulse amperometry, the potential of the working electrode is stepped between

the potentials for detection, cleaning, and reactivation [30]. The main advantage of such way of carrying measurements is a significant enhancement of the detection sensitivity for compounds considered as non-electroactive for detection under constant applied potential and with poor optical properties for spectrophotometric detection. This concerns numerous polar aliphatic compounds (carbohydrates, amines, thiols) and macromolecules of biological importance (peptides, proteins) [31].

A potentiometry is the next detection electrochemical method, which is used in flow systems. It has a wide applicability especially in flow injection analysis and numerous applications in capillary electrophoresis. The main directions of development of potentiometric detection in modern flow analysis are construction of new chemical sensors (indicating electrodes) and biosensors for flow applications, development of new designs of flow-through detectors, and whole flow measuring systems (manifolds) ensuring appropriate sample processing prior to reaching the detector. The use of ion-selective electrodes coupled with flow systems have found wide applications because these methods are usually fast, highly selective, highly sensitive, portable, and do not require extensive training of the personal [32, 33].

Application of voltammetric (and amperometric) measurements in flow systems are even more broadly described in analytical literature than potentiometric measurements. One of the reasons is that voltammetry offers several techniques from which we can choose the best one for determination of concrete analyte(s). For example, square-wave and pulse voltammetry is often used in connection with flow-based techniques. The stripping voltammetry allows very sensitive detection especially of trace amount of metals and some organic compounds. In general, flow voltammetric methods offer some significant advantages compared to traditional batch design. Due to the nonzero flow rate of the sample, the convection importantly contributes to the total mass transfer and the signal is higher. Reproducibility can be better because continuous streaming of the solution can facilitate the removal of interfering products of electrode reactions from the working electrode surface. In one part of this thesis (chapter 4.1., 8. Appendixes/Publication 2), using of non-stop-flow differential pulse voltammetry for determination of chemotherapeutic drug lomustine is described. Results showed that the signal of analyte considerably increases with increasing flow rate. It is the confirmation of the fact that the voltammetry under hydrodynamic conditions is more sensitive that stationary one. In practice, hydrodynamic voltammetry is employed in combination with FIA and rarely with HPLC or capillary electrophoresis. Applicability of the tubular gold electrode is evaluated in the speciation of Sb(III) and Sb(V) using anodic stripping voltammetry in a single flow manifold [34]. In order to reduce the sample consumption and waste generation for electrochemical purposes, a screen-printed electrode used for electrodeposition of bismuth film and a thermostated electrochemical flow cell were developed for determination of Cd^{2+} and Pb^{2+} ions in natural, waste and tap water samples by square-wave anodic stripping voltammetry [35]. Silva and col. described the construction of a Nafion coated glassy carbon tubular electrode, coupled to a multi-commutated flow system, for the voltammetric determination of acetaminophen (paracetamol) in serum and pharmaceutical formulations. The modification of the electrode enhanced the analytical signal intensity and, simultaneously, prevented the electrode surface fouling [36]. Another work described the optimization of a sequential injection method to automate the determination of paraquat by square-wave voltammetry employing a hanging mercury drop electrode [37].

On previous examples we wanted to demonstrate an extensive application of electrochemical methods in flow systems in different fields. Another examples of electrochemical detection coupled with high-performance liquid chromatography, capillary electrophoresis, flow injection analysis and other flow techniques such coulometry or conductimetry are described in reviews [30-33, 38-40].

Except working electrode, the electrochemical cell also contains a reference and an auxiliary electrode for three-electrodes arrangement or only the reference electrode in a two-electrodes arrangement. While the working electrode is the electrode at which the reaction of interest occurs, the reference electrode provides a stable and reproducible potential (independent of the sample composition), against which the potential of the working electrode is compared [41]. So there is no doubt that the reference electrode is the important part of each two- or three electrode system. It plays an important role in ensuring proper device operation, reliability of the results, and it has a pronounced influence on the accuracy of measurements. The requirements for functional and robust reference electrode can be summarized as follows [42]:

1. The reference electrode must have a high exchange current density, must be reversible and non-polarizable.
2. The electrode reaction should not consume the electrode or change its surface area in any way, so as not to change the reaction area and hence the current.
3. The inner filling solution in contact with the reference electrode should be saturated. It is mainly because evaporation of the solvent and any minute fluctuation in the concentration of a saturated solution will not change the electrochemical potential.
4. The reference electrode should be constructed so that it does not contaminate the analyzed solution by compounds disturbing a measurement.
5. The liquid junction potential should be the minimum possible, and should be constant in time.
6. Characteristics of the reference electrode must be reproducible.

The primary reference electrode in electrochemistry is the standard hydrogen electrode (SHE). The standard potentials of all other reference electrodes are linked to that of the SHE at the same temperature. The SHE contribution to the cell potential is by convention zero at all temperatures [43]. It consists of a platinum electrode in contact with a solution of H^+ at unit activity and saturated with H_2 gas with a fugacity referred to the standard pressure of 10^5 Pa [44]. The scheme and half-cell reaction of the SHE is as follows:

$$Pt\,|\,H_2\ (10^5\ Pa),\ H^+\ (aq)\ (a = 1)$$

$$2\ H^+\ (aq) + 2\ e^- \leftrightarrow H_2\ (g)$$

However, due to the complexity and fragility of its construction as well as the necessity to keep all the conditions strictly constant, SHE is not convenient for practical measurements and it is applied only when determination of reliable thermodynamic values, for example the standard potentials, is necessary [45].

For common measurements there are so called second kind electrodes which are composed of metal covered by layer of its hardly soluble salt immersed into a solution contained an anion of this salt (filling solution). The potential of these reference electrodes depends on the concentration of the filling solution and temperature.

One of the most frequently used reference electrodes in modern electrochemistry is probably silver–silver chloride–potassium chloride electrode (Ag|AgCl, KCl) due to its simple

construction. It consists of a silver wire covered by solid silver chloride which is immersed into the solution of potassium chloride. The potentials of Ag|AgCl, KCl depending on the concentration of the filling solution are given in Table 1. In a highly concentrated KCl, the flow of current which causes a change in chloride content, will not affect its potential and it is the reason for this electrode's stability.

Another commonly used reference electrode is a calomel electrode ($Hg|Hg_2Cl_2|KCl$). The calomel electrode belongs to the group of reference electrodes formed from mercury and an excess of sparingly soluble mercurous salt. It was introduced by Ostwald in 1890 and it is now the most popular mercury reference electrode [46]. The calomel electrode is composed of metallic mercury and mercury(I) chloride Hg_2Cl_2 in contact with KCl solution. Mercury has properties which are desirable for setting up well-behaved electrode systems. It is noble, liquid metal, easy to purify, and therefore easy to obtain in a standard state with properties quite independent of its chemical, mechanical or thermal history. This is a considerable advantage over any solid metal, even a soft one such as lead. Mercury also forms a large number of mercurous compounds, suitably well-crystallized and of low solubility in water, with properties that fit them to act as the second phase of electrodes of the second kind [47]. If it is necessary to avoid the presence of chloride anions in the analyzed solution, it can be used mercury–mercurous sulfate electrode ($Hg|Hg_2SO_4|K_2SO_4$ sat.) or mercury–mercury oxide electrode ($Hg|HgO|1$ mol dm^{-3} NaOH). The mercury–mercurous sulfate electrode consists of metallic mercury coated with slurry of mercury(I) sulfate. This reference electrode is frequently used due to its outstanding reproducibility, following only the hydrogen electrode [46].

Mercury-mercury oxide electrode (Hg|HgO|NaOH) is the next mercury reference electrode has been known for rather long time. It is used as a reference, especially for higher concentrations of alkali solutions including higher temperatures. The use of such an electrode, for instance, in the study of corrosion, fuel, and storage cells, is especially advised [46]. Many others types of reference electrodes are described in monographs [47, 48].

The reference electrodes contain metallic mercury which is considered as a dangerous toxic compound and therefore its use is prevented or even prohibited in some states. On the other side, amalgams are used as a dental material for many decades and they are non-toxic. Following previous studies of amalgam electrodes and the voltammetric/potentiometric sensors, the reference electrodes of second kind based on silver solid amalgams have been successfully designed and tested in two publications [49, 50]. In this Ph.D. thesis, the reference electrodes based on silver and copper paste amalgam are presented. The principal difference between classical mercury reference electrodes and reference electrodes based on paste amalgams is that liquid mercury has been substituted by the paste of non-toxic silver and copper amalgam. The preparation and testing of: i) saturated calomel electrode based on silver paste amalgam, ii) silver paste amalgam–mercurous sulfate electrode, iii) silver paste amalgam–mercuric oxide electrode and iv) copper paste amalgam–copper(II) oxide electrode will be discussed in chapter 4.2., 8. Appendixes/Publication 3. Due to the current trend towards miniaturization of the working electrodes and detection cells also the reference electrodes have to adapt to this fact. Although the development of new technological approaches such as the thick film and thin film techniques supported arrangements with planar reference electrodes, special problems with miniaturized reference electrodes still exist, and there is extensive search for the conception of an integrated micro reference electrode [42, 51]. In our work, each of reference electrode based on paste

amalgam could be prepared in miniaturized version when the body of electrode is about 2 cm long. Even though such miniaturization is not radical, the most important is that they are a very stabile in time. The laboratory made saturated calomel electrode of silver paste amalgam is then used in all experiments carried out in this work.

Table 1. Overview of selected reference electrodes [48, 51]

Reference electrode	Half-cell reaction	Filling solution	Temperature, ° C	Potential vs. SHE, V
Silver–silver chloride–potassium chloride electrode	$Ag \mid AgCl, KCl ([Cl^-])$ $AgCl + e^- \leftrightarrow Ag^0 + Cl^-$	3 mol dm^{-3} KCl	20	+0.2105
			25	+0.2070
			40	+0.1961
		sat. KCl	20	+0.2019
			25	+0.1970
			40	+0.1814
Calomel electrode	$Hg\|Hg_2Cl_2\|KCl ([Cl^-])$ $Hg_2Cl_2 + 2\ e^- \leftrightarrow 2\ Hg^0 + 2\ Cl^-$	1 M KCl	25	+0.280
		sat. KCl	20	+0.244
			25	+0.241
			40	+0.231
Mercury–mercurous sulfate electrode	$Hg\|Hg_2SO_4\|K_2SO_4$ sat. $Hg_2SO_4 + 2\ e^- \leftrightarrow 2\ Hg^0 + SO_4^{2-}$	sat. K_2SO_4	25	+0.6125
			35	+0.6039
			45	+0.5947

Nowadays modification procedures of working electrodes play a very important role. It is partly due to effort to improve the properties of electrodes such as selectivity, sensitivity or corrosion prevention and partly because it enables the preparation of biosensors which are indispensable in clinical, environmental and others routine analysis requiring high selectivity. Literature offers numerous modification techniques depending on the type of modified surface (mercury, carbon, metals, amalgams, glass, etc.) or on the chemical substances by which the surface will be modified (fatty acids, organosilicon derivatives, thiols, etc). On next few pages the formation of thiols monolayers on the different metal electrode materials by self-assembly method and electrodeposition will be discussed.

Substances those containing the –SH group (thiols, R–SH) or –S–S– group (disulfides, $R_1S–SR_2$) can spontaneously adsorb on various surfaces and form stable monolayer films (SAM – self-assembled monolayer) [52]. The self-assembly method has been recognized as one of the most attractive approaches to create well-defined functional molecular layers on electrode surfaces [53]. Monolayers can be prepared using physisorption or chemisorption. In the latter case, monolayer is strongly bonded with the electrode material by covalent bonds and it is long-term stable. It could be realized at gold [54-57], silver [58, 59], platinum [60, 61], palladium [62, 63], copper [64-66], mercury [67-70] and others surfaces. Thiols have a high affinity for the surfaces of noble and coinage metals makes it possible to generate well-defined organic surfaces with useful and highly alterable chemical functionalities displayed at the exposed interface [71].

The most studied thiols SAMs are at the gold. Due to its ease of preparation and well-defined order and also the relative inertness of the substrate, which makes it comparatively easy to clean, thiols on gold have become a model system for SAMs [72]. The most studied substrate for SAMs of thiolates next to gold is silver. It gives high-quality SAMs with simpler structure than gold, but it also oxidizes readily in air and it is toxic for cells [71]. Other substrates suitable for formation of SAMs are amalgams. Amalgam electrodes of different metals were first used by us to create monolayer thiol films [52]. For these purposes, solid

[19, 73] or paste [74-76] amalgams are the most convenient. Electrodes of solid amalgams (MeSAE; where Me is Ag, Au, Cu, Bi, etc.) can be well polished, they do not contain liquid mercury, and they also have similar properties as mercury [19, 77]. Solid amalgams are well wetted by mercury and hence they can be easily covered by mercury meniscus (m-MeSAE) [19, 78] or by mercury film (MF-MeSAE) [79, 80]. These electrodes have ideally smooth and easily renewable surfaces, which ensure preparing a monolayer with a small number of defects. Similar surface is provided by mercury itself, for example in the form of hanging mercury drop electrode (HMDE). Moreover, while mechanical instability of the liquid mercury drop limits the applicability of HMDE [81], the mercury-covered solid amalgams appear to be suitable for the preparation of MLs-based biosensors. In this Ph. D. thesis we have studied monolayer formation of 11-mercaptoundecanoic acid at polished, mercury meniscus and mercury film covered amalgam electrodes (p-AgSAE, m-AgSAE, MF-AgSAE, m-CuSAE, m-BiAgSAE, m-CdSAE). Then p-AgSAE with thiol monolayer was used for preparation of biosensor for determination of biotin and biotin-labeled substances (chapter 4.3., 8. Appendixes/Publication 4). Also further biosensors prepared in this Ph. D. thesis are based on immobilization of enzymes at the surfaces of porous and powder silver solid amalgams modified by 11-mercaptoundecanoic acid and 4-aminothiophenol, respectively (chapter 4.4. and 4.5., 8. Appendixes/Publication 5 an 6).

SAMs are typically prepared by immersing cleaned substrate into the solution of thiols of ~ millimolar concentration over night at lab temperature. Dense coverages of thiol forms relatively quickly, but organization process between molecules can takes hours. Another way of thiol monolayer formation is electrodeposition using cathodic stripping voltammetry (CSV). In general, CSV is a very sensitive electrochemical method by which it is possible to determine number of thiosubstabces. But the similar approach also can be used for a deposition of a compact monolayer of thiols on amalgams, gold [82], silver [83] and mercury [84]. In this method, the thiosubstance is accumulated at the electrode surface by oxidation of the electrode material at a suitable potential, and the created metal (M) cations bind covalently with sulfur in –SH group(s) [52] according equation [71]:

$$R–SH + M \rightarrow RS–M + H^{+} + e^{-}$$

This process is reversible thus depending on conditions it is possible both to create and to remove the monolayer from electrode surface. Even though the self-assembled method is most frequently used, both the contact angle and the infrared reflection spectroscopic characterization of the complete thiolate monolayers at gold show qualitative similarities of the electrodeposited and the self-assembled monolayers [85]. Moreover, the electrochemical deposition is very quickly and it ensures a good repeatability of the monolayer formation due to a computer driven appropriate electrodeposition program. In this work we have used both methods for biosensor preparation. The thiol monolayer was formed at electrode or in porous and powder reactors which do not have an electric contact.

Sullivan and col. published an overview about classes of organic reactions that have been performed on SAMs for modifying the terminal groups, e.g. oxidation, nucleophilic substitution, acylation, nucleophilic addition ant others [86]. Reactions on monolayers have already been commercially exploited. Work of Pirrung describes one of the widely used way to synthesize so-called DNA chips by multistep reactions on self-assembled monolayers [87]. Modification of monolayers allows to prepare various types of biosensors based on a selective interaction

between a compound, featuring a biorecognition element, linked to a SAM surface and an analyte. Such compounds can be DNA or RNA [70, 88, 89], enzyme [90-92], antibody [93, 94], (strept)avidin or biotin [95], etc. Monolayers can serve as first layer of model phospholipid membranes [81, 96]. A broad overview of the use of MLs at electrodes is given in reviews [96-98].

According to a recently proposed IUPAC definition, the biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured [99]. A transducer is used to convert (bio)chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. The intensity of generated signal is directly or inversely proportional to the analyte concentration.

Biosensors can be categorized depending on the used transducer or the biochemical receptor. According to the transducing elements, biosensors are classified as electrochemical (amperometric, potentiometric, conductimetric or impedimetric), optical (fluorescent, luminescent, refractive), piezoelectric (acoustic, microcantilever) and thermal [100]. As biochemical receptors enzymes, proteins, nucleic acids, antigens/antibodies, whole cells or plant and animal tissues are widely used [101].

One of the founding fathers of the field of biosensors was American biochemist Leland C. Clark Jr. In 1962 he with Lyons made the first prototype glucose biosensor by immobilizing the glucose oxidase on his oxygen electrode – Clark electrode which he has invented in 1956 [102]. Since that, biosensors have faced a massive growth in usage for various applications such as diagnostic, quality control, agriculture and veterinary medicine, environmental protection, medical applications, drag production and others.

Among electrochemical biosensors the amperometric ones are the most widespread [101] and the use of enzymes as biorecognition element is most frequent. Nevertheless, the use of enzymes has some disadvantages such as high price of some enzymes, relatively short operation lifetime, or negative effect of inhibitors. On the other side, the immobilized enzymes provide one very important advantage – depending on enzyme's specificity, they have ability to recognize monitored analyte or group of analytes (substrates) with high selectivity and catalyze their transformation. These unique properties make the enzymes powerful tools to develop analytical devices [103-106]. The amperometric biosensors find employment in various fields. From a practical and commercial point of view four enzymatic biosensors have been widely used [107]: for determination of glucose (diagnosis and treatment of diabetes, food science, biotechnology) [108-110], lactate (sports medicine, critical care, food science, biotechnology) [111-114], urea (clinical applications) [115-118], and glutamate/glutamine (food science, biotechnology) [119-124].

In publications presented in this Ph. D. thesis (chapter 4.4.–4.6., 8. Appendixes/Publication 5-7) we have paid attention to amperometric biosensors with enzymes as biological recognition systems. An example of impedimetric biosensor can be found in chapter 4.3. (8. Appendixes/Publication 4). The main concept of our first type of enzymatic biosensors consists

in the use of two parts – a flow reactor where the enzymatic reaction takes place, and a tubular flow detector where increasing or decreasing of concentration of some component of this enzymatic reaction is measured. Both the reactor and the detector are based on silver solid amalgam but of different types. While tubular detector is made from compact smooth amalgam, the consistence of silver solid amalgam of the reactor is porous or powdered. It demonstrates wide possibilities of solid amalgams. It is necessary to note that the large surface of the enzymatic reactor can be provided by various porous or powdered materials, e.g., by porous carbon felt [125], by small particles of organofunctionalized silica [126], by aminoaryl [127] or by aminopropyl [128, 129] controlled pore glass, by the immobilizing agent Eupergit C 250 L (oxirane acrylic beads) [130], by chitosan porous beads [131, 132], by porous agarose beads [133], by nylon membrane coated with a thin layer of gold [134] or by microporous gold [135]. Detailed overview of utilized reactors and biosensors is given in the paper [136]. Two above mentioned biosensors based on porous and powdered amalgams were applied for determination of glucose, sodium ascorbate, hydrogen peroxide, catechol, pyrogallol, and dopamine. Second type of electrochemical flow biosensor is also based on silver solid amalgam which is shown to be reliable and easy to prepare. It consists of the electrode of polished compact silver solid amalgam and as in previous case it is also intended for analysis in flow systems, namely in flow injection analysis with wall-jet cell. The main part of this biosensor is the classic pen-type electrode mechanically covered by layer of chitosan in which the enzyme sarcosine oxidase is immobilized via crosslinking agent glutaraldehyde.

The amperometric biosensors can be divided into mediatorless amperometric biosensors and mediator amperometric biosensors. In the mediatorless amperometric biosensors the concentration of electroactive reagents or products of an enzymatic reaction are directly measured at the electrode surface amperometrically. Biosensors containing oxidoreductases belong to this group.

For example in our work we have used ascorbate oxidase (AscOx), glucose oxidase (GOx), sarcosine oxidase (SOx), catalase (Cat), laccase (Lac) and tyrosinase (Tyr). The simplified equations of enzymatic reactions of these enzymes are following:

2 L-Ascorbic acid + $\mathbf{O_2} \xrightarrow{AscOx}$ 2 L-Dehydroascorbic acid + 2 H_2O

D-Glucose + H_2O + $\mathbf{O_2} \xrightarrow{GOx}$ D-Gluconic acid + H_2O_2

Sarcosin + $\mathbf{O_2}$ + $H_2O \xrightarrow{SOx}$ Glycine + Formaldehyde + H_2O_2

2 $H_2O_2 \xrightarrow{Cat}$ $\mathbf{O_2}$ + 2 H_2O

Phenol containing compound + $O_2 \xrightarrow{Lac}$ **corresponding Quinone** + H_2O

Catechols or cresol + $O_2 \xrightarrow{Tyr}$ **corresponding Quinone** + H_2O

During enzymatic reaction of AscOx, GOx and SOx the concentration of substrates can be determined via decreasing concentration of oxygen, in the case of Cat via increasing concentration of oxygen, and in the case of Lac and Tyr the concentration of substrates are

directly proportional to the concentration of quinones which are easily reducible compounds and can be determined by silver solid amalgam detector. In general, determination via the increasing concentration of hydrogen peroxide by oxidation at potential about +600 mV at for example carbon or platinum electrodes is also possible, but there are some metabolites such as uric acid, ascorbic acid, glutathion etc. which are also oxidized and interfere with the electrochemical signal. It is therefore essential to apply an electrode potential as low as possible. Therefore, electrochemically active electron acceptors to which the enzyme can donate electrons were looked for. In this context, some artificial electron acceptors having low oxidation potentials were discovered and they are commonly called mediators. This approach leads to a considerable reduction of electrochemical interferences and the development of mediated biosensors [137]. In general, a mediator is a low-molecular weight particle, which transfers electrons between redox center of an enzyme and a working electrode. During the catalytic reaction, the mediator first reacts with the reduced enzyme and then diffuses to the electrode surface to undergo rapid electron transfer [137]. The most common and well-known mediators are ferricyanide and ferrocene. Liao and col. presented a disposable amperometric ethanol biosensor based on screen-printed carbon electrodes mediated with ferricyanide-magnetic nanoparticle mixture. Results showed that the presence of ferricyanide nanoparticles could enhance the peak currents of redox species [138]. A biosensor based on a horseradish peroxidase enzyme-capsaicin reaction mediated by ferrocene has been developed for the amperometric determination of capsaicin. With mediation by ferrocene, the biosensor could measure capsaicin concentrations at a potential 0.22 V (vs. Ag/AgCl), which prevented potential interference from other electroactive species in the sample [139]. Another examples of mediators are organic dyes such as Prussian blue or methylene blue. A biosensor based on screen-printed carbon electrode for the determination of hydrogen peroxide was fabricated with Prussian blue-modified hydroxyapatite (PB-HAP). PB-HAP was incubated with horseradish peroxidase. The synergistic effect between horseradish peroxidase and Prussian blue facilitated the electron-transfer process and retained the bioactivity of horseradish peroxidase [140]. Yao and col. reported a nano-array sensor for hydrogen peroxide where horseradish peroxidase and mediator methylene blue were co-immobilized on the surface of an indium tin oxide electrode. Methylene blue was capable efficiently shuttle electrons between horseradish peroxidase and the electrode [141].

There are also amperometric biosensors based on direct electron transfer between enzyme and electrode. The key features of these biosensors setting them apart from other amperometric sensors are catalytic nature of the process as a whole as well as direct electron transfer from the electrode toward the substrate molecule and vice versa across active center of an enzyme without any carrier [142]. The direct electron transfer of enzymes (proteins) with electrodes can be applied to study enzymes-catalyzed reactions in biological systems and this has developed into an electrochemical basis for the investigation of the structure of enzymes (proteins), mechanisms of redox transformations of enzyme (protein) molecules, and metabolic processes involving redox transformations. If an enzyme immobilized on an electrode surface is capable of the direct electron transfer and keeping its bioactivity, it can be used in biosensors and biofuel cells without the addition of mediators or promoters onto the electrode surface or into the solution [143]. This issue is described in detail in papers and reviews [143-146].

In literature, we can find variety of techniques of immobilization of enzymes on the solid surface, which is possible to categorize as physical and chemical interactions [105, 147]. The first type is based on the weak physical sorption between a supporting layer and enzyme. While

chemical interactions are performed by covalent bonds. Chemical methods offer more stable immobilized product than physical ones. They can be achieved for example by cross-linking. Crosslinking is a process of chemically joining two or more molecules by a covalent bond. Crosslinking agents (or cross-linkers) are molecules that contain two or more reactive ends capable or chemically attaching to specific functional groups on proteins or other molecules. In fact, protein chemical targets account for the vast majority of crosslinking and chemical modification techniques: primary amines ($–NH_2$, this group exists at the N-terminus of each polypeptide chain and in the side chain of lysine residues), carboxyls (–COOH, at the C-terminus of each polypeptide chain and in the side chains of aspartic and glutamic acids), sulfhydryls (–SH, in the side chain of cysteine), and carbonyls (–CHO, ketone or aldehyde groups can be created in glycoproteins by oxidizing the polysaccharide post/translational modifications). Cross-linkers are selected on the basis of their chemical reactivity (i.e., specificity for particular functional groups), compatibility of the reaction with the application, chemical specificity, spacer arm length, water solubility, and cell membrane permeability. The same (homobifunctional) or different (heterobifunctional) reactive groups, spontaneously reactive or photoreactive, can be used. EDC (*N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimid hydrochloride) and other carbodiimides are zero-length cross-linkers. They cause direct conjugation of carboxylates (–COOH) to primary amines ($–NH_2$) without becoming part of the final crosslink (amide bond) between target molecules. NHS esters contain reactive groups formed by EDC-activation of carboxylate molecules. NHS ester-activated cross-linkers and labeling compounds react with primary amines under slightly alkaline conditions (pH 7.2–8.5) to yield stable amide bonds. Although NHS (or sulfo-NHS) is not required for carbodiimide reactions, their use greatly enhances coupling efficiency [148]. The stability of the created NHS-ester is dependent on the acidity of the solution. The half-time of the ester hydrolysis is 4–5 h at pH 7. We were used EDC/NHS chemistry to attaching avidin and streptavidin (with $–NH_2$ groups) on the monolayer of 11-mercaptoundecanoic acid to prepare impedimetric biosensors based on mercury film modified silver solid amalgam electrode and mercury meniscus modified silver solid amalgam electrode for determination of biotin and biotin-labeled albumin, respectively (chapter 4.3., 8. Appendixes/Publication 4). The similar approach was applied to immobilize enzyme glucose oxidase on the 11-mercaptoundecanoic acid monolayer covered porous silver solid amalgam in reactor of biosensor for determination of glucose (chapter 4.4., 8. Appendixes/Publication 5).

Other examples are imidoester cross-linkers which react with primary amines to form amidine bonds. Maleimide-activated cross-linkers and labeling reagents react specifically with sulfhydryl groups (–SH) at near neutral conditions (pH 6.5–7.5) to form stable thioether linkages. Another examples of cross-linkers are haloacetyls, pyridyl disulfides, hydrazides or diazirines. Among the many available protein crosslinking agents, glutaraldehyde (1,5-pentanedial) has undoubtedly found the widest application in various fields [149, 150] such as cytochemistry [151], enzyme and protein technology [150, 152], immunochemistry [153-155], and many others. Glutaraldehyde is symmetrical linear molecule with two aldehyde groups. It is soluble in all proportions in water and alcohol, as well as in organic solvents. It reacts with several functional groups of proteins, such as amino, thio, phenolic and imidazole rings [156]. Wide-ranging detailed information about crosslinking can also be found in reviews [157-159].

Another method of covalent (or non-covalent) attachment is immobilization of the enzyme within the polymeric gel matrix. For these purposes the natural polymers such alginate, collagen, cellulose, pectin or chitosan as supports are commonly used [160]. Chitosan is widely used immobilization matrix. It is natural polyaminosacharide obtained by deacetylation of chitin which is the major constituent of the shells of crustaceans, the exoskeletons of insects and the cell walls of fungi [147]. Chitosan's linear polyglucosamine chains of high molecular weight have reactive amino and hydroxyl groups, amenable to chemical modifications. For electrochemical detection, chitosan has two very important advantages. Firstly, the chitosan layer is not electrochemical active in negative region and it is possible to apply potential up to about –2.0 V without influencing chitosan chemical structure. Second advantage is the electrolytic permeability of the chitosan hydrogel which allows diffusion of some components of the enzymatic reaction to the electrode surface. The preparation of the biosensor based on chitosan monolayer with immobilized of enzyme sarcosine oxidase via cross-linker glutaraldehyde is described in our last publication (submitted) (chapter 4.6., 8. Appendixes/Publication 7).

The presented Ph. D. thesis is focused on the construction of new type of the detectors/electrodes and enzymatic biosensors based on solid amalgams for detection of biological important compounds in flow systems. For these purposes, different techniques of modification of the electrode surfaces and enzymatic attachment were used. All results were published in following six scientific articles and in one submitted paper, which are attached as appendixes:

1. **Yosypchuk, O.**; Barek, J.; Yosypchuk, B.: *Tubular Detector of Silver Solid Amalgam for Electrochemical Measurements in Flow Systems*. Electroanalysis 24, 2230 – 2234 (2012).

2. **Josypčuk O.**, Barek J., Josypčuk B.: *Application of Non-stop-flow Differential Pulse Voltammetry at a Tubular Detector of Silver Solid Amalgam for Electrochemical Determination of Lomustine (CCNU).* Electroanalysis 26, 306–311 (2014).

3. Yosypchuk, B.; Barek, J.; **Yosypchuk, O.**: *Preparation and Properties of Reference Electrodes based on Silver Paste Amalgam*. Electroanalysis 23, 2226–2231 (2011).

4. Yosypchuk, B.; Fojta, M.; **Yosypchuk, O.**: *Thiolate Monolayers Formed on Different Amalgam Electrodes. Part II: Properties and Application*. Journal of Electroanalytical Chemistry 694, 84–93 (2013).

5. Josypčuk B., Barek J., **Josypčuk O.**: *Flow Electrochemical Biosensors Based on Enzymatic Porous Reactor and Tubular Detector of Silver Solid Amalgam.* Analytica Chimica Acta 778, 24–30 (2013).
6. **Josypčuk O.**, Barek J., Josypčuk B.: *Electrochemical Biosensors Based on Enzymatic Reactor of Silver Solid Amalgam Powder for Measurements in Flow Systems.* Electroanalysis 26, 1729–1738 (2014).

7. **Josypčuk O.**, Barek J., Josypčuk B.: *Construction and Application of Flow Enzymatic Biosensor Based of Silver Solid Amalgam Electrode for Determination of Sarcosine.* Electroanalysis (submitted).

3. Aims of the Work

The main aim of this Ph. D. thesis was a development and fabrication of electrochemical flow enzymatic biosensors based on silver solid amalgam. To meet the target it was necessary to realize the following steps:

- Design and preparation of the tubular detector based on silver solid amalgam
- Preparation and construction of minireactors filled by porous and powdered silver solid amalgam where enzymes would be attached
- Development of a methodology for a) covalent modification the silver amalgam surface by thiols; b) Covalent immobilization of enzymes at the thiol layer in the reactors and at the surface of the pen-type electrode
- Connection of enzymatic reactor with tubular detector to create a first type of biosensors
- Preparation of the second type of biosensors based on polished silver solid amalgam covered by chitosan with covalently immobilized enzyme
- The application of biosensors for the determination of biological active species in model and real samples in flow systems (flow injection analysis and voltammetry in flow)
- Design and construction of all flow cells and miniaturized reference electrode based on paste silver solid amalgam.

4. Results and Discussion

4.1. Tubular Detector of Silver Solid Amalgam

There is no doubt that electrochemical detection plays important role in combination with flow systems as FIA, HPLC or electromigration methods. For this purposes silver solid amalgam electrodes (AgSAEs) are commonly used in thin-layer, microcylindric or wall-jet arrangement. In this chapter an AgSAE in new tubular shape is presented. like to present, This novel type of electrode is designed just for electrochemical measurements in flow.

The scheme and photo of tubular detector is depicted in Fig. 1A and its preparation is described in detail in attached publication (chapter 8. Appendixes/Publication 1). Briefly, a silver powder was firmly pressed into the Teflon tube and a platinum contact was fixed in this silver powder column. A hole of the same diameter as the inner diameter of the inlet capillary was formed along the central part of the silver column. Into the Teflon tube with the silver column liquid mercury was added and after ca two hours an excess of mercury was removed. The piece of the inlet capillary was fixed by epoxy resin with the Teflon tube to touch the silver amalgam column. On outside of the amalgam column, 2 mm long piece of the Teflon capillary was glued. The inner diameter of the used tubular detector is the same (0.5 mm) as the inner diameter of connecting capillaries. It is necessarily to note that the solid amalgam forms a continuation of the inlet capillary, thereby any turbulent flow is effectively minimized.

Firstly, the tubular detector was tested under flow injection analysis conditions in amperometric mode. For this purpose model samples of Zn^{2+} and Cd^{2+} were selected as representatives of inorganic reducible analytes and 4-nitrophenol (4-NPh) as an organic one. In three-electrode system the tubular detector represented a working electrode, a saturated calomel electrode based on silver paste amalgam was a reference electrode (preparation and characteristics of this new reference electrode will be described in next chapter), and platinum wire was an auxiliary one. All three electrodes were placed in lab-made small flow glass cell (see Fig. 1B). During experiment injected sample was pumped through tubular detector where a reduction of analytes took place at the amalgam surface. Products of the reaction were flown away to a glass cell with reference and auxiliary electrodes and then to waste.

As it is typical for other arrangement of silver solid amalgam electrodes, at the beginning of each working day, an electrochemical activation of electrode surface was carried out in used supporting electrolyte at potential –2.200 V for 300 s. Then it was necessary to find a convenient composition of supporting electrolytes. Zn^{2+} and Cd^{2+} were determined in acetate buffer (AcB) (pH 4.8) while for reduction of nitro group of 4-NPh more acidic medium was needed so the solution of 0.01 mol dm^{-3} HCl was used. It was found that the concentration of supporting electrolyte plays an important role. FIA-ED recordings of Cd^{2+} obtained in 0.02, 0.10 and 0.50 mol dm^{-3} AcB (pH 4.8) showed that with increasing concentration of the buffer, the background current significantly increased (–4.7, –13.3, –27.5 μA) but the peak height (charge) decreased almost three times. It is supposed that the increasing of the background current is caused by increasing conductivity of the more concentrated buffer, whereas diffusion coefficient of the analyte decreases resulting in the decrease of the peak height (charge). Similar results were

obtained for HCl and 4-NPh. Due to this fact, 0.02 mol dm^{-3} AcB (pH 4.8) for Zn^{2+} and Cd^{2+} and 0.01 mol dm^{-3} HCl for 4-NPh were used for further measurements.

The interesting parameter and in the same time the main advantages of tubular detector is potential of detection. The optimal values for Zn^{2+}, Cd^{2+} and 4-NPh were –1.500 V, –1.700 V, and –1.200 V respectively. As it can be seen, the tubular detector can operates at relatively high negative potential without any problems. That was proved by good repeatability of the peak heights of 10 consecutive injections of each analyte. The RSD for Zn^{2+} and Cd^{2+} and 4-NPh were 0.8 %, 0.9 % and 0.8 % ($c_{Zn} = 7.7 \cdot 10^{-5}$ mol dm^{-3}, $c_{Cd} = 4.5 \cdot 10^{-5}$ mol dm^{-3} and $c_{4\text{-NPh}} = 3.6 \cdot 10^{-5}$ mol dm^{-3} (e. i. 5 ppm for all substances)).

Under the optimum conditions the calibration dependences were obtained over the concentration range $1.5 \cdot 10^{-6}$–$1.5 \cdot 10^{-4}$ mol dm^{-3} (0.10–10.0 ppm) for Zn^{2+}, $8.9 \cdot 10^{-7}$–$8.9 \cdot 10^{-5}$ mol dm^{-3} (0.10–10.0 ppm) for Cd^{2+} and $1.8 \cdot 10^{-6}$–$3.6 \cdot 10^{-5}$ (0.25–10.0 ppm) for 4-NPh. The dependences were linear for Zn^{2+} and Cd^{2+} in the whole concentration range with high correlation coefficients ($R^2 = 0.999$ for both cations). In the case of 4-NPh, the narrower linear range was obtained (only up to the concentration of 5.00 ppm ($3.6 \cdot 10^{-5}$ mol dm^{-3}; $R^2 = 0.999$). The calculated limits of detection were $1.4 \cdot 10^{-6}$ mol dm^{-3} (0.09 ppm) for Zn^{2+}, $7.0 \cdot 10^{-7}$ mol dm^{-3} (0.08 ppm) for Cd^{2+}, and $5.0 \cdot 10^{-7}$ mol dm^{-3} (0.07 ppm) for 4-NPh, respectively.

The effect of a length of the amalgam column on the signal was also investigated. Zinc cations were measured by tubular detector of three different lengths – 6.0, 7.5, and 13.5 mm. RSD of 10 consecutive measurements using tubular detector of each length were 0.3 % (6.0 mm), 0.6 % (7.5 mm), and 0.6 % (13.5 mm), respectively. Average values for all detectors ($I_p = -0.750 \pm 0.037$ μA; RSD = 4.9 %) indicate that the detector length does not have any significant effect on the peak height in the length range 6.0–13.5 mm.

For all substances degrees of conversion γ at the highest and at the lowest flow rates were calculated. Considering that Zn^{2+} and Cd^{2+} are reduced with the exchange of 2 electrons and 4-NPh of 6 electrons, following degrees of conversion were obtained (the results are shown in the form: γ(analyte; v[ml min^{-1}])): γ(Zn^{2+}; 3.00) = 0.2 %, γ(Zn^{2+}; 0.04) = 11.9 %, γ(Cd^{2+}; 3.00) = 0.6 %, γ(Cd^{2+}; 0.04) = 19.8 %, γ(4-NPh; 3.00) = 1.7 % and γ(4-NPh; 0.10) = 13.1 %. Therefore, it can be concluded that the tubular detector operated in the amperometric mode because in contrast to coulometric detectors (γ~100 %), only relatively low fraction of the analyte was electrochemically converted.

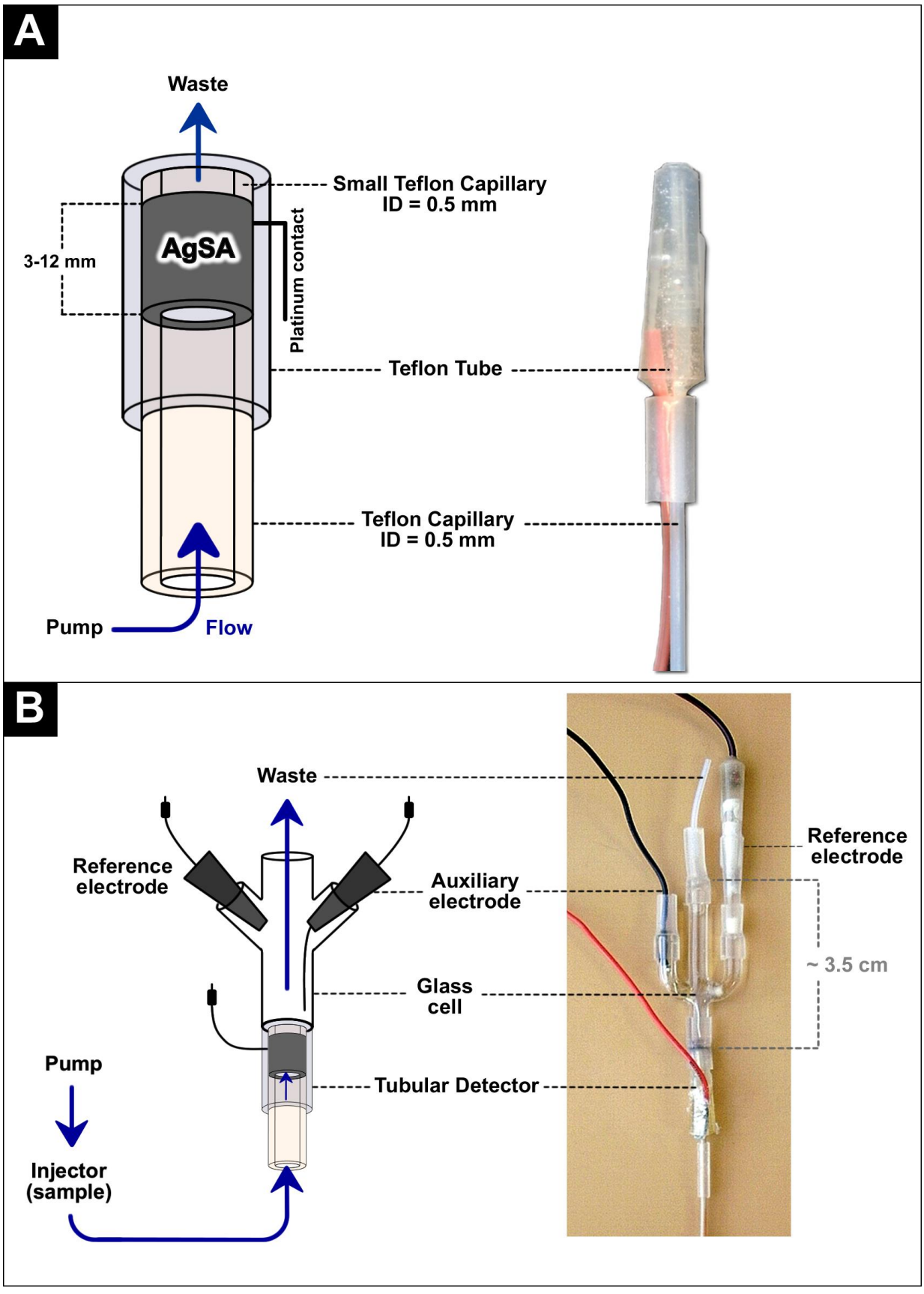

Fig. 1. (A) The tubular detector of silver solid amalgam (AgSA) and (B) experimental arrangement for amperometric measurements under conditions of flow injection analysis.

After previous experiments confirming that the prepared tubular detector provides a reliable and well repeatable signal, it was used for control of pharmaceutical preparation CeeNU® Lomustine (chapter 8. Appendixes/Publication 2). An active ingredient of this drug is lomustine which structural formula is depicted in Fig. 2A. Lomustine is an alkylating agent used in the treatment of brain tumors, lymphomas (including Hodgkin's disease), myeloma, lung cancer and melanoma [161]. In this case the voltammetric technique – non-stop-flow differential pulse voltammetry under conditions of flow injection analysis was used (Flow-DPV). In our arrangement of Flow-DPV, one of the six possible measured solutions was continually sucked by pump (withdraw regime) through tubular detector and the glass cell into the pump syringe (Fig. 2B). During measurements the sample flows with constant rate without stopping and DP-voltammograms are recorded. Flow-DPV or other voltammetric methods offer some significant advantages compared to traditional batch voltammetry. Due to the non-zero flow rate of the sample, the convection importantly contributes to the total mass transfer and the signal is higher. Reproducibility can be better because continuous flowing of the solution can facilitate the removal of interfering products of electrode reactions from the working electrode surface. This arrangement is saving time of the analysis partly due to relatively short distance between vessel with the sample and the tubular detector and partly due to continuous flow without stopping during measurement. Moreover, there is the possibility of rapid switch among six analyzed samples.

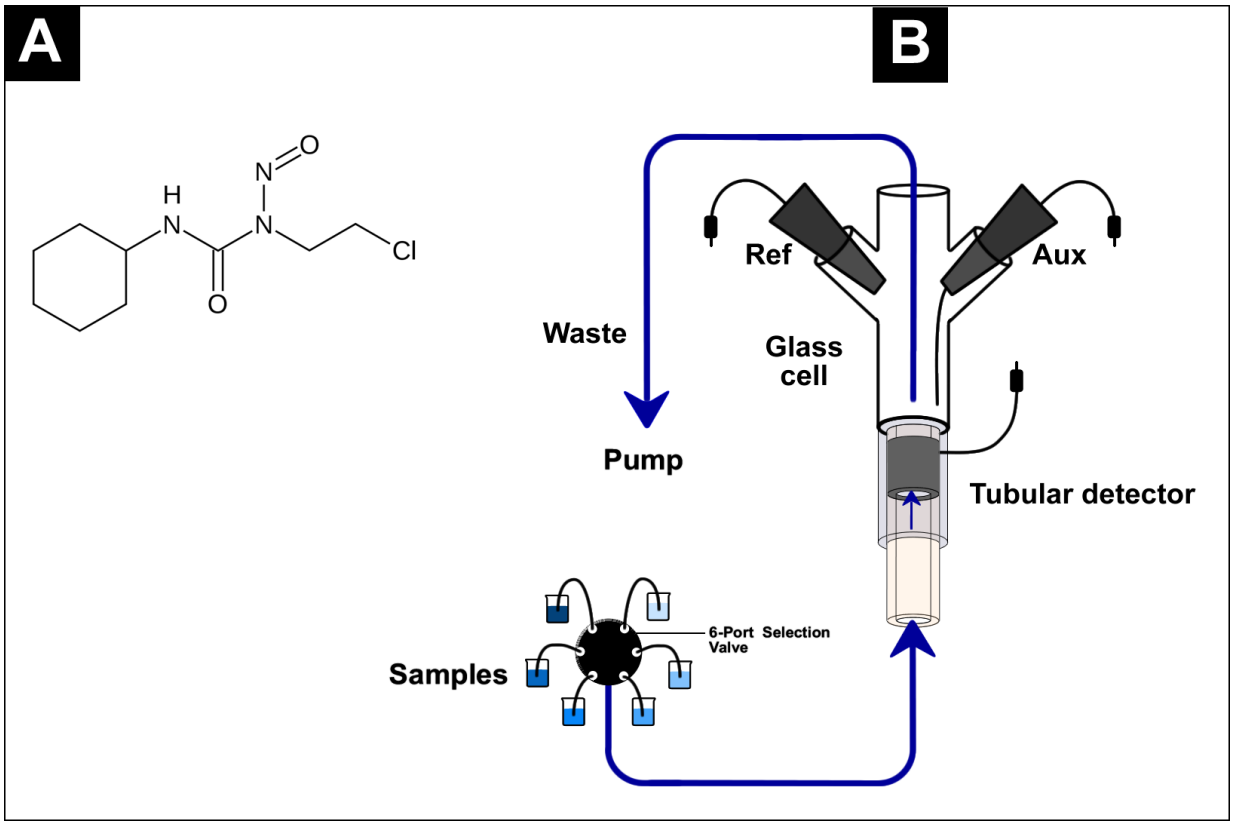

Fig. 2. (A) Structural formula of lomustine and (B) experimental arrangement for non-stop-flow differential pulse voltammetry.

In the search for optimum conditions for determination of lomustine, attention was paid to the dependence of electrochemical signal on composition of the supporting electrolyte (pH of the buffer, concentration of NaCl, amount of ethanol), the flow rate of the sample solution and the modulation amplitude. Changing conditions did not affect the noise of the signal.

In the whole studied pH range (6.0–8.0) only one reductive peak was observed which presumably corresponds to the two-electron reduction of N-nitroso group to hydroxylamino group [162]:

$$R–NO + 2\ e^- + 2\ H^+ \rightarrow R–NHOH$$

The highest peak was obtained at pH 6.0 which was chosen as optimal for following measurements. The mobile phase as well as sample contained as high concentration of NaCl to ensure a constant ionic strength of measured sample. Therefore, and the influence of NaCl concentration in buffer solution was investigated. FIA-DPV curves were recorded in solutions containing 0.5–3.0 mol dm^{-3} NaCl. The peak height increased up to the concentration of NaCl 2.5 mol dm^{-3} and afterwards it was constant at concentrations 2.5 and 3.0 mol dm^{-3}. With increasing concentration of the NaCl, the background current increased from ~ –4.5 to ~ –9.0 μA. It is supposed that the increase of the signal was caused by increasing conductivity of the measured solutions. For further experiments the concentration of 2.0 mol dm^{-3} was chosen. Because both the model and real samples of lomustine were prepared by dissolving of pure lomustine and/or contents of the drug capsules in ethanol, the effect of amount of ethanol was studied. The analyzed model samples contained 1, 5, 10, 25 and 50 % of ethanol. Up to 10 % of ethanol, the peak heights have the similar value but at 25 and 50 % of ethanol there was 1.8 times and 5.4 times decrease of the peak current, respectively. The solution of 10 % of ethanol was chosen as optimal because of higher peaks and higher resistance to electrode passivation in the presence of ethanol. So the optimal composition of mobile phase/sample was [0.10 mol dm^{-3} MES; 2.00 mol dm^{-3} NaCl; pH 6.0]:EtOH, 9:1.

Flow rate of the mobile phase/sample seemed to be very important parameter which has significant influence on the signal. As can be seen in Fig. 3A, when the flow rate was increased from 0.10 ml min^{-1} to 1.00 ml min^{-1} the peak height increased by 78 %. Since a mass transfer in the solution occurs by diffusion, migration, and convection [163], it is assumed that the signal growth is caused by the increase of the convection as a consequence of the increased flow rate. The appearance of active convection constituent of the mass transport considerable increases a sensitivity of the determination of this analyte. It is one of the main advantages of voltammetry in flow systems in comparison of voltammetry in batch arrangement.

No optimization of electrochemical method can do without studying of electrode passivation and repeatability of measurements. In this case results shown good repeatability with RSD = 1.6 % of 25 consecutive measurements of model samples of lomustine ($c = 1 \cdot 10^{-6}$ mol dm^{-3}). Before each Flow-DPV recording, the regeneration program (E_1 = –1.300 V, t_1 = 60 s; E_2 = –0.100 V, t_2 = 10 s) was applied. Regeneration of the tubular detector surface was very important, because without it the strong passivation was observed. Then, the signal of analyte was recorded every day for 5 working days (under the same conditions). The RSD of the mean of the peak height was 0.3 %. The tubular detector has also shown a good long-term stability – it is actively used in our lab for about 2 years and it provides stable and repeatable signals.

The calibration dependences were obtained over the concentration range $1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ mol dm^{-3} at three different combinations of the modulation amplitude (*Amp*) and the flow rate: 1) –0.050 V and 0.10 ml min^{-1}; 2) –0.070 V and 0.10 ml min^{-1} and 3) –0.070 V and 0.50 ml min^{-1} for demonstration of different sensitivity at different values of these parameters (Fig. 3B). Concentration dependences are linear in the whole range with high correlation coefficients. Predictably, the most sensitive is combination of higher modulation amplitude and higher flow rate: –0.070 V and 0.50 ml min^{-1} (LOD = $1.5 \cdot 10^{-7}$ mol dm^{-3}). Thus the choice of the values of these two parameters depends on our requirements. For the determination of low concentration of lomustine (the concentration of 10^{-6} and lower) it is better to apply the amplitude about –0.050 V and the flow rate about 1 ml min^{-1}. Due to high flow rate the obtained peaks will be higher, whereas the relative small amplitude prevents peak widening. And vice versa, for controlling of amount of lomustine in drugs, when the concentration of lomustine is relatively high (it depends how the sample will be diluted), the flow rate about 0.1 ml min^{-1} is absolutely sufficient and high value of modulation amplitude (–0.070 V) can be used (LOD = $1.9 \cdot 10^{-7}$ mol dm^{-3}). Moreover, the low flow rate requires substantially less amount of the sample.

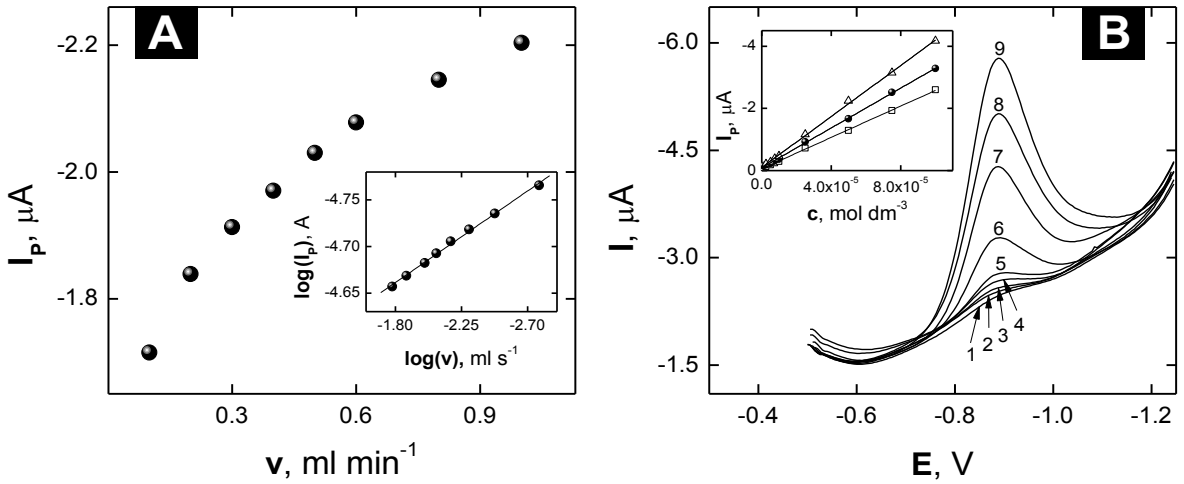

Fig. 3. **(A) Dependence of the lomustine peak height on the flow rate of the model sample. (B) FIA-DP voltammograms of lomustine** in [0.1 mol dm^{-3} MES, 2.0 mol dm^{-3} NaCl, pH 6.0]:EtOH (9:1); flow rate (*v*) 0.10 ml min^{-1} and modulation amplitude (*Amp*) of –0.070 V; concentration of lomustine 1) $1.0 \cdot 10^{-6}$, 2) $2.5 \cdot 10^{-6}$, 3) $5.0 \cdot 10^{-6}$, 4) $7.5 \cdot 10^{-6}$, 5) $1.0 \cdot 10^{-5}$, 6) $2.5 \cdot 10^{-5}$, 7) $5.0 \cdot 10^{-5}$, 8) $7.5 \cdot 10^{-5}$, and 9) $1.0 \cdot 10^{-4}$ mol dm^{-3}. **Inset: calibration straight line of lomustine**. *Experimental conditions: c*(lomustine) = $1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ mol dm^{-3}; supporting electrolyte: [0.1 mol dm^{-3} MES, 2.0 mol dm^{-3} NaCl, pH 6.0]:EtOH (9:1); △) *v* = 0.10 ml min^{-1}, *Amp* = –0.050 V; ●) *v* = 0.10 ml min^{-1}, *Amp* = –0.070 V; □) *v* = 0.50 ml min^{-1}, *Amp* = –0.070 V.

Finally, optimized method was successfully applied for the determination of lomustine in chemotherapy drug CeeNU® Lomustine 40 mg. The measured concentration of lomustine found

by our method was 103.2 % ± 2.4 % ($\tilde{x}$ ± 1 SD) with RSD 2.3 % (10 tablets) which corresponded to the value declared by manufacturer.

In summary, it was demonstrated that tubular detector based on silver solid amalgam used in presented works is an effective and low cost device for monitoring reducible organic and inorganic analytes in an amperometric mode with possibility of measurement at highly negative potentials (up to −2 V in aqueous solutions). It is Also suitable for rapid and sensitive control of the amount of the active ingredient in drugs, in this case lomustine, by DPV in flow system without separation steps. Tubular detector has a good measurements repeatability and a long-term stability at least 2 years. Moreover, as will be described in chapter 4.4. and 4.5., the tubular detector plays important role as an inseparable part of two types of flow enzymatic biosensors.

4.2. Reference Electrodes Based on Silver Paste Amalgam

In this chapter the preparation and testing of new reference electrodes based on paste silver amalgam (AgPA) is described.

The principle of these electrodes is the substitution of metallic mercury three commonly used reference electrodes [1) calomel (Hg|Hg_2Cl_2|KCl sat.), 2) mercury-mercurous sulfate (Hg|Hg_2SO_4| K_2SO_4 sat.), and 3) mercury-mercuric oxide (Hg|HgO|1 mol dm^{-3} NaOH)]by AgPA (Fig. 4).

For example, for the preparation of the saturated calomel electrode (SCE-AgPA), a small tube was used, a platinum wire was soldered with a cable and it was sealed by epoxy resin in the upper part of the tube. The place of soldering must be covered by theresin. Then the silver paste amalgam (AgPA; weight ratio: 13 % Ag and 87 % Hg) was prepared. Mixture of mercury and of fine silver powder was vigorously mixed for 60 s in dental amalgams preparation unit. The mixture of the silver paste amalgam with calomel Hg_2Cl_2 (weight ratio 1:1) and a small amount of saturated KCl solution was thoroughly homogenized in an agate mortar. The tube was almost filled by this paste mixture and then the tube was clogged by a plug of a filter paper. Thus prepared tube was immersed into saturated solution of KCl in a bigger tube or pipette tip with a porous membrane in its lower part. The same procedure was followed to prepare silver paste amalgam-mercurous sulfate electrode (MMSE-AgPA) and silver paste amalgam-mercury oxide electrode (MMOE-AgPA). For MMSE-AgPA AgPA, crystalline Hg_2SO_4 and a saturated solution of K_2SO_4were used. MMOE-AgPE was prepared from AgPA, mercuric oxide HgO (red modification) and 1 mol dm^{-3} NaOH. A high concentration of NaOH and KCl were purposely used to prevent the change of electrochemical potentials in the case of possible evaporation of the solvent or any minute fluctuation in the concentration of these solutions.

The amalgam paste is not liquid, it is soft but a long-term stable and dense enough to keep the allocated shape. This enables to prepare the reference electrode in small size. Nowadays, a miniaturization of detectors/electrodes and cells is one of the main trends in electroanalytical chemistry. That is why also the reference electrodes have to be smaller. In Fig. 4 it can be seen that the body of prepared reference electrode is made of 10 μl pipette tip and it is about 2 cm long. A manipulation with so small reference electrodes is much easier then with ones of typical size, and it is possible to put them to small electrochemical (flow) cells.

From an electrochemical point of view, AgPA is very similar to pure mercury. For example, if we connect SCE-AgPA as the anode, the following reaction occurs:

$$2\ Hg\ (Ag) + 2\ Cl^- - 2\ e^- \rightarrow Hg_2Cl_2 + (Ag);$$

connecting this amalgam as the cathode changes of the reaction direction:

$$Hg_2Cl_2 + 2\ e^-\ (Ag) \rightarrow 2\ Hg\ (Ag) + 2Cl^-$$

Electrode processes on other prepared reference electrodes take place according to a similar scheme. Experimental confirmation of the electrochemical similarity of metallic mercury and silver paste amalgam is given by the close values of potentials of electrodes prepared from these two materials (Table 2).

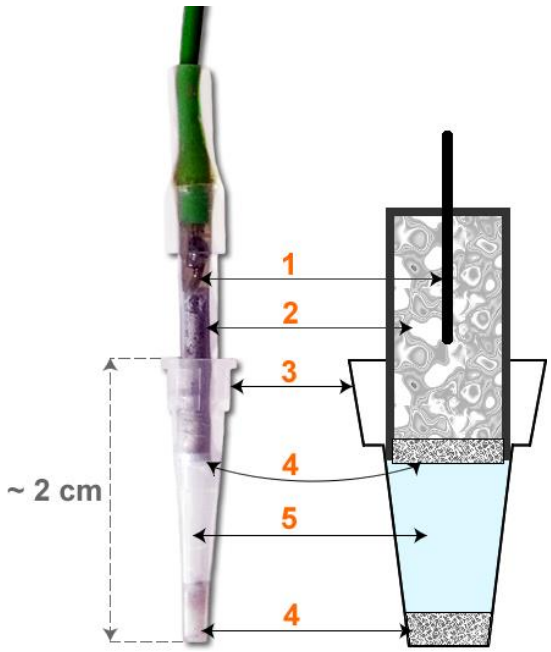

Fig. 4. Photo and scheme of reference electrode based on silver paste amalgam.
1) platinum wire; 2) a) mixture of silver paste amalgam (AgPA; 13 % Ag), Hg_2Cl_2 and saturated KCl in the case of SCE-AgPA; b) mixture of AgPA, Hg_2SO_4 and saturated K_2SO_4 in the case of MMSE-AgPA; c) mixture of AgPA, HgO and 1 mol dm^{-3} NaOH in the case of MMOE-AgPA; 3) pipette tip; 4) porous matter; 5) a) saturated KCl in the case of SCE-AgPA; b) saturated K_2SO_4 in the case of MMSE-AgPA; c) 1 mol dm^{-3} NaOH in the case of MMOE-AgPA.

Table 2. Potentials of prepared reference electrodes in comparison with commonly used ones.
Electrolyte: 0.1 mol dm^{-3} KCl; *x*-AgPA were connected with the negative and classical SCE with the positive connector of the digital millivoltmeter.

Prepared reference electrode	**E vs. SCE, mV**	**E vs. SHE, mV**	**Commonly used reference electrode**	**E vs. SHE, mV**
SCE-AgPA saturated, ~ 22–25 °C	–0.56	+244.44	SCE saturated, 25 °C	+ 244
MMSE-AgPA saturated, ~ 22–25 °C	+391.5	+635.5	MMSE saturated, 25 °C	+ 640
MMOE-AgPA 1 mol dm^{-3} NaOH, ~ 22–25 °C	–104.9	+139.1	MMOE 1 mol dm^{-3} NaOH, 25 °C	+ 140

SHE – standard hydrogen electrode
SCE-AgPA – silver paste amalgam-calomel electrode
MMSE-AgPA – silver paste amalgam-mercurous sulfate electrode
MMOE-AgPA – silver paste amalgam-mercury oxide electrode

In a 3-electrode arrangement the reference electrode is in an almost currentless state but in 2-electrode system the ability of it to maintain its potential sufficiently stable depends on the current flowing through it. Each reference electrode has some limited value of current above which it starts to become polarized, so it is necessary that this value is much higher then the current measured during an experiment.

Prepared reference electrodes were tested with respect to possible polarization in a two-electrode mode when each tested reference electrode was connected as working electrode and a large classic SCE was connected as a reference one (it was assumed that SCE does not get polarized). The cyclic voltammograms (CV) were recorded from –2.0 V to +2.0 V. The value of the current flowed through the circuit was regulated by connecting optional standard resistors. If tested reference electrode does not polarize, the cathodic and anodic branches of CV are identical and the CV has the shape of two overlapping lines (in fact it is a record of Ohm's law). On the other side, the electrode polarization results in the separation of both curves of CV. Results of this experiment showed that MMSE-AgPA has the lowest resistance and it begins to polarize when additional resistor 60 kΩ is connected to the circuit, which corresponds to maximum current around 15 μA. For MMOE-AgPA the polarization is obvious when the resistor value is 40 kΩ (max. current is about 40 μA). In other words, these electrodes would not work properly if the current passed through them would be at those levels. SCE-AgPA is much more resistant to the polarization which was only observed when no additional resistor was used and when maximum current was larger than 2000 μA. So, the calomel electrode based on the silver paste amalgam has proved to be the best one for work with large currents.

For the sake of comparison, this experiment has been performed also with the commonly used commercial silver-silver chloride reference electrode (Trutnov, Czech Republic). It was already polarized if 1 MΩ resistor was connected (max. current about 2 μA) and it was the least resistant electrode to polarization in our tests.

As was mentioned in introduction, the reference electrodes should show minimal changes of potential in time. In Table 3 the results of a long-term and short-term stability are presented. The obtained data show that all tested reference electrodes maintain their stable potentials and potential fluctuations are usually within 1 mV during one day and the maximum 8 mV during 14 months.

Table 3. Potential stability of reference electrodes based on the silver paste amalgam. Electrolyte: 0.2 mol dm^{-3} KCl for a long-term stability test and sat. KCl for a short-term stability test; Reference electrodes based on silver paste amalgam were connected with the negative and classical SCE with the positive connector of the digital millivoltmeter. The results are presented in following form: average ± 1 SD.

Electrode	E, mV
A long-term stability (14 months); N = 120	
SCE-AgPA	−0.56±1.9
MMSE-AgPA	+391.5±7.8
MMOE-AgPA	−104.9±3.8
A short-term stability (8 hours); N = 8	
SCE-AgPA	+0.33±0.70
MMSE-AgPA	+401.9±1.1
MMOE-AgPA	−106.8±0.3
A short-term stability (60 minutes); N = 13	
SCE-AgPA	+1.58±0.18
MMSE-AgPA	+403.6±0.2
MMOE-AgPA	−106.3±0.05

SCE-AgPA – silver paste amalgam-calomel electrode
MMSE-AgPA – silver paste amalgam-mercurous sulfate electrode
MMOE-AgPA – silver paste amalgam-mercury oxide electrode

In this chapter three new reference electrodes based on silver paste amalgam were designed, prepared and tested. Calomel, mercury–mercurous sulfate and mercury–mercuric oxide electrodes based on silver paste amalgam were shown to be stable from both long-term and short-term point of view. These electrodes have potentials very close to their metallic mercury analogues and thus they can replace them. The calomel reference electrode based on silver paste amalgam has proved to be the most resistant to polarization and thus its miniaturized version (in a pipette tip) was used in all next experiments in this thesis.

4.3. Electrochemical Modifications of Solid Amalgam Electrodes

Thiol monolayers (ML) at electrodes were commonly used for the preparation of various types of biosensors based on a selective interaction between a biorecognition element, linked to a ML surface, and an analyte. In our work (8. Appendixes/Publication 4), the formation of 11-mercaptoundecanoic acid monolayer on HMDE and on the electrodes based on solid amalgams: the polished silver solid amalgam electrode (p-AgSAE), the mercury film covered silver solid amalgam electrode (MF-AgSAE), the mercury meniscus covered silver solid amalgam electrode (m-AgSAE), the mercury meniscus covered bismuth-silver solid amalgam electrode (m-BiAgSAE), the mercury meniscus covered copper solid amalgam electrode (m-CuSAE) and the mercury meniscus covered cadmium solid amalgam electrode (m-CdSAE) was studied. It is necessary to point out that used MF-AgSAE, m-AgSAE, m-BiAgSAE, m-CuSAE and m-CdSAE are not covered in fact by pure mercury but a saturated liquid amalgam of metal(s) of which electrode material consists. That can substantially influence electrochemical parameters of ML preparation and the properties thiol deposited at the electrode surface. Thus, the accumulation potential, the desorption potential, and the potential range in which ML is stable can differ for various amalgam electrodes.

Procedure of forming monolayer of 11-mercaptoundecanoic acid at the electrodes mentioned above includes creation of the mercury meniscus or the mercury film at the surface of polished AgSAE, the electrochemical activation of the electrode, the electrochemical accumulation of thiol for a given time at the optimal potential for each concrete working electrode in the thiol solution [0.5 mol dm^{-3} NaOH, 48 % EtOH, $1 \cdot 10^{-3}$ mol dm^{-3} $HS(CH_2)_{10}COOH$], and registration and evaluation of cyclic voltammogram(s). During our previous research [52] the optimal accumulation potentials for all electrodes were found. These data are summerised in Table 4. The principle of thiol layer formation is that metals of the electrode material are electrochemically oxidized during the potential application and create covalent bonds with sulfur. Since the thiol deposition is potential-controlled, this process on mercury and amalgam electrodes is much faster than self-assembly method of the ML formation.

The dependence of the amount of the chemisorbed compound on the deposition time (1–300 s) was investigated. A monolayer was prepared in $1.0 \cdot 10^{-3}$ mol dm^{-3} thiol solution at an optimal accumulation potential at all electrodes within 1−2 min. Areas of reductive desorption peaks were practically unchanged with longer deposition time and hence, short accumulation times (t_{ac} up to 120 s) were used in our experiments. Not only accumulation time but also the concentration of the thiol solution is important. When the 11-mercaptoundecanoic acid monolayer deposition on m-AgSAE was performed within 1 min from $1.0 \cdot 10^{-3}$ mol dm^{-3} thiol solution, the thiol surface concentration was calculated as $\Gamma = 9.5 \times 10^{-10}$ mol cm^{-2}. Value $\Gamma = 5.9 \times 10^{-10}$ mol cm^{-2} was reached when $1.0 \cdot 10^{-4}$ mol dm^{-3} 11-mercaptoundecanoic acid solution was used. Extension of the accumulation time even decreased this value (t_{ac} = 5 min, $\Gamma = 5.6 \times 10^{-10}$ mol cm^{-2}; t_{ac} = 60 min, $\Gamma = 5.2 \times 10^{-10}$ mol cm^{-2}). So, it can be concluded that in the case of 11-mercaptoundecanoic acid it is better to create the monolayer electrochemically in a short time and from higher concentration of the thiol solution. However, a literature states that full coverage of self-assembled monolayer of

Table 4. Potentials of reductive desorption peaks (E_p) of 11-mercaptoundecnoic acid on different types of electrodes and electrochemical conditions of 11-mercaptoundecnoic acid monolayer formation.

Supporting electrolyte with 11-mercaptoundecnoic acid: [0.5 mol dm^{-3} NaOH, 48 % EtOH, $1 \cdot 10^{-3}$ mol dm^{-3} $HS(CH_2)_{10}COOH$], scan rate 1 V s^{-1}. E_{ac} – accumulation potential, t_{ac} – accumulation time.

Working electrode	E_p, V	E_{ac}, V	t_{ac}, s
p-AgSAE	–0.835	–0.350	
MF-AgSAE	–0.859	–0.350	
m-AgSAE	–0.863	–0.350	
m-BiAgSAE	–1.079	–0.800	60
m-CuSAE	–1.136	–0.800	
m-CdSAE	–1.248	–1.050	
HMDE	–0.864	–0.350	

p-AgSAE – polished silver solid amalgam electrode
MF-AgSAE – mercury film covered silver solid amalgam electrode
m-AgSAE – mercury meniscus covered silver solid amalgam electrode
m-BiAgSAE – mercury meniscus covered bismuth-silver solid amalgam electrode
m-CuSAE – mercury meniscus covered copper solid amalgam electrode
m-CdSAE – mercury meniscus covered cadmium solid amalgam electrode
HMDE – hanging mercury dropped electrode

uncharged n-decanethiol at HMDE was achieved after a short time (less than 5 min) for concentrations above 10^{-5} mol dm^{-3} of thiol [164]. The reason of different behavior of MUA is probably the mutual repulsion of negatively charged carboxyl groups arising due to their dissociation in the strongly alkaline supporting electrolyte. After the start of the 11-mercaptoundecanoic acid deposition, the thiol film is created very quickly and its density is proportional to the 11-mercaptoundecanoic acid concentration in solution. Then the surface concentration varies substantially less because the arising negatively charged film prevents the passage of the negatively charged 11-mercaptoundecanoic acid molecules to the electrode surface.

The concentration dependence of 11-mercaptoundecanoic acid was studied in the range $2.0 \cdot 10^{-6}$–$1.1 \cdot 10^{-3}$ mol dm^{-3}. It is known that molecules of thiols in monolayer form lateral interactions and thus Frumkin isotherm is often used to describe such adsorption/desorption processes. However, the obtained experimental data were fitted much better to Langmuir isotherm which is used in the case where the adsorbed molecules do not interact among themselves. It is assumed that the lateral interactions among 11-mercaptoundecanoic acid molecules are compensated by a repulsion of the negatively charged carboxyl groups and the system therefore behaves as if the molecules at the electrode surface do not interact among themselves. The adsorption isotherms obtained with those working electrodes were similar as well as calculated values of adsorption free energy. The lowest saturated surface coverage was observed for HMDE (8.5×10^{-10} mol cm^{-2}) and the highest one for MF-AgSAE (9.8×10^{-10} mol cm^{-2}).

The dependence of the peak current on the scan rate (v) was investigated from 20 to

1000 mV s^{-1}. If the reduction of a compound is run from the adsorbed state and the adsorbed molecules do not interact with each other, the dependence i_p–v is linear [165-168]. The nonlinearity of this dependence in our experiments shows that among the adsorbed molecules lateral interactions exist. In general, the 2/3 power ($= 0.\bar{6}$) dependence is consistent with voltammetric behavior associated with dissolution of two-dimensional molecular films [168]. In our experiments, the highest correlation coefficients were obtained for $x = 0.60$ (R_{HMDE} = 0,991; $R_{m\text{-}AgSAE}$ = 0,998; $R_{MF\text{-}AgSAE}$ = 0,991; $R_{m\text{-}CuSAE}$ = 0,994), which confirms that 11-mercaptoundecanoic acid forms a two-dimensional monolayer film with lateral interactions at the surface of the studied WEs.

The trends of important parameters such as the peak widths at its half height and potentials of the desorption peaks were always investigated and they are described in detail in our publication (8. Appendixes/Publication 4).

Finally, the biosensors based on MF-AgSAE and m-AgSAE which use the avidin-biotin and streptavidin-biotin labeled albumin interactions, respectively, were prepared according following procedure: 1) 11-mercaptoundecanoic acid was covalently bonded at the electrode surface by electrochemical deposition, 2) carboxylic groups of the deposited acid were activated by EDC/NHS technology, 3) such prepared electrodes were incubated with avidin (streptavidin) to create the amide bonds and immobilize the protein. The described biosensor structure is based on covalent bonds which makes it stable over long time. Each avidin or streptavidin molecule attached at the electrode has 4 binding sites for biotin or biotin labeled substances. The surface of the used biosensor is effectively blocked by the thiol monolayer and therefore voltammetric measurements cannot be performed. The connection of avidin or streptavidin with biotin change thickness and permeability of the biosensor surface layer which change capacitance and resistance of the whole construction at the electrode. These changes were detected by electrochemical impedance spectroscopy.

The preparation of the biosensor started with electrochemical renewal of the surface of MF-AgSAE and m-AgSAE directly in the 11-mercaptoundecanoic acid solution by imposition of the potential –2.200 V for 180 s. Then the thiol monolayer is electrochemically formed at –0.350 V during 300 s. Avidin and streptavidin are bonded with 11-mercaptoundecanoic acid layer via amide bond between –COOH groups of 11-mercaptoundecanoic acid and $–NH_2$ groups of avidin or streptavidin. For this reaction to occur the carboxyl groups must first be activated. The activation is performed by immersing electrode covered by 11-mercaptoundecanoic acid into the aqueous solution of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimid hydrochloride (EDC) with N-Hydroxysuccinimide (NHS) for 15 min. The last step was the transfer of the electrodes into avidin (streptavidin) solution for 30–60 min. Under optimal conditions for impedance measurements, the response of the prepared biosensors with avidin or streptavidin to additions of biotin or biotin labeled albumin was linear in the logarithmic scale in the range 0.58–4.03 μg ml^{-1} of biotin and 1.96–20.0 μg ml^{-1} of biotin labeled albumin.

A good reproducibility of repeated formation and desorption of thiol monolayers attests that the different types of amalgam are convenient electrochemical materials for modification by thiols as well as for preparation of biosensors based on thiol monolayers.

4.4. Flow Electrochemical Biosensor Based on Enzymatic Porous Reactor of Silver Solid Amalgam

In our previous work we have demonstrated that silver solid amalgam is a suitable base for creating of thiol monolayers. On the ground of this finding we have constructed and tested a flow biosensor based on enzymatic porous reactor of silver solid amalgam (chapter 8. Appendixes/Publication 5).

The biosensor consists of two parts – an flow enzymatic reactor and a tubular detector which was described in chapter 4.1 (Fig. 5A). The flow enzymatic reactor was prepared from a porous silver solid amalgam. We have found no information in literature on the previous use of this material for biosensors. The porous silver amalgam was modified by a thiol (11-mercaptoundecanoic acid) and an enzyme was covalently immobilized at the modified surface of porous amalgam using EDC/NHS chemistry. For our experiment we selected the glucose oxidase (GOx) as one of the most studied and stable enzymes. When the glucose (= analyte) passed through the reactor, the enzymatic reaction occurs according to equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOx} \text{Gluconic acid} + H_2O_2$$

As it can be seen, during this reaction oxygen (dissolved in the mobile phase) is consumed. Decrease of O_2 concentration is directly proportional to the concentration of glucose and it is measured amperometrically by the detector. This decrease of oxygen concentration results in the decrease of current and it is graphically represented as a reverse peak (Fig. 6).

The preparation of porous reactor is as follows: a piece of Teflon capillary with the same outer diameter as the inlet Teflon capillary was inserted into the Teflon tube in the place of the planned beginning of the silver solid amalgam column. A silver powder was gradually added into the upper hole of the tube and was mildly pressed by another piece of the Teflon capillary. As soon as the length of the silver powder column was 10 mm (or other if it was needed), it was rinsed by 2 mol dm^{-3} $HClO_4$ and then mercury was added into this tube to convert silver powder into amalgam. After about 5 min, an excess of mercury was carefully removed from the reactor. At both ends of the Teflon tube, two long silicone tubes were fixed. These tubes seal the inlet and outlet capillary of the reactor body. The prepared porous reactor can be used immediately, although it is preferable to let the solid amalgam to be stabilized until the next day. Porosity of the reactor depends on the size of the silver particles and on the degree with which the powder was compressed before amalgamation.

Prior to the self-assembled monolayer (SAM) formation of 11-mercaptoundecanoic acid, reactor was cleaned and dried. Next, the solution of ([0.01 mol dm^{-3} 11-mercaptoundecanoic acid; 0.01 mol dm^{-3} HClO4, 70% EtOH] was passed through reactor with slow flow rate (0.016 ml min^{-1}) for 30 min at room temperature to ensure reliable SAM formation (Fig. 5B). It is known that some proteins denature when they get into contact with metal surface. Chemisorbed thiol monolayer not only prevents a contact of enzyme with the metal surface but it (electro)chemically blocks this surface. This substantially reduces releasing of mercury and silver cations which can be inhibitors of the enzymatic reaction. Moreover, the mobile phase and samples also contain $1 \cdot 10^{-3}$ mol dm^{-3} Na_2EDTA for bonding silver and mercury cations which could be released from porous amalgam reactor.

The immobilization of GOx was carried out using coupling agents EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and NHS ((2-(N-morpholino)ethanesulfonic acid). Carboxylic groups of 11-mercaptoundecanoic acid can react with NHS in the presence of carbodiimide such as EDC, resulting in a NHS ester which may then react with amine groups of GOx. Whole immobilization process took about 1 hour and 40 minutes. In general, one reactor could be used for repetitive preparation of the biosensor, where many other enzymes could be attached.

Finally, the porous reactor with attached GOx was connected with the tubular detector and placed into the miniature lab-made glass cell.

The following parameters of glucose determination were optimized with respect to amperometric response: composition of the mobile phase, its concentration, the potential of detection, and the flow rate.

Various aqueous solutions were tested to find the optimal composition and concentration of the mobile phase. 0.1 mol dm^{-3} acetate buffer of pH 6.5 was shown as the best. As was mentioned above, a complexing agent Na_2EDTA ($1 \cdot 10^{-3}$ mol dm^{-3}) was also added.

Effect of detection potential on the biosensor response was investigated from −0.100 to −1.500 V. The peak current increased with increasing negative potential up to −1.00 V, and then it remained practically unchanged up to −1.5 V. Detection potential −1.100 V was chosen as the optimum for maximum sensitivity and for the best repeatability of measurements. At higher potentials, the repeatability of measurements was substantially worse probably due to the evolution of hydrogen
which forms microbubbles on the surface of the detector and uncontrollably changes the active area of the detector.

The choice of the flow rate has effect especially on two important parameters of the analysis – sensitivity and time of one measurement. With increasing flow rate (0.04–0.40 ml min^{-1}) of the mobile phase injected glucose was in contact with enzyme for shorter time which lowered the yield of enzymatic reaction r and thus decreased the signal. On the other hand, at low flow rates the sensitivity is higher but the analysis time is longer. Therefore, as the compromise the value of the flow rate 0.10 ml min^{-1} was chosen when one measurement takes about 3.5 minutes and sensitivity is high enough. At work with enzymatic reactors a recovery time of the enzyme activity after each measurement had also to be taken into account. It means that in the case of GOx it was necessary to wait about 240 seconds between each injections of glucose. Otherwise the biosensor response gradually decreased.

The biosensor had a good repeatability of 11 consecutive injections of glucose ($c = 4.0 \cdot 10^{-4}$ mol dm^{-3}) with relative standard deviation 1.83 %. Before each measurement the tubular detector was electrochemical cleaned by imposing of –0.100 V for 10 s at first; then the potential was switched to 1.100 V for 10 s.

One of the most important characteristic of the biosensor is long-term stability. It depends partly on strength of bonds amalgam–thiol and thiol–enzyme, and partly on the stability of the enzyme under measured conditions which is given by natural character of the particular enzyme and also could depends on storage conditions. We assume that covalent bonds between amalgam–thiol–enzyme should ensure a good stability. Also when the reactor with the immobilized enzyme was not used, it was stored in mobile phase at 4 °C. The result of this monitoring during 35 days indicated that 77% of the current response of glucose was retained.

Nevertheless, despite this decrease of the response the biosensor could be successfully used further, but for evaluation of the data a standard addition method or comparison the results of measurements of samples with the measurement of the glucose standard solution should be applied.

The calibration dependence of model sample of glucose was linear in the range $2.0 \cdot 10^{-5}$–$8.0 \cdot 10^{-4}$ mol dm^{-3} with limit of detection of $1.0 \cdot 10^{-5}$ mol dm^{-3}. Finally, the biosensor was used for the determination of glucose in real sample – commercial honey with declared glucose concentration 32–37 mass %. Using our method glucose concentration 35.5±1.0 mass % (n = 7, RSD = 3.2 %) was found which agrees well with the declared and literature values.

In this work, it was demonstrated that proposed biosensor has shown a good repeatability, long-term stability and sufficient sensitivity for determination glucose in the real sample of honey. Moreover, the ability to work at so high negative potential makes it relatively exceptional.

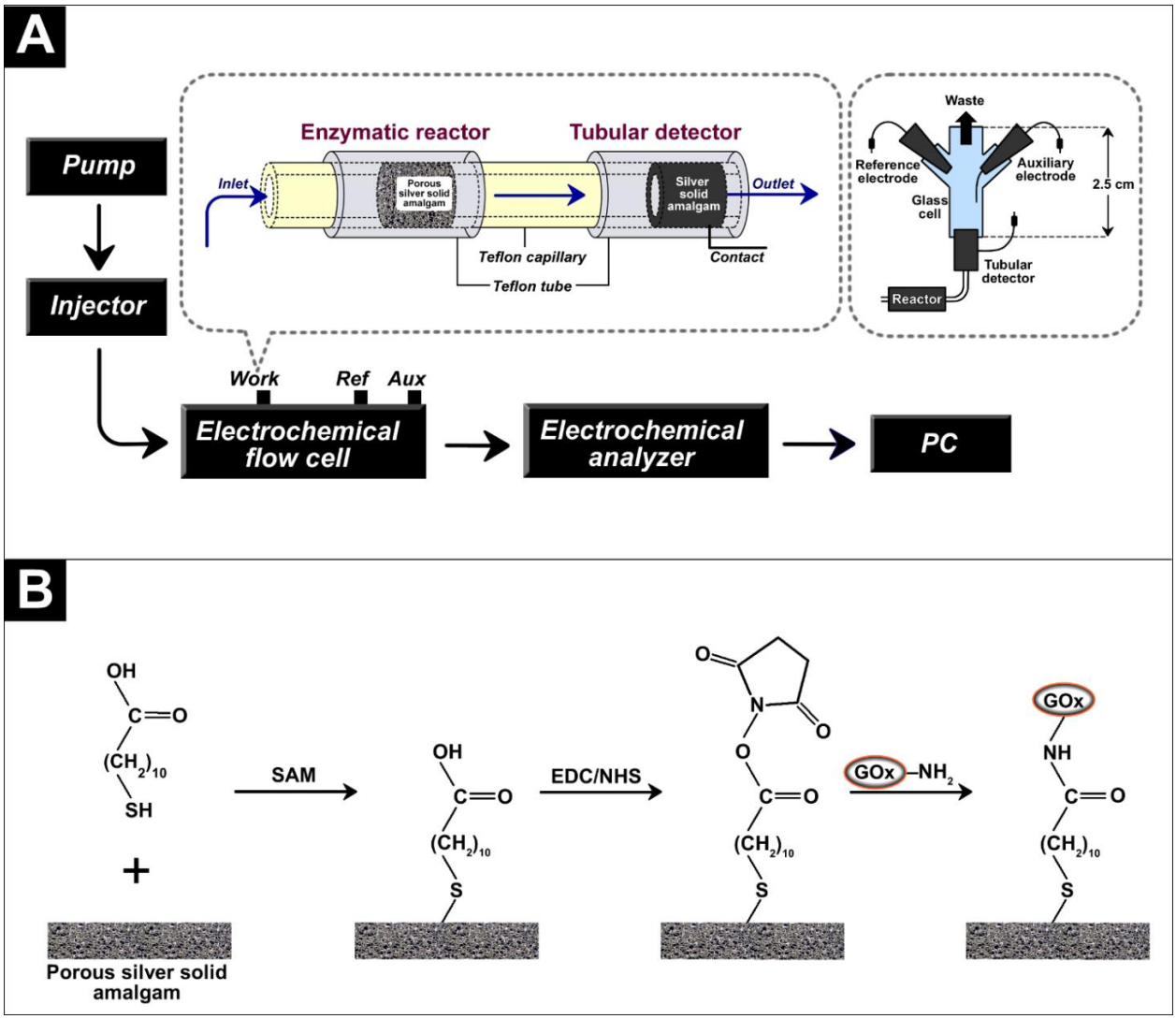

Fig. 5. (A) Scheme of experimental arrangement for amperometric measurements under conditions of flow injection analysis. (B) Modification process of the reactor based on porous silver solid amalgam. EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; NHS – (2-(N-morpholino)ethanesulfonic acid); GOx – glucose oxidase.

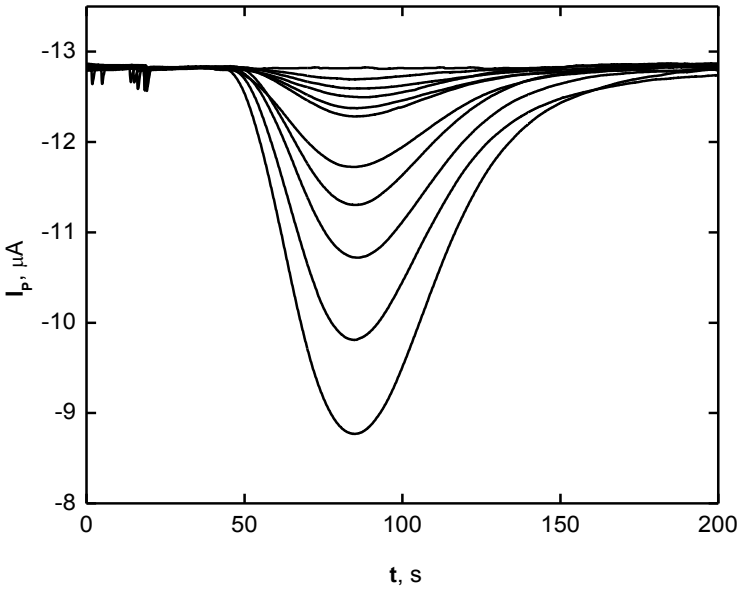

Fig. 6. FIA-ED recordings of glucose in the mobile phase and in the model samples in concentration range $2.0 \cdot 10^{-5}$–$2.0 \cdot 10^{-3}$ mol dm^{-3}.
Experimental conditions: mobile phase [0.10 mol dm^{-3} acetic buffer; 0.001 mol dm^{-3} Na_2EDTA, pH 6.5]; injected volume 50 μl; potential of detection −1.100 V, flow rate 0.10 ml min^{-1}.

4.5. Flow Electrochemical Biosensor Based on Enzymatic Reactor of Silver Solid Amalgam Powder

While in the previous chapters we have prepared and tested the biosensor with reactor of porous silver solid amalgam, in this chapter the reactor filled by silver solid amalgam powder will be introduced (Fig. 7A) (8. Appendixes/Publication 6). The powder was modified by thiol 4-aminothiophenol and enzyme was covalently immobilized at the surface of thus modified powder via crosslinking agent glutaraldehyde. The experimental arrangement for amperometric measurements under conditions of flow injection analysis remains the same as in the case of the biosensor with porous reactor.

The preparation of the powder reactor included next several steps. Firstly, silver solid amalgam powder was prepared from the metals with mercury/silver powder ratio 70/30 (w/ w) in a dental amalgamator and then it was pulverized to the fine powder in an agate mortar. Then, at the surface of amalgam powder particles a self-assembled monolayer of 4-aminothiophenol was formed (Fig. 7B). The immobilization of enzyme was carried out via crosslinking agent glutaraldehyde. Glutaraldehyde is symmetrical linear molecule with two aldehyde groups. One group reacts with NH_2-group of 4-aminothiophenol, and second one reacts with NH_2 group of enzyme. Thus glutaraldehyde figures as a connection between thiol and enzyme. The amalgam powder could be regenerated and used for repetitive preparation of the biosensor, in which many other enzymes could be immobilized. Finally, the silver amalgam powder modified by enzyme was added into the Teflon tube. At the beginning and at the end of the powder column a small amount of the wet cotton wool or a filter paper was placed to prevent powder to be washed away. Thus prepared powder reactor can be used immediately. In this way a few reactors containing enzyme ascorbate oxidase (AscOx), glucose oxidase (GOx), catalase (Cat), tyrosinase (Tyr) and laccase (Lac) were prepared. The complete flow enzymatic biosensor consists of this powder reactor and the tubular detector.

Concentration of substrates in the model/real samples was measured amperometrically by the detector under flow injection analysis conditions from the change of oxygen concentration during enzymatic reaction or from measuring of the reduction of an appropriate product. The substrates of enzymes (stated in brackets) are sodium ascorbate (AscOx), glucose (GOx), hydrogen peroxide (Cat), catechol (Tyr, Lac), pyrogallol (Tyr, Lac) and dopamine (Lac).

During enzymatic reactions of GOx and AscOx oxygen dissolved in solution is consumed. Decrease of oxygen concentration is shown on the amperometric record as the downward peak. The current of this peak is directly proportional to the concentration of the substrate. The detection potentials for these enzymes should be more negative than –1000 mV where the four-electron reduction of oxygen takes place on the amalgam electrode. Thus the optimal potentials were –1.100 V (GOx) and –1.300 V (AscOx). At higher potentials the sensitivity was lower and the repeatability of measurements was substantially worse. It was also verified that neither glucose nor sodium ascorbate were reduced at chosen potentials without enzymatic reactors (they are reduced at –1.700 V or more negative potentials and they cannot affect these measurements).

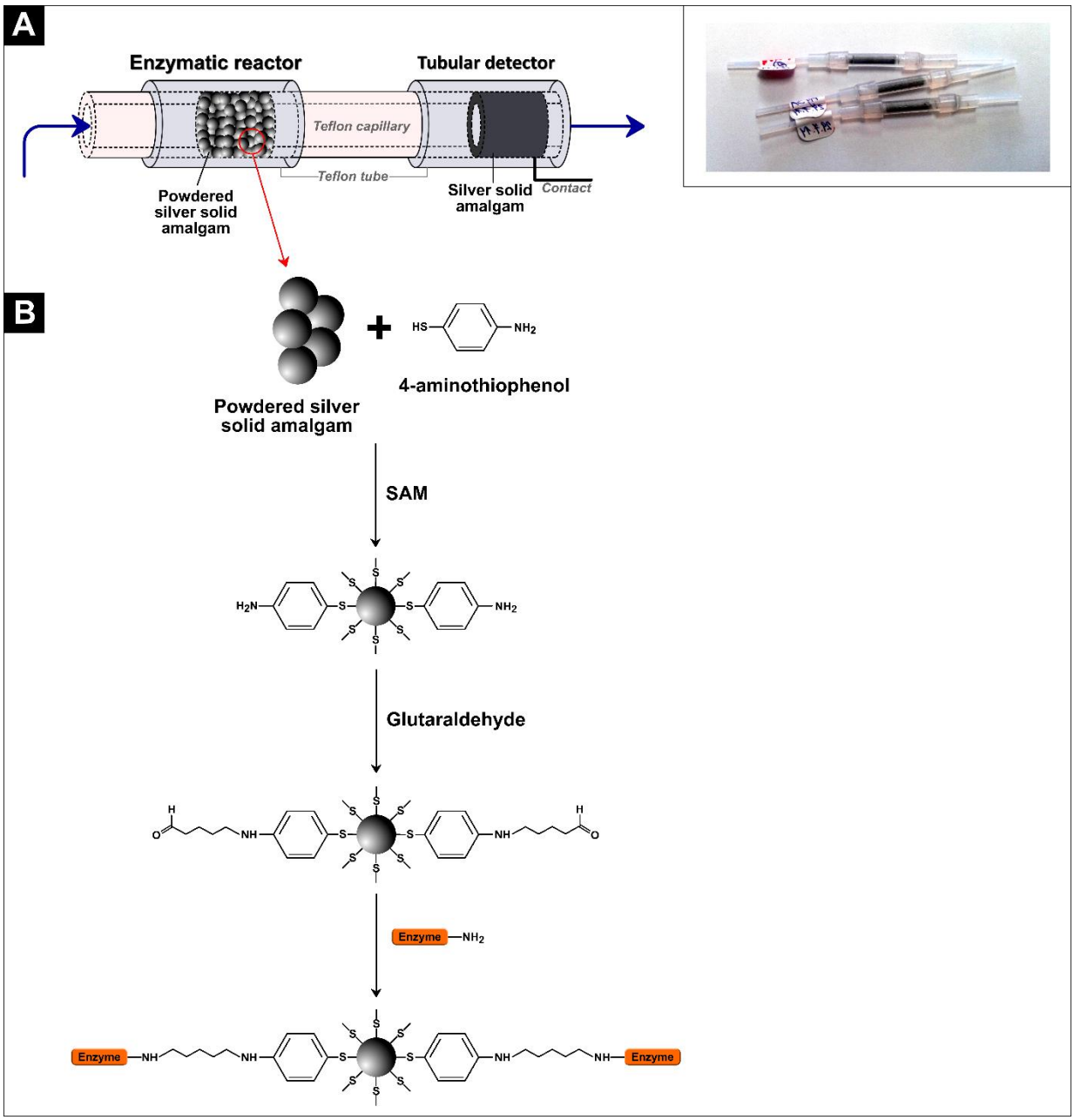

Fig. 7. (A) The biosensor based on silver solid amalgam powder reactor and silver solid amalgam tubular detector, (B) modification process of silver solid amalgam powder.

Catalase is used in living tissues to convert hydrogen peroxide to oxygen and water. Connecting the reactor with catalase to the flow system causes an increase of oxygen concentration in the solution due to the enzymatic reaction and to subsequent growth of the oxygen reduction current on the detector. Hydrogen peroxide was also reduced without Cat-reactor at potentials higher than –0.900 V. So, for the reactor with catalase it was important to find such detection potential at which the response corresponding to the oxygen reduction would be maximal, but at which hydrogen peroxide is not yet reduced. The detection potential of –0.900 V was chosen as optimal because the ratio of the detection current with catalase reactor to such current without reactor was the highest one.

Unlike the GOx, AscOx and Cat, Tyr and Lac catalyze oxidation of a large number of phenolic compounds (dopamine, catechol, pyrogallol, etc.). During enzymatic reactions with such phenolic compounds oxygen is consumed and the substrate is oxidized to the corresponding quinone. Quinones are easily reducible compounds and this property is often used for their electrochemical determination. The peak current of the quinone reduction is proportional to the concentration of phenol(s) in the measured solution. The optimal potential for reduction of quinones generated during the enzymatic reaction was found to be –0.050 V.

The optimization of remaining parameters of the method, namely a flow rate of the mobile phase and an injection volume of the sample are described in detail in attached publication (8. Appendixes/Publication 6).

Concentration dependence of Asc–biosensor was linear from 0.02 to $0.6 \cdot 10^{-3}$ mol dm^{-3} with limit of detection $12 \cdot 10^{-6}$ mol dm^{-3}. This linear part was used as basic information for preparing the solution of vitamin tablet Celaskon® for determination of ascorbic acid in these tablets. The content of ascorbic acid found in one tablet was 104.9 ± 2.2 mg (N = 9; SD = 2.9 mg; RSD = 2.8 %) what corresponds well with the manufacturer´s declared value (95–105 mg per 1 tablet). The limit of detection of other analytes were in the range $0.51–14.10^{-6}$ mol dm^{-3}.

A volume of the enzymatic reactor was the last investigated parameter. For this study the amalgam powder with the enzyme laccase was used. Parallel measurements with catechol solution were carried out with Lac–reactors of different volumes (length): 5.6 µl (2.8 mm), 10.3 µl (5.2 mm), 13.5 µl (6.8 mm), 18.1 µl (9.1mm), 21.4 µl (10.8 mm), and 29.8 µl (15.0 mm). The reduction peak current of quinone (which was generated during the enzymatic reaction) depended on the volume of the reactor linearly while peak height increased 2.2-times when the reactor volume was changed from 5.6 µl to 29.8 µl (5.4 times). It is important that the peak width at half-height was almost unchanged. So, the choice of optimal volume should be in agreement with required sensitivity and with the accessible amount of the enzyme, and thus for most of our experiments the reactor volume of about 20 µl have been used. A high enzymatic capacity of the reactor can be very useful for enzymes that have so-called "suicidal" behavior. Such the enzyme is tyrosinase. Its activity decreases after each contact with the substrate [169]. In our other experiment at the surface of the pen-type silver solid amalgam electrode, a thin layer of chitosan was applied and tyrosinase was covalently bound with it. The response of this biosensor rapidly decreased after each measurement in the solution with catechol, and after 5–6 measurements the amperometric peak completely disappeared. When using Tyr–reactor (20 µl) which contained a much larger amount of enzyme tyrosinase, the first 10–12 measurements were reproducible, and then the response of the biosensor slowly declined. However, this decrease of the biosensor response is not too pronounced. When using the procedure in which a sample is measured immediately after the standard solution (analyte concentration can be calculated by comparing peak currents), such Tyr–reactor can work for the whole day.

The repeatability of each biosensor was determined by evaluation of 11 consecutive measurements of model samples of the studied substrates based on peak height under optimal conditions. The values of RSD ranged from 0.8 to 2.1 % what indicates a good repeatability of measurements. It is necessarily to note that tubular detector was electrochemically cleaned before each measurement by imposing an appropriate potential(s) for 10–20 s.

According to our experience, it can be generally said that the working temperature have the biggest influence on the progressive decrease of the enzyme activity. Therefore, enzymatic reactors should be stored in the refrigerator at 4 °C. The amalgam powder covered by enzyme should be stored at −18 °C. Such powder after unfreeze is active even after storing for several months.

The proposed powder reactor is an improved version of porous silver amalgam reactor described in the previous chapter. We did a comparative experiment when in both the porous and powder reactors enzyme glucose oxidase was immobilized. It was found that sensitivity of powder reactor was 2–3 times higher than the porous one.

4.6. Flow Enzymatic Biosensor Based on Polished Silver Solid Amalgam Electrode

In this chapter a different type of biosensor which is more suitable for enzymes with lower activity in comparison with previously described biosensors is presented. It consists of a classical pen-type electrode of polished compact silver solid amalgam covered by layer of chitosan in which the enzyme sarcosine oxidase (SOx) is immobilized via crosslinking agent glutaraldehyde (Fig. 8A) (8. Appendixes/Publication 7). The biosensor was place into the lab-made wall-jet cell (Fig. 8B) and it worked in amperometric mode under conditions of the flow injection analysis. A substrate of SOx (and at the same time an analyte) is natural amino acid sarcosine (Fig. 8C). Sarcosine pays important role as a differential metabolite concentration of which is highly increased during prostate cancer progression to metastasis and it also was studied in the connection with the treatment of schizophrenia and depression.

The preparation of the biosensor is simple and includes next four steps: 1) polishing and activation of the electrode, 2) formation of the chitosan layer at the electrode surface, 3) modification of the chitosan layer by glutaraldehyde, and finally 4) covalent immobilization of sarcosine oxidase at modified chitosan layer.

Chitosan is widely used immobilization matrix. It is natural polyaminosacharide obtained by deacetylation of chitin which is the major constituent of the shells of crustaceans, the exoskeletons of insects and the cell walls of fungi. Its linear polyglucosamine chains of high molecular weight have reactive amino and hydroxyl groups amenable to chemical modifications. The layer of chitosan was formed by the application of a small amount of chitosan at the electrode surface. However, due to hydrophobicity of amalgam and Teflon body of the electrode and hydrophily of the chitosan, an adhesion of the chitosan layer to electrode is very low. Fortunately, this problem can be solved by fixing a commonly available ferrule on the electrode body as it is depicted in Fig. 8A. Then the layer is mechanically catch in the ferrule and does not drop during analysis. For electrochemical detection chitosan has two very important advantages. Firstly, the chitosan layer is not active at high negative potentials so it is possible to work at about ~ –2 V without affect on the chemical structure. Second advantage is the electrolytic permeability of the chitosan hydrogel which allows easy diffusion of some compounds of the enzymatic reaction to the electrode surface.

For immobilization of enzyme on the chitosan, a crosslinking agent glutaraldehyde was used. One aldehyde group reacts with amino group of chitosan, and the other one with amino group of enzyme, thus the connection between enzyme and chitosan matrix is ensured. All bonds are covalent. Whole preparation process of the biosensor took about 3 hours which is relatively short period in comparison with the preparation procedure of some biosensors which take tens of hours.

The enzymatic reaction takes place in the following way [170, 171]:

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{SOx} \text{formaldehyde} + \text{glycine} + H_2O_2$$

According this reaction oxygen dissolved in the mobile phase and sample is consumed. This decrease of oxygen concentration, which is directly proportional to the concentration of sarcosine, was amperometrically measured directly at the electrode surface of the biosensor.

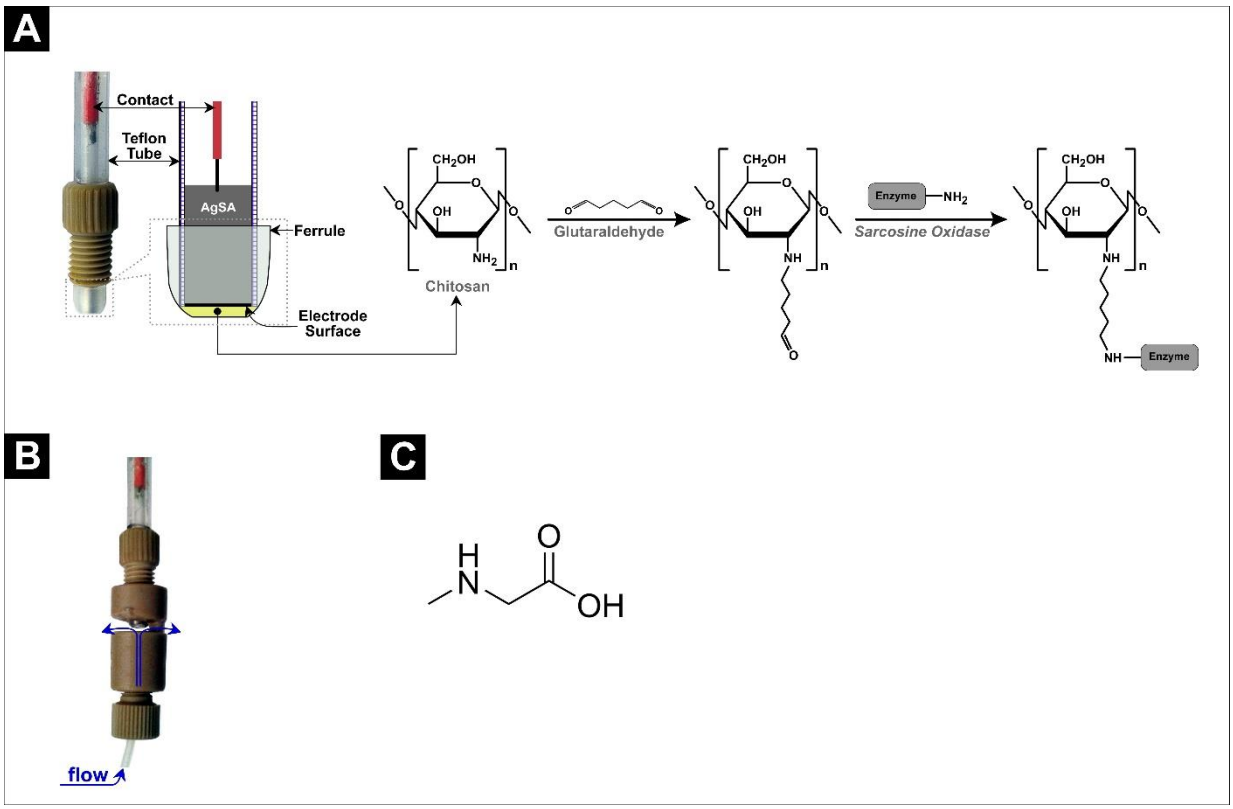

Fig. 8. (A) Design of flow enzymatic biosensor based of silver solid amalgam electrode (AgSA), (B) wall-jet arrangement, (C) structural formula of sarcosine.

The method for the determination of sarcosine was optimized with respect to amperometric response and following parameters were investigated: pH of the mobile phase, potential of the working electrode, the flow rate of the measured sample, and the volume of the injected loop.

As the mobile phase 0.1 mol dm^{-3} phosphate buffer with 0.001 mol dm^{-3} Na_2EDTA was used. The enzyme has shown the highest activity at pH 8.0. pH values higher than 8.5 and lower than 7.0 were not use to avoid damage of the enzyme. The potential of detection was investigated from −0.400 V up to −1.500 V. The peak height was increasing up to −1.300 V and then practically did not change. The shape of this dependence was very similar to ones in the case of glucose oxidase and ascorbate oxidase. Hence, it is assumed that at this type of biosensor oxygen reduces in the same way. The potential −1.400 V was chosen as optimal and used for all further measurements. Also the dependence on the flow rate looked similarly – the signal decreased with increasing flow rate from 0.04 to 0.25 ml min^{-1}. The value 0.10 ml min^{-1} was chosen as a compromise between sensitivity and time of the scan. Thus one measurement takes about 5 minutes.

The volume of injection loop was the last studied parameter. For this purpose the home-made injection loops 50, 80, 100, 150, and 200 µl were tested. For the determination of high concentrations of sarcosine the loop of volume >100 µl is recommended. On the other side, the concentration around limit of detection is better to determine with loop about 100 µl because the higher volume of the sample causes spreading of even small signal.

Under optimal conditions two concentration dependences of model sample were measured. First one with 50 µl injection loop and second one with 100 µl injection loop. As it was supposed,

the determination of sarcosine with loop of 100 μl was more sensitive (1.5×) and the limit of detection was 3.5× lower. The corresponding calibration curve was linear in interval $7.5 \cdot 10^{-6}$–$5.0 \cdot 10^{-4}$ mol dm^{-3} with correlation coefficient R^2 = 0.998 and limit of detection $2.0 \cdot 10^{-6}$ mol dm^{-3}.

The biosensor had also a good repeatability. The relative standard deviation of 10 consecutive injections of sarcosine at the concentration of $1 \cdot 10^{-4}$ mol dm^{-3} was 1.6 %. No significant passivation was observed and so no regeneration steps were used. The life-time of the biosensor was investigated for 14 days. During this period, it was used at least 3 hours per day and then stored in mobile phase at laboratory temperature. Sarcosine oxidase lost about 50 % its activity after 8 days (100 % is the peak height in the first day i.e. in a day when biosensor was prepared) and after two weeks about 22 % remained. However, the progressive loss of the signal is not critical because the concentration of sarcosine can be found out by a method of standard additions, and a bigger injection loop can be used to obtain higher signal.

5. Conclusions

Newly developed tubular detector based on silver solid amalgam (TD-AgSA) was prepared for the determination of reducible compounds in flow systems. Firstly, it was tested in model solutions of Cd^{2+}, Zn^{2+} and 4-nitrophenol in amperometric mode under conditions of flow injection analysis. The newly developed tubular detector is a simple, robust and inexpensive device with good repeatability and sensitivity. One of its main advantages is applicability for cathodic measurements in aqueous solutions at potentials up to –2 V. The developed tubular detector has also a good long-term stability – it is actively used in our laboratory for about 2 years and it provides stable and repeatable signals.

After pilot experiments, the tubular detector was successfully used for the determination of an active ingredient lomustine in pharmaceutical preparation CeeNU® Lomustine. As a detection method the non-stop-flow differential pulse voltammetry was used. Voltammetry in a flow system, where the analyte is sucked by pump through the tubular detector and the electrochemical cell, was found to be a suitable method for this type of determination. Moreover, the flow arrangement of this common voltammetric method is more sensitive than in batch arrangement. It is supposed that it is caused by convection which importantly contributes to the total mass transport. In this experiment it was demonstrated that the tubular detector is suitable for rapid and sensitive controlling of the amount of the active ingredient in drugs, in this case lomustine. The mean value of lomustine measured by proposed method well corresponded to the value declared by manufacturer. TD-AgSA has also good measurements repeatability.

For measurements in the flow system it was also necessarily to design and fabricate a small flow glass cell with miniature auxiliary and reference electrode. The reference electrodes (saturated calomel electrode, mercury-mercurous sulfate, and mercury-mercuric oxide electrode) in which metallic mercury was replaced by paste silver solid amalgam were proposed, fabricated, and tested for 14 months. All newly developed reference electrodes have proved a very stable in both long-term and short-term tests. Their potentials are almost identical with their metallic mercury analogues. The saturated calomel electrode based on paste silver solid amalgam was shown to be the most resistant to polarization and it was used as a reference electrode in all further experiments described in this thesis.

The formation of 11-mercaptoundecanoic acid monolayer on HMDE and on the electrodes based on solid amalgams (MF-AgSAE, m-AgSAE, m-BiAgSAE, m-CuSAE,m-CdSAE) was studied. The thiol formed the two-dimensional molecular films with lateral interactions between molecules. The created films completely blocked the surface of all electrodes. The lowest saturated surface coverage was observed at HMDE and the highest one at MF-AgSAE. Next, MF-AgSAE and m-AgSAE were used for preparation of several biosensors. First, monolayer of 11-mercaptoundecanoic acid was electrochemically formed at both electrodes. Then, avidin was bonded at MF-AgSAE and streptavidin at m-AgSAE via EDC/NHS chemistry. Thus prepared biosensors were successfully tested for the determination of biotin and biotin labeled albumin, respectively, by electrochemical impedance spectroscopy. From carried out experiments and from our previous experiences it could be concluded that the amalgam surface is suitable for modification by thiols and they can be used for the preparation of biosensors based on thiol monolayers.

The flow enzymatic biosensors based on the enzymatic reactor and the tubular detector were proposed. In the first case, the enzymatic reactor based on *porous* silver solid amalgam was prepared. The silver amalgam was modified by thiol 11-mercaptoundecanoic acid. The immobilization of enzyme glucose oxidase at thiol layer was carried out using EDC/NHS chemistry. The biosensor (porous reactor + tubular detector) was then successfully used for the determination of glucose in commercial honey as a real sample.

In the second case, the enzymatic reactor contained *powdered* silver solid amalgam. The amalgam powder was modified by 4-aminothiophenol and enzyme was attached via crosslinking agent glutaraldehyde. Five different enzymes were used (ascorbate oxidase, glucose oxidase, catalase, tyrosinase, and laccase), so five different biosensors were prepared for the determination of ascorbic acid, glucose, hydrogen peroxide, catechol, pyrogallol, and dopamine. The biosensor with ascorbate oxidase was used for the determination of ascorbic acid in the vitamin tablets Celascon®. In general, it was found that sensitivity of biosensors with powder reactor is 2–3 times higher than that of the biosensors with porous reactor.

The biosensors with porous reactor as well as powder ones were shown to be suitable for amperometric measurements in flow system under conditions of flow injection analysis. Their further advantages are reliability, relatively fast and simple preparation (1 hour 40 minutes), hogh sensitivity and good repeatability.

The last biosensor was constructed using polished silver amalgam electrode which was covered by layer of chitosan. Then, the enzyme sarcosine oxidase was immobilized at the surface of the chitosan via crosslinking agent glutaraldehyde. The covalent bonding of sarcosine oxidase on chitosan layer was found to be an effective and relatively fast procedure (3 hours) of enzyme attachment. This technique is suitable especially for enzymes with lower activity. Thus prepared biosensor was used for determination of biologically important substance – sarcosine in model sample by amperometry in wall-jet arrangement.

6. References

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Confirmation of Participation

1. **Yosypchuk, O.**; Barek, J.; Yosypchuk, B.: *Tubular Detector of Silver Solid Amalgam for Electrochemical Measurements in Flow Systems*. Electroanalysis 24, 2230 – 2234 (2012).
Impact Factor: **2.502**; percentage of participation of Mgr. Oksana Josypčuk ~ **75 %**.

2. **Josypčuk O.**, Barek J., Josypčuk B.: *Application of Non-stop-flow Differential Pulse Voltammetry at a Tubular Detector of Silver Solid Amalgam for Electrochemical Determination of Lomustine (CCNU).* Electroanalysis 26, 306–311 (2014).
Impact Factor: **2.502**; percentage of participation of Mgr. Oksana Josypčuk ~ **75 %**.

3. Yosypchuk, B.; Barek, J.; **Yosypchuk, O.**: *Preparation and Properties of Reference Electrodes based on Silver Paste Amalgam*. Electroanalysis 23, 2226–2231 (2011).
Impact Factor: **2.502**; percentage of participation of Mgr. Oksana Josypčuk ~ **50 %**.

4. Yosypchuk, B.; Fojta, M.; **Yosypchuk, O.**: *Thiolate Monolayers Formed on Different Amalgam Electrodes. Part II: Properties and Application*. Journal of Electroanalytical Chemistry 694, 84–93 (2013).
Impact Factor: **2.871**; percentage of participation of Mgr. Oksana Josypčuk ~ **50 %**.

5. Josypčuk B., Barek J., **Josypčuk O.**: *Flow Electrochemical Biosensors Based on Enzymatic Porous Reactor and Tubular Detector of Silver Solid Amalgam.* Analytica Chimica Acta 778, 24–30 (2013).
Impact Factor: **4.517**; percentage of participation of Mgr. Oksana Josypčuk ~ **50 %**.

6. **Josypčuk O.**, Barek J., Josypčuk B.: *Electrochemical Biosensors Based on Enzymatic Reactor of Silver Solid Amalgam Powder for Measurements in Flow Systems.* Electroanalysis 26, 1729–1738 (2014).
Impact Factor: **2.502**; percentage of participation of Mgr. Oksana Josypčuk ~ **75 %**.

7. **Josypčuk O.**, Barek J., Josypčuk B.: *Construction and Application of Flow Enzymatic Biosensor Based of Silver Solid Amalgam Electrode for Determination of Sarcosine.* Electroanalysis (submitted).
Impact Factor: **2.502**; percentage of participation of Mgr. Oksana Josypčuk ~ **75 %**.

I declare that the percentage of participation of Mgr. Oksana Josypčuk at the above given papers corresponds to above given numbers.

Prague, 03. 11. 2014

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Prof. RNDr. Jiří Barek, CSc.

List of Publications

1. Yosypchuk, O.; Pecková, K. a Barek,J.: *Voltametrické stanovení 1-nitropyrenu a 1-aminopyrenu na borem dopované diamantové filmové elektrodě.* **Chemické listy** 104, 186–190 (2010).

2. Yosypchuk, O.; Barek, J.: *Elektrochemická detekce karcinogenních derivátů pyrenu a jejich metabolitů.* **Chemické listy** 104, s61–s64 (2010).

3. Yosypchuk, O.; Barek, J.; Vyskočil, V.: *Voltammetric Determination of Carcinogenic Derivatives of Pyrene Using a Boron-Doped Diamond Film Electrode.* **Analytical Letters** 45, 449–459 (2012).

4. Yosypchuk, B.; Barek, J.; Yosypchuk, O.: *Preparation and Properties of Reference Electrodes based on Silver Paste Amalgam*. **Electroanalysis** 23, 2226–2231 (2011).

5. Yosypchuk, O.; Jiří Barek, Vyskočil, V.: *Determination of 1-Hydroxypyrene in Human Urine by HPLC with Electrochemical Detection at a Boron-doped Diamond Film Electrode.* **Analytical and Bioanalytical Chemistry** 404, 693-699 (2012).

6. Yosypchuk, O.; Karásek, J.; Vyskočil, V.; Barek, J.; Pecková, K.: *The Use of Silver Solid Amalgam Electrodes for Voltammetric and Amperometric Determination of Nitrated Polyaromatic Compounds Used as Markers of Incomplete Combustion*. **The Scientific World Journal** 2012, Article ID 231986, 12 pages, doi:10.1100/2012/231986

7. Yosypchuk, O.; Barek, J.; Yosypchuk, B.: *Tubular Detector of Silver Solid Amalgam for Electrochemical Measurements in Flow Systems*. **Electroanalysis** 24, 2230 – 2234 (2012).

8. Yosypchuk, B.; Fojta, M.; Yosypchuk, O.: *Thiolate Monolayers Formed on Different Amalgam Electrodes. Part II: Properties and Application*. **Journal of Electroanalytical Chemistry** 694, 84–93 (2013).

9. Josypčuk B., Barek J., Josypčuk O.: *Flow Electrochemical Biosensors Based on Enzymatic Porous Reactor and Tubular Detector of Silver Solid Amalgam.* **Analytica Chimica Acta** 778, 24–30 (2013).

10. Josypčuk O., Barek J., Josypčuk B.: *Application of Non-stop-flow Differential Pulse Voltammetry at a Tubular Detector of Silver Solid Amalgam for Electrochemical Determination of Lomustine (CCNU).* **Electroanalysis** 26, 306–311 (2014).

11. Josypčuk O., Barek J., Josypčuk B.: *Electrochemical Biosensors Based on Enzymatic Reactor of Silver Solid Amalgam Powder for Measurements in Flow Systems.* **Electroanalysis** 26, 1729–1738 (2014).

12. Josypčuk O., Barek J., Josypčuk B.: *Construction and application of flow enzymatic biosensor based of silver solid amalgam electrode for determination of sarcosine.* **Electroanalysis** (submitted).

Oral and Poster presentations

1. **XXIX. Modern Electroanalytical Methods, Jetřichovice, Czech Republic, 25.–29. 5. 2009**
   Oral presentation: *Voltametrické stanovení směsi 1-nitropyrenu a 1-aminopyrenu na borem dopované diamantové filmové elektrodě.*

2. **61. zjazd chemických společností, Vysoké Tatry, Tatranské Matliare, Slovakia, 7.–11. 9. 2009**
   Poster presentation: *Stanovení 1-nitropyrenu, 1-aminopyrenu a 1-hydroxypyrenu vysokoúčinnou kapalinovou chromatografií s elektrochemickou detekcí.*

3. **Modern Electroanalytical Methods 2009, Prague, Czech Republic, 9.–13. 12. 2009**
   Poster presentation: *Determination of 1-hydroxypyrene and 1-aminopyrene in human urine by HPLC with electrochemical detection based on boron doped diamond film electrode.*

4. **XXXI. Modern Electroanalytical Methods, Jetřichovice, Czech Republic, 23.–27. 5. 2011**
   Oral presentation: *Amperometric Determinations of Nitrocompounds with Flow Detector of Silver Solid Amalgam.*

5. **63. zjazd chemických spolecností, Vysoké Tatry, Slovakia, 5.–9. 9. 2011**
   Poster presentation: *HPLC stanovení nitrosloučenin pomocí průtokového detektoru ze stříbrného pevného amalgámu.*

6. **7th International Students Conference "Modern Analytical Chemistry", Prague, Czech Republic, 29.–30. 9. 2011**
   Oral presentation: *Electrochemical biosensor for determination of nucleic acid bases.*

7. **14th International Conference on Electroanalysis, Portorož, Slovenia, 3.–7. 6. 2012**
   Poster presentation: *Electrochemical biosensor based on the mercury covered solid silver amalgam electrode for determination of cytosine.*

8. **12th International Conference on Flow Analysis, Thessaloniki, Greece, 23.–28. 9. 2012**
   Poster presentation: *Tubular Detector of Silver Solid Amalgam for Electrochemical Measurements in Flow Systems.*

9. **XXXIII. Modern Electroanalytical Methods, Jetřichovice, Czech Republic, 20.–24. 5. 2013**
   Oral presentation: *Construction and Application of Tubular Detector and Porous Flow-through Detector/Reactor of Silver Solid Amalgam for Electrochemical Measurements in Flow Systems.*

10. **Euroanalysis XVII., Warsaw, Poland, 25. 8.–29. 8. 2013**
    Poster presentation: *Tubular Detector Based on Silver Solid Amalgam as a New Construction Arrangement of the Amalgams Electrodes Designated for Flow Measurements.*

11. **18th International Conference on Flow Injection Analysis, Porto, Portugal, 15.–20. 9. 2013**
    Poster presentation: *Electrochemical Flow Biosensor Based on the Silver Solid Amalgam Electrode for the Determination of Cancer Marker Sarcosine.*

12. **9th International Students Conference 'Modern Analytical Chemistry', Prague, Czech Republic, 23. 9.–24. 9. 2013**
    Oral presentation: *Electrochemical flow biosensors based on the silver solid amalgam electrodes.*

13. **24th Anniversary World Congress on Biosensors, Melbourne, Australia, 27. 5.–30. 5. 2014**
    Poster presentation: *Amperometric Flow Enzymatic Biosensor for Detection of Sarcosine Based on Polished Silver Solid Amalgam Electrode.*

Achievements

1. **2nd prize in the competition for the best scientific work in analytical chemistry**, Bratislava, Slovakia, 2008

2. **2nd prize in the 13th statewide competition for the prize of Merck company for the best student scientific work in analytical chemistry**,
   České Budějovice, Czech Republic, 2010

3. **Prize of the Metrohm company 2013 for the best publication** *Flow electrochemical biosensors based on enzymatic porous reactor and tubular detector of silver solid amalgam* (Anal. Chim. Acta 778, 24 (2013).

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• *New Electrode Materials and Its Modification for Analysis of Biological Active Substances*, Grant Agency of Charles University in Prague, 2011–2013.

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