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# Journal of Metallomics and Nanotechnologies

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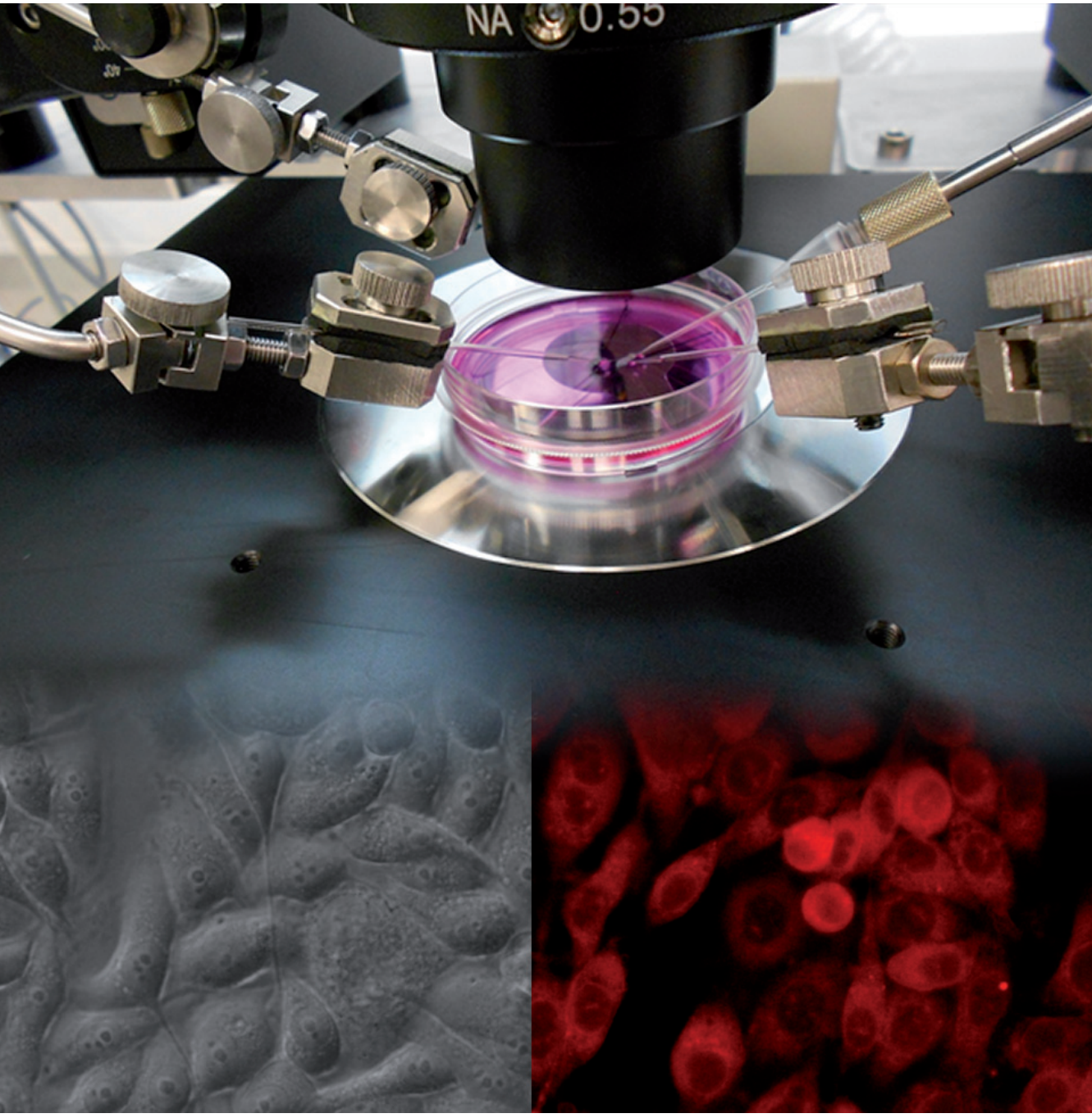

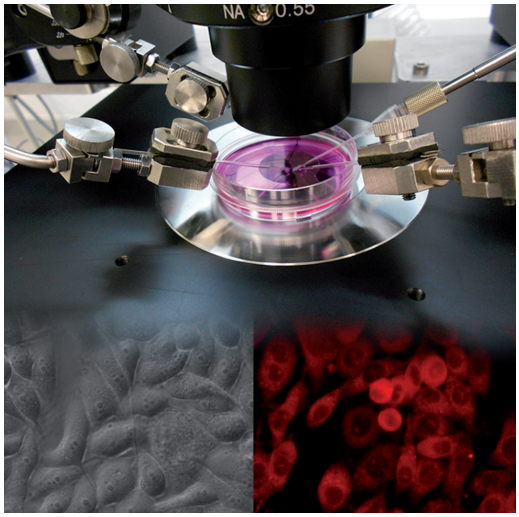

The journal of Metallomics and Nanotechnologies is exclusively in ellectronic form and is published quarterly. The thematic conent is focused on nano-biochemistry, nanotechnology, biomedicine and nanomedicine. The journal is published without regional mutations in Czech, Slovak and English language. Publisher: Laboratory of metallomics and nanotechnologies, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic.

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**on the cover:**

A set of 4 micromanipulators prepared for manipulation with cells (upper image) and for measurement of electrochemical changes in cell cultures. A micrograph of PC-3 cells in an ambient light (lower left image) and a micrograph of PC-3 cells fluorescence after their incubation with doxorubicin (lower right image).

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Journal of Metallomics and Nanotechnologies
**Publisher:** Mendel University in Brno, **Chief editor**: Ondrej Zitka, **Edition**: First 2014
**Number of pages:** 60, based electronically, ISSN 2336-3940

Dear readers,

The fourth number of the „Journal of Metallomics and Nanotechnologies“ is the very first which is completely in English. Editorial board is composed from members of various countries (Fig. 1). In the end of the year 2014 the last members who have been included were: Dr. Carlos Fernandez, Robert Gordon University, United Kingdom and Dr. Sarmistha Raychaudhuri, University of Calcutta, India. Now the JMN is also part of the DOAJ database which is increasing the visibility and ease of use of open access scientific and scholarly journals.

This number contains three reviewes and five articles. The reviews are focused on the area of detection of papilomavirus, clinical application of capillary electrophoresis and the role of phytochelatins in plant and animals. All the publications also makes a part of the output of research projects as CEITEC, IGA MZ - Spincancer, GACR NANOCHEMO and GACR NanoBioTeCell and thus it shows the versatility of the scope of the laboratory of Metallomics and Nanotechnologies. Also one scientific abstract and two laboratory reports are included in this issue. The first report shows the progress of reconstruction of the laboratory space and the second-one reminds us the birthday of Assoc Prof. Libuše Trnková which is the person which actualy iniciated the genesis of the „Laboratory of Metallomics and Nanotechnologies“ by inspiring and the enthusiasm couple years before.

Next number of the first issue of 2015 will be focused on the viruses. Particularly it will be based on five currently most known viruses as Influenza, Ebola, HIV and also HPV virus.

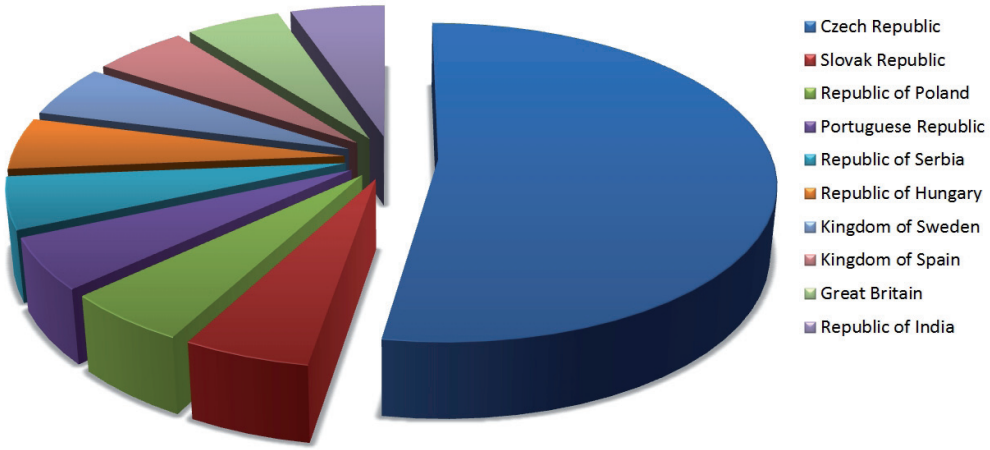


**Figure 1.** Constitution of editorial board of the JMN in the end of the year 2014.

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## Review



## Article



## Scientific abstract



## Laboratory reports

REVIEW

# Human papilloma virus (HPV) and methods for its identification in head and neck cancers

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**Human papilloma virus (HPV) and methods for its identification in head and neck cancers**

Human papillomaviruses (HPV) are small circular, double-stranded DNA viruses infecting epithelial tissues. The viruses have been detected in humans and a variety of mammalian and avian species. The papillomavirus family that affects human skin and the moist membranes that line the body, such as the throat, mouth, feet, fingers, nails, anus and cervix. There are over 100 types, of which 40 can affect the genital area. Most known HPV types cause no symptoms to humans. Some, however, can cause verrucae (warts), while a small number can increase the risk of developing several cancers, such as that of the cervix, penis, vagina, anus and oropharynx. Researches are focused on the development of HPV detection assays specially designed for head and neck squamous cell carcinoma (HNSCC). Many different methodologies have been utilized to identify the HPV virus including polymerase chain reaction (PCR) testing, real time PCR, in situ hybridization analysis (ISH), immunohistochemical (IHC) staining for tumor suppressor protein p16, and southern blotting assays. The objective of this review is to explain HPV virology, the molecular mechanisms of carcinogenesis in head and neck cancer and the main methodology for HPV detection in these types of cancer

**Accepted:** 5. 12. 2014
**Keywords:** human papillomavirus, cancer, head, neck, PCR, In situ hybridization, immunohistochemical staining, southern blotting

## 1. Introduction

Human papillomavirus (HPV) selectively infects the epithelium of the skin and mucous membranes. Specific HPV types are associated with squamous cell carcinoma, adenocarcinoma, and dysplasias of the cervix, penis, anus, vagina and vulva[1]. The term head and neck cancer includes malignancy in an area that comprises the skin, oral cavity, salivary glands, lip, pharynx, larynx, nasal cavity, paranasal sinuses and soft tissues of the neck and ear[2].

The first association of HPV with head and neck cancer was published in 1985[3]. HPV was also shown to play a role in the pathogenesis of a subset of head and neck squamous cell carcinomas (HNSCCs)[4]. Almost 650,000 patients worldwide are diagnosed with head or neck cancer each year and 350,000 patients die of this disease as this cancer is the sixth most prevalent type of cancer worldwide.

The ratio of males to females is approximately 2:1[2, 3]. From the point of view of the infection, HPVs have developed several molecular mechanisms to enable infected cells to suppress apoptosis[5, 6]. Based on their potential for oncogenesis, HPV types can be classified both as high-risk or low-risk[7]. Precancerous lesions of the oral mucosa are epithelial changes that are able to undergo malignant transformation more likely than normal tissue at other mucosal sites[8].

A total of 150 HPV genotypes have been identified[9]. The HPV 16 and 18 strains, which are known to cause nearly all cases of cervical cancer, also raise the risk of developing oro-

pharyngeal cancer[10]. The evident similarities between both cervical and head and neck tumors prompted the utilization of the same HPV diagnostic procedures. There is now compelling evidence that specially designed methodologies must be employed for prognosis[11, 12].

## 2. HPV virology

HPVs are quite small, non-enveloped double--stranded circular DNA viruses, that has diameter of 55 nanometers. The DNA of HPV has 8,000 nucleotide base pairs associated with histones. HPV genome is enclosed in an icosahedral capsid shell comprised of major and minor capsid proteins [2, 13] and can be divided into 3 domains: an early region with 6 E genes E1, E2, E4, E5, E6 and E7; a late region with 2 L genes, L1 and L2; and a non-coding regulatory region (NCR) of approximately 1 kb. Organisation of HPV 16 genome is shown in **Fig. 1** [1].

The L1 and L2 late proteins form capsomers of the virus that encapsidate the viral DNA. The early E proteins have different functions. The E1 protein binds to the origin of replication. The E2 protein binds to E1 protein and stimulates viral DNA replication. The E2 protein also acts as a transcriptional repressor of HPV E6 and E7 gene expression. The E4 protein is expressed in an later phase with a role in the assembly and release of the viral particle. E5 protein stimulates the transforming activity of the epidermal growth factor receptor resulting in the increased cell proliferation[14, 15].

The viral protein E6 and E7 are implied in tumorigenesis and are known to induce degradation of the tumor suppressors p53 and pRB, respectively[16]. They can suppress apoptosis and alter the function of factors involved in cell-cycle regulation, thereby facilitating prolongation of the proliferative stage of keratinocyte differentiation[17]. The E6 protein of HPV-16 is a small polypeptide of 150 amino acids. This protein can inactivate p53 by targeting the protein for ubiquitination and consequent degradation[18]. The HPV-16 E6 protein also activates the telomerase, an enzyme that maintains the telomeric DNA at the ends of linear chromosomes. Almost all human cancers and immortalized cell lines have highly active telomerase[19-21].

The E7 protein of HPV-16 is a small, nuclear polypeptide of 100 amino acids. E7 binds to retinoblastoma protein (pRb)[20]. Besides pRb, E7 also interacts with various other proteins, most of which are important regulators of the cell growth The E7 protein induces abnormal centrosome duplication, resulting in multipolar, abnormal mitoses, aneuploidy and genomic instability[17, 22].

The majority of cervical carcinomas caused by the two most common HPV types, HPV-16 and HPV-18, contain integrated viral sequences that express E6-E7 protein[23]. E6-E7 oncogene expression is considered necessary for carcinogenesis, and maintenance of the malignant phenotype of these cancers[2, 24].

## 3. Methods for detection and identification of HPV

Some of the main methodologies for detection of HPV virus: southern blotting assay, polymerase chain reaction (PCR) testing, real time PCR, in situ hybridization (ISH) analysis, immunohistochemical (IHC) staining for p16 are shown in Table 1[26].

### Southern Blotting Assay

Southern blotting is an assay that has long been one of the standard techniques for the detection of HPV DNAs, and it has the ability to differentiate between episomal and integrated DNA and can detect as little as 0.1 copies of viral DNA per cell[27].

Southern blot has a theoretically higher specificity but is less sensitive than PCR. This method cannot be applied to formalin-fixed, paraffin--embedded tissue samples because they contain cross-linked, degraded nucleic acids. Nevertheless, the Southern blotting assay can be useful for comparing results of other methods for detection of viral integration, though this method has no practical or clinical utilization[27, 28].

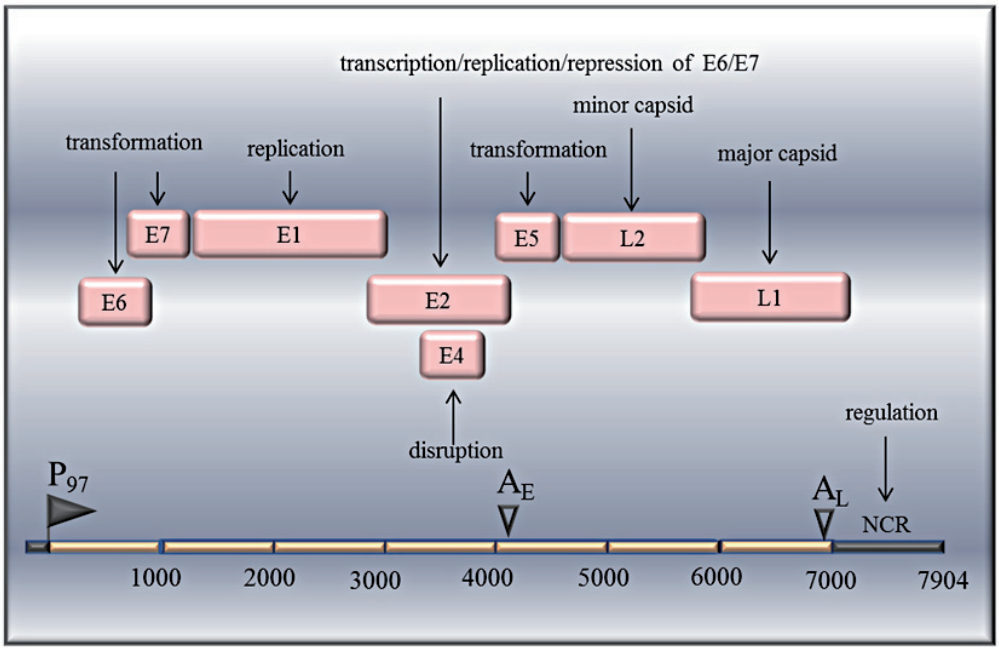


**Figure 1.** Genetic Map of the human papillomavirus (HPV) type 16. HPV genome is a double-stranded circular DNA molecule. Early genes from the DNA sequence are designated E1 to E7 and late genes, L1, and L2, indicated in pink boxes. The non-coding region (NCR) is indicated by the black box. Adopted and modified according Chen et al [25].

### Polymerase Chain Reaction (PCR) and real-time PCR

Polymerase chain reaction (PCR) is a sensitive marker for HPV DNA detection and RT-PCR may be a sensitive marker for HPV mRNA quantification[29]. PCR represents a highly-sensitive and cost-effective method for HPV detection. In theory, it can be used to detect as little as one copy of a DNA sequence and can be utilized in paraffin-embedded tissue or fresh tissue from biopsies[27]. The PCR techniques have a number of drawbacks in comparison to in situ hybridization (ISH); they have lower specificity, they do not allow distinction between HPV that is present in the neoplastic and non-neoplastic cells and they cannot distinguish between episomal and integrated HPV DNA[30].

The presence of latent virus leads to false positive results due to the ability of PCR to detect just a few copies of HPV DNA per cell. Attempts have been made to resolve this issue through use of real-time (RT)-PCR, which provides a quantitative analysis of viral load. RT-PCR amplification of viral E6/E7 mRNA is considered for the detection of clinically significant HPV infection within tumor specimens as it detects transcriptionally active HPV[31, 32]. Nonetheless, PCR and RT-PCR cannot localize HPV in the area of neoplasia and other techniques like ISH can provide this information together with a higher clinical specificity[33].

### *In Situ* Hybridization for HPV

*In situ* hybridization (ISH) is the only molecular method allowing reliable detection and identification of HPV in topographical relationship to their pathological lesions. Unlike in other molecular methods, in ISH the whole HPV detection procedure occurs within the nucleus of infected cells[34]. The result of the hybridization reaction is evaluated microscopically and the appearance of an appropriate precipitate within the nucleus of epithelial cells is indicative for the presence of HPV in the specimen. In addition, the physical state of the virus can be evaluated by the presence of punctuate signals for integrated virus and diffuse signals for

| METHODS | ADVANTAGES | DISADVANTAGES |
|---|---|---|
| **Southern Blotting** | High specificity<br><br>Differentiation between episomal and integrated DNA<br><br>Detects 0.1 copies of DNA per cell | Not easily applied to FFPE samples<br><br>Practical/clinical utilization |
| **PCR** | High sensitivity<br><br>Cost effective<br><br>Several commercially available primer sets | Low specificity<br><br>No quantitative measure of viral load<br><br>No distinction between episomal and integrated DNA |
| **Real time-PCR** | High sensitivity<br><br>High specificity<br><br>Ability to differentiate between episomal and integrated DNA | False positive and false negative products<br><br>No direct evidence of viral integration |
| **ISH** | High specificity<br><br>High sensitivity<br><br>Ability to differentiate between episomal and integrated DNA | Technically difficult to be use in routine screening |
| **p16 Immunostaining** | High Sensitivity<br><br>Easily applied to FFPE tissue | Surrogate marker<br><br>Specificity not ideal |

**Table 1.** Possible methods and techniques of HPV detection. Adopted and modified according Venuti and Paolini[26]

episomal virus. In this way, ISH may overcome some of the limitations of PCR by detecting only clinically relevant infection. Although the specificity of this method is high, the sensitivity is not ideal[35]. The sensitivity of ISH is still less than that seen in PCR analysis. However, ISH is more specific for HPV infection than p16 immunohistochemical staining[30]. In addition, current ISH-based assays are considered by many experts in the field to be too laborious and to have insufficient clinical sensitivity to be used in routine screening[34].

**Immunohistochemical Staining for p16**

The transcription of the viral oncoprotein E7 inactivate the function of the pRB gene, causing perturbation of other key components of the retinoblastoma pathway, and to induce upregulation of P16 protein expression, reaching levels that can be detected readily by immunohistochemistry. Accordingly, P16 immunohistochemistry is sometimes advocated as a surrogate marker of HPV infection[36].

P16 immunostaining is an appropriate assay for elimination of HPV negative cases from any additional analysis. Since specificity is almost 100%, a finding positive for HPV 16 on in situ hybridization reduces the number of false--positive cases by P16 immunostaining alone. Whatever the method is used to establish the presence of non-HPV 16 virus types, upfront use of P16 immunostaining and HPV 16 in-situ hybridization accurately establishes the HPV status of most oropharyngeal cancers[37].

Alternatively, P16 overexpression could suggest pRB pathway disturbances unrelated to HPV. Using E6 and E7 mRNA levels as conclusive evidence of HPV involvement, P16 immunostaining is 100% sensitive but only 79% specific as a surrogate marker of HPV infection[36].

## 4. Conclusion

Many different HPV tests exist, and much more information about their analytical and clinical properties. All of the methods are able to give information about the presence of HPV in biological samples. At the present time, IHC staining for p16 and PCR appear to be the most sensitive markers for HPV, while ISH confers the great specificity. The combination of a sensitive test, p16 IHC and a specific test, ISH could allow for the best potential to accurately establish the presence or absence of HPV.

This information is crucial for the prognostic assessment of these patients but it is ineffective for the individuation of people at risk of tumor after HPV infection. Thus, there is a need for improving these HPV diagnostic tools by detection not just of the virus, but also simultaneous detection of other biological markers like alterations in tumor suppressor gene pathways and the modification of the gene expression profiles.

*This work was financially supported by project SPINCANCER NT/14337.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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# Clinical application of capillary electrophoresis – determination of free amino acids in body fluids

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**Clinical application of capillary electrophoresis – determination of free amino acids in body fluids**

The determination of free amino acids (AAs) levels in body fluids presents routine biochemical analysis providing information about pathophysiological pathways in AAs metabolism or evaluation of nutritional state of organism. For the AAs quantification a number of method based on chromatographic separation such as high pressure liquid or most widespread ion-exchange chromatography are used. After popularization the capillary electrophoresis (CE) as routine laboratory technique, it has become a useful alternative for determination of AAs, biogenic amines or peptide to chromatographic methods. CE is characterized by low sample and solution consumption, short analysis time and separation efficiency. Due to the high resolving power of CE is used for analysis of complex physiological fluids. The main body fluids for AAs detection are for example blood, cerebrospinal fluid, urine, saliva or amniotic fluid.

**Accepted:** 3. 12. 2014
**Keywords:** amino acids, body fluids, capillary electrophoresis

## 1. Introduction

Determination of biomarkers in body fluids provides information about the overall state of the organism[1]. Among commonly determined markers in clinical laboratories belong AAs. The AAs (**Fig. 1**), basic structural units of peptides, as well as their derivatives occupy many metabolic and biochemical roles in human body [2,3]. The AAs belong to zwitterions containing acidic and basic functional groups (carboxyl groups and amino, respectively). Zwitterions have neutral character at a certain pH known as the isoelectric point due the dissociation of acidic and basic groups. Therefore, the charge of AAs depend on the number of functional groups and pH environment [4].

Determination of AAs levels in the body fluids such as blood and/or plasma [5,6], cerebrospinal fluid [6,7], urine [6,8], saliva [6,7] or amniotic fluid [9] represents the significant clinical indicator not only for control of the nutritional state of the organism, but for a number of metabolic disorders[10]. Examples can be inherited metabolic disorders such as phenylketonuria (PKU) (an enzyme deficiency in phenylalanine hydroxylase (PAH) (**Fig. 2**))[11] or maple syrup urine disease (an enzyme deficiency in branched-chain α-ketoacid dehydrogenase (BCKD) (**Fig. 3**))[12].

There are many methods for AAs determination employing various techniques such as high pressure liquid chromatography (HPLC)[13] or gas chromatography (GC) [14]. During the 80's of the 20th century CE became a routine laboratory technique[15]. Recently, this method has ranked among very useful tools for separation and determination of AAs [2,9,16,17], biogenic amines[18] or peptides [19] and is often used as an alternative to HPLC

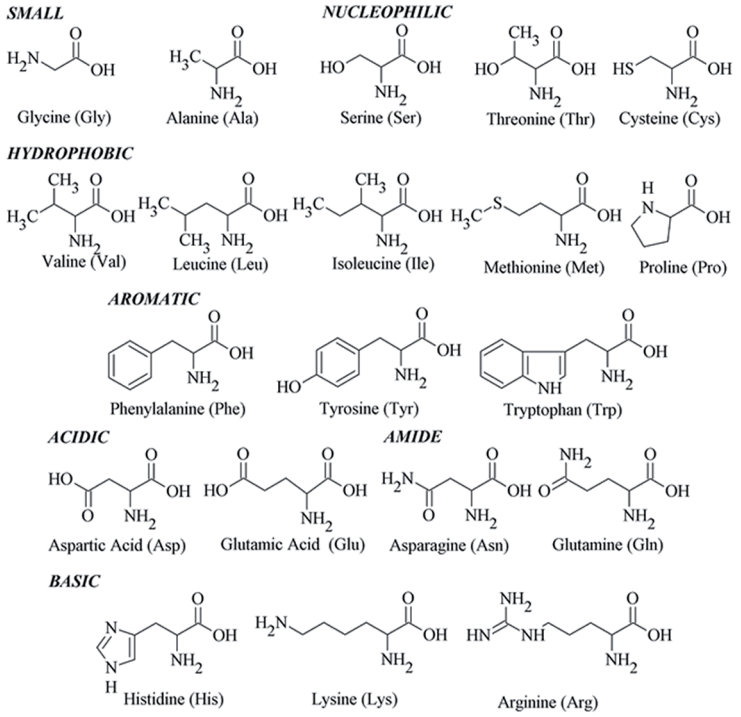


**Figure 1.** Twenty commonly occurring amino acids.

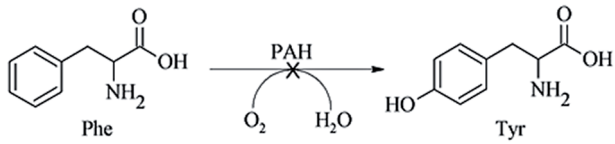


**Figure 2.** Enzyme defect in PKU disease.

or GC due to its electrophoretic separation mechanism [16,20]. In addition, a main advantage is very low sample and solvent consumption, speed of analysis and excellent separation efficiency [2,16,21-23]. Besides, due to the high resolving power is CE a promising method for the analysis of biological fluids (complex mixtures of many metabolites)[2,22].

The essential part of the CE instrumentation is a detector. In photometric and fluorescence detection is problem with a limited presence of chromophores and fluorofores. Because of that, the derivatization procedures prior CE are usually used, which is the one of the limiting factors [24]. Therefore, there are attempts to analyze AAs without derivatization employing various strategies such as indirect detection [25], electrochemical detection (conductometric detection [6,8], amperometric detection [26]) or CE in combination with mass spectrometry (MS) [5,11].

## 2. Determination of AAs in body fluids

### 2.1 Blood and/or plasma

The plasma concentration of AAs represents not only an important indicator of the intermediary metabolism, but plays a very important role in many diseases such as diagnostic biomarker, evaluating the disease progression, and monitoring response to the drug therapy[27]. Recent clinical studies have shown an association between the high level

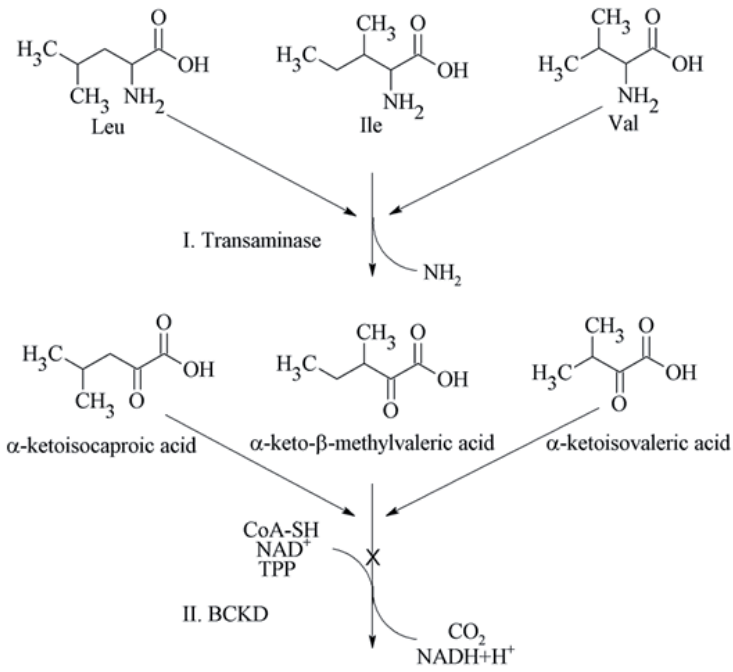


**Figure 3.** Enzyme defect in maple syrup urine disease.

of homocysteine (hCy) and Cys and vascular diseases[28,29]. hCy and Cys belong to the low-molecular-weight biothiols, which play an important role in metabolism. To this group belongs also significant tripeptide, the glutathione (GSH), composed of the glutamic acid, cysteine and glycine. GSH occurs in two forms - reduced triol (GSH) and oxidized disulfide (GSSH). The antioxidant function and detoxification processes belong between the important roles of GSH[30]. Zunic and Spasic focused their study on determination of GSH as reflect of oxidative status and aromatic and sulfur containing AAs as good indicators of protein metabolism. They analyzed human capillary blood using CE with direct photometric detection[24].

As it was mentioned, the plasma concentration of AAs also plays an important role as a biomarker for a number of diseases. The association with a number of neuropsychiatric diseases was demonstrated. For example, the association between plasma level of Glu and Gly and psychotic symptomatology in bipolar disorder (BD) [27] or the correlation between plasma levels of Glu, Ala and Ser with severity of depression and schizophrenia [31,32] was observed. Lorenzo and co-workers focused their attention on determination the AAs concentration in human plasma from patients with BD. They developed a sensitive CE coupled to laser-induced fluorescence (LIF) detection. The method permitted to quantify fourteen AAs and the obtain results were consistent with published values for patients with BD. This method is suitable for studies that use the AAs as biomarkers in the monitoring of diseases. In addition, chiral CE-LIF method permits separation of L- and D- AAs, which cannot be detected with other general methods based on GC and HPLC [10].

In the introduction, the inherited metabolic disorders characterized by an enzyme defect were mentioned. An important role plays detection and diagnosis of these diseases in the

neonatal period as part of newborn screening, because the enzyme defect may cause an accumulation of toxic metabolites that leads to serious and irreversible complications[33]. Jeong and co-workers focused on diagnosis of PKU by determination of Phe and Tyr from dried blood spots using capillary CE-MS with electrospray ionization[11]. A routine clinical laboratory detection can determine semi-quantitatively the Phe using a bacterial inhibition assay [34] and enzymatic colorimetric assay [35] in the form of commercial kits. But these kits may lead to relatively high rates of false-positive results. Between the instrumental methods as the conventional PKU screening method belongs HPLC-MS[36]. Jeong and co-workers examined CE-MS as an alternative for routine analytical methods for clinical applications and applied the optimized CE-MS method to real samples of Korean newborns. After comparison with the above common techniques the results showed potential of method as a routine analytical methods for clinical applications due to its different separation mechanism, speed, high separation efficiency, and extremely low sample consumption[11].

Attractive alternative to detection without the interaction of electromagnetic radiation with the analyte is electrochemical detection in the form of conductometry, amperometry or potentiometry. Samcova and Tuma focused their attention on CE using contactless conductivity detection (CCD) method[23]. This alternative brings analysis of AAs without a presence of chromofore and without derivatization step prior CE. The optimized method determined 18 AAs from 20 proteinogenic AAs, 3 nonproteinogenic AAs and creatinine from plasma samples prepared from the arterial blood of 9 healthy humans and the ranges for the AAs levels in human plasma samples agreed well with those determined by the liquid chromatographic method in two other laboratories. LODs for individual AAs vary in an acceptable range from 4.3 mM for Arg to 42.9 mM for Cys [23].

### 2.2 Cerebrospinal fluid (CSF)

As a plasma concentration of AAs also a concentration of AAs in CSF plays an important role in a number of physiological and pathophysiological processes [37]. CSF surrounds the brain and the spinal cord and it is produced from the choroid plexuses. CSF circulates in the central nervous system (CNS) and provides a constant chemical environment for brain cells. This body fluid can be used most likely to detection disturbances of the CNS due to the close relationship between CSF and the CNS[7,38]. Significant indicators for the control of various functions in the central and peripheral nervous system are the AAs neurotransmitters[39]. Among the most studied AA neurotransmitters include excitatory AAs such as Glu or Asp playing a role in learning and memory[7,40], the inhibitory neurotransmitters such as γ-aminobutyric acid (GABA), Gly or taurine (Tau) [41] and AA Gln closely linked to the metabolic turnover of Glu and GABA[17]. In recent studies correlation between the changes in AAs level and a number of neurological diseases was found [42]. For example Alzheimer's disease, where anatomical and biochemical evidence suggests the dysfunction of excitatory AAs pathways[40]. Another diseases which has been confirmed by the association between changes in AAs level and diseases was Parkinson's disease [43], stroke [44,45] or epilepsy [46]. Deng and co-workers developed a new CE-LIF method for the determination of AAs neurotransmitters [7]. The practical utility of developed method was carried out on human CSF and saliva samples. This method achieved detection of Gln, GABA, Gly, Tau, Glu and Asp with the LOD as low as 0.06 nM and the outlook for this method includes detection of AA neurotransmitters released from a single cell and their in vivo monitoring by microdialysis sampling.

### 2.3 Saliva

Saliva represents one of the other significant informative body fluids primarily due to easy and noninvasive approach. This fluid permits the early gathering information about the human health[7,20]. Many studies have confirmed the presence of free AAs in saliva[47,48]. Association between levels of Lys and Arg and their cariostatic effect and caries was observed. By caries-free adults have been showed elevated

levels of these AAs in the saliva, as compared with caries-susceptible adults[49]. An involvement of Gly in the pathogenesis of parodontitis was suggested due its stimulatory effects of production interleukin-1beta-induced prostaglandin E2 [50]. In addition, this AA is together with Glu involved in the metabolism of microorganism. Besides, the presence of Pro indicates putrefaction[16,51].

Deng and co-workers employed the saliva for practical utility their developed CE-LIF method for determination of AA neurotransmitters[7]. This method has been mentioned by determination of AA neurotransmitters in CSF. Due to this method have been detected main AAs occurring in saliva from two volunteers (Pro, Ser, Gly and Glu) with the LODs in the range from 0.1 to 2.4 nM. The promising results of AAs quantification was in good agreement with data in earlier studies. For the elimination of derivatization step by determination of AAs, Coufal and co-workers employed CE-CCD [21]. The detection limits of individual AAs ranged within a concentration interval from 9.1 μM for Lys to 29 μM for Asp. This method was tested on more natural samples as beer, yeast or urine and in the saliva was successfully detected Gly and Pro.

### 2.4 Urine

Between the other important biological fluids belongs the urine. This complex mixture presents an essential part of clinical routine for the screening of the intermediary metabolism in the body. As well as in saliva the big advantage of urine is its non-invasive approach[52]. In the previous section was mentioned Coufal and co-workers CE-CCD method with elimination the derivatization procedure. One of the tested samples was urine where managed to identify nine amino acids and creatinine. Five years later, Chen and co-workers employed for determination of AAs in urine cyclodextrin--modified CE-LIF [22]. This method permitted the successful determination of seven amino acids (Phe, Glu, Pro, Gly, Ser, Ala, Val) with the LODs in range 160~330 nM and without complicated pretreatment procedures. Another method for AAs determination introduced Ramautar and co-workers. They present improved CE-TOF-MS method achieving the LODs down to 20 nM and permitting the selective detection of all commonly occurring amino acids, except for the isobaric amino acids such as leucine and isoleucine. This method is suitable for the metabolic profiling of urine and other complex fluids.

### 2.5 Others

The detection and determination of biomarkers play also the important role in prenatal diagnostic. For these purposes amniotic fluid (AF) serves and the monitoring of this fluid brings information about the fetus health and maturity. AF forms a sac around the fetus and creates a protective environment from mechanical and thermal shock [9,53,54]. In addition to this role, is AF involved in development of the lungs, kidneys, and gastrointestinal tract [9]. The volume of AF varies according week of pregnancy and is in the correlation with the growth of fetus. The volume ranges from about 50 mL at 12 weeks to 1 L at 38 weeks. After this week, the volume decreases to a volume of about 600 mL [9,54]. The biochemical investigation of the AF is done in the case of the semblance of the abnormal fetal development or by the pregnant woman age over 35 years. Because of the AAs monitoring various metabolic disorders can be detected, for example mentioned PKU associated with abnormal metabolism of AAs [9]. Tuma and co-workers employed CE-CCD method and applied it on the AF from 20 pregnant women aged over 35 years and 24 pregnant women with abnormal fetal development for the whole profile of AAs [9]. This method permitted the sensitive determination of 20 proteinogenic AAs and 12 other biogenic compounds with LODs between 1.5~6.7 μM. The obtained results showed systematically higher mean concentration of majority of AAs for women suspect of abnormal fetal development.

Summary of CE conditions for analysis of AAs in various body fluids is given in **Table 1**.

**Table 1**: Table summarizing some experimental conditions for determination of AAs using CE analysis.

| DETECTION METHOD | SAMPLE | DERIVATIZATION STEP | BGE | CAPILLARY | VOLTAGE | REF |
|---|---|---|---|---|---|---|
| **UV** | Human capillary blood | No | 10 mmol/l phosphate buffer (pH 2.8) | 47 cm, 75 µm ID | 15 kV | 24 |
| **Indirect-UV** | Human plasma, supernatant of macrophage cultures | No | p-Aminosalicylic acid buffered with sodium carbonate (pH 10.2 ± 0.1) | 87 cm, 75 µm ID | 15 kV | 3 |
| **Indirect-UV** | Human urine | No | 5.0 mM carbonate, 2.0 mM salicylate, 0.15 mM myristyltrimethyl-ammonium bromide (pH 10.7) | Unknown length, 100 µm ID | 15 kV | 55 |
| **LIF** | Human plasma | NBD-F | 175 mM borate buffer, 12.5 mM β-cyclodextrin (pH 10.25) | 60 cm, 75 µm ID | 21 kV | 10 |
| **LEDIF** | Human cerebrospinal fluid | NDA | 10 mM tetraborate, 0,6% PEO (pH 9.3) | 50 cm, 75 µm ID | 15 kV | 38 |
| **LIF** | Human cerebrospinal fluid, saliva | SIFA | 100 mM SDS, 100 mM boric acid (pH 9.6) | 60.2 cm, 75 µm ID | 15 kV | 7 |
| **LIF** | Human saliva | FITC | 20mM borate buffer (pH 9.5) | 58 cm, 75 µm ID | 20 kV | 16 |
| **LIF** | Human urine | FITC | 80 mM borate buffer (pH 9.2) containing 45 mM α-cyclodextrin | 60 cm, 50 µm ID | -15 kV | 22 |
| **LIF** | HUVEC, ECV304, R1 stem cells | 5-IAF | 20 mmol/l sodium phosphate, 16.5 mmol/l boric acid, 100 mmol/L N-methyl-D-glucamine (pH 11.2) | 60 cm, 50 µm ID | 30 kV | 30 |
| **CCD** | Human plasma | No | 1.7 M acetic acid containing 0.1% hydroxyethylcellulose (pH 2.2) | 80 cm, 75 µm ID | 20 kV | 23 |
| **CCD** | Human urine and saliva, beer, yeast, herb extracts | No | 2.3 M acetic acid and 0,1% hydroxyethylcellulose | 80 cm, 50 µm ID | 30 kV | 21 |
| **CCD** | Amniotic fluid | No | 1.7 M acetic acid and 0.1% hydroxyethylcellulose (pH 2.15) | 80 cm, 75 µl ID | 20 kV | 9 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **MS** | Human dried blood spots | No | 3 mM $NH_4Ac$ (pH 10.7) | 40 cm, 50 µm ID | 25 kV | 11 |
| **MS** | Child plasma | No | 50 mM aqueous formic acid (pH 2.5) | 105 cm, 50 µm ID | 30 kV | 56 |
| **MS** | Human urine | No | 20% methanol, 2M formic acid | 100 cm, 50 µm ID | Unknown | 52 |
| **MS** | Human urine | No | 1 M formic acid (pH 1.8) | 130 cm, 50 µm ID | 30 kV | 57 |

**Abbreviations**: 5-IAF 5-iodoacetamidofluorescein; **NBD-F** 4-fluoro-7-nitro-2,1,3-benzoxadiazole; **SIFA** N-hydroxysuccinimidyl fluorescein - O--acetate; **NDA** naphthalene dicarboxaldehyde; **FITC** fluorescein isothiocyanate; **PEO** poly(ethylene oxide)

## 3. Conclusion

The CE represents a powerful tool for determination of amino acids in complex body fluids due the high resolving power, small reagents consumption and separation efficiency. There are a number of detection methods such as optical method, electrochemical method or CE in combination with MS. Problem occurs in photometric and fluorescence detection by analytes without the presence of chromophore or fluorophore. In this case derivatization procedures prior CE are used. Effective alternative without derivatization procedure is CE-CCD or CE-MS. CE-MS represents powerful analytical tool with highly sensitive for selective quantification and determination relative molecular weights and molecular structure of substances. However, the limiting factor in the use of this method in routine practice can be demanding requirements to use interface.

Whereas, CE-CCD permits the analysis of the whole profile of proteinogenic AAs in native form without derivatization and demanding requirements.

*Financial support from CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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REVIEW

# The role of phytochelatins in plant and animals: A review

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**The role of phytochelatins in plant and animals: A review**

Phytochelatins (PCs) are thiol-containing oligomers formed in post-translational synthesis from glutathione. They were firstly described in yeasts Schizosaccharomyces pombe. Subsequently their presence was monitored in plants, microorganisms, but also in many animal species. It is well known, that in plants PCs exhibit significant function in manner of chelating of metals. Since they contain thiol functional groups originated from cysteine moieties they keep a metal homeostasis balanced. Although the presence of genes encoding PCs was confirmed in a few animal species, their function in these organisms was not satisfactorily elucidated. Some studies revealed that PCs in animal species are closely linked with detoxification processes in similar way as in plants. It was also shown that thiols in invertebrates are utilized as the biomarkers of heavy metals contamination.

**Accepted:** 21. 11. 2014
**Keywords:** phytochelatin; phytochelatin synthase; heavy metal; plants; animals

## 1. Phytochelatins and phytochelatin synthase

Increasing emissions of heavy metals such as cadmium, mercury, and arsenic into the environment pose an acute problem for all organisms. As a mass of protection, many of them, develop mechanisms of full resistance or at least exhibit partially resisting toward these effects. In this way, based on the chemical similarity of the involved metallic species, they are able, to replace them with viable metals necessary for the effective functioning of the cell. These heavy metals may be bound to the functional groups of proteins and modify their structure and through this also affect their physiological function[1,2]. Higher plants, algae, certain yeasts and animals are able to respond to heavy metals by synthesizing phytochelatins (PCs) and related cysteine-rich polypeptides. Phytochelatin synthases are γ-glutamylcysteine (γ-Glu-Cys) dipeptidyl transpeptidases that catalyze the synthesis of heavy metal-binding PCs[3,4]. PCs, cysteine-rich peptides, are produced from glutamine, cysteine and glycine. Unlike commonmetal-binding structures, MT and GSH, PCs are not gene-encoded, but enzymatically synthesized peptides[5]. PCs have been identified in a wide variety of plant species, microorganisms and some invertebrates[6-10]. They are structurally related to glutathione (GSH) and were presumed to be the products of a biosynthetic pathway. Numerous physiological, biochemical and genetic studies have confirmed GSH as the substrate for PCs biosynthesis[11,12]. The general structure of PCs is (c-Glu-Cys)*n*-Gly, with increasing repetitions of the dipeptide Glu-Cys linked through a c-carboxylamide bond (**Fig 1**), where n varies from 2 to 11, but typically reaching not further than five[13]. Except glycine, also other amino acid residues can be found on C-terminal end of $(\gamma\text{-Glu-Cys})_n$ peptides. Examples of which, like Ser, Glu, Gln and Ala are often found at

this position in some plant species, and they are assumed to be functionally analogous and synthesised via essentially similar biochemical pathways[14, 15]. *In in vitro* studies of PC synthase expressed in *E. coli* or in *S. cerevisiae*, the enzyme was activated to varying extents by Cd, Cu, Ag, Hg, Zn and Pb ions[16-18]. PC synthase genes were also isolated in *A.thaliana*[16] and *T.aestivum*[18]. Genes homologous to those from A.thaliana and *T.aestivum* were also found in *S.pombe* and *C.elegans*, suggesting the existence of PC synthase genes in more species[19].

## 3. Phytochelatins in plants

Contamination of soils with toxic metals has often resulted from human activities, especially those related to mining, industrial and emissions. In this context, phytoremediation has been developed as a cost effective and environmentally friendly remediation method of contaminated soils[22, 23]. In recent years many studies showed the mechanisms of chelation of metals-PC[24-28]. Chelation and sequestration of metals by particular ligands are also mechanisms used by plants to cope with metal stress.

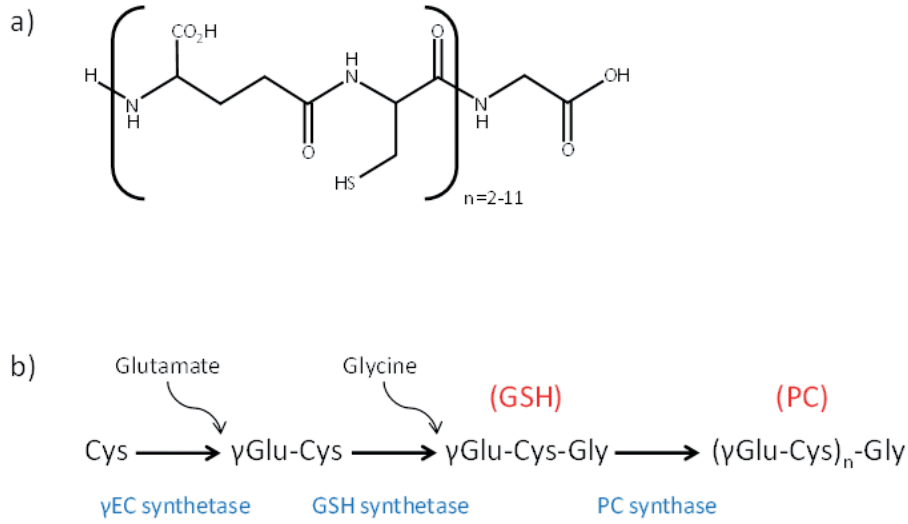


**Figure 1.** a) The general structure of PCs. b) Biosynthesis of phytochelatins.

## 2. Phytochelatins inmicroorganisms

Interestingly, although PC(*n*=2) has been described in the yeast *S. cerevisiae*, there is no homologue of the PC-synthase genes in the *S. cerevisiae* genome. An alternative pathway for PCs biosynthesis which has been in *S. pombe* has been proposed. However, it can be a similar pathway that functions in *S. cerevisiae* too[20]. A study shows that the two vacuolar serine carboxypeptidases are responsible for PC synthesis in *S. cerevisiae*. Therefore, the finding of a PCS-like activity of these enzymes in vivo discloses another route for PC biosynthesis in eukaryotes[21].

The two best-characterized metal-binding ligands in plant cells are the PCs and metallothioneins [29-31]. Naturally hyperaccumulating plants do not overproduce PCs as part of their mechanism against toxic metals and this feature appears to be an inducible rather than a constitutive mechanism, observed especially in metal non-tolerant plants[8].

Several studies of plants that overexpressed γ-glutamyl-cysteine synthetase or transgenic plants expressing bacterial γ-glutamyl-cysteine synthetase evaluated its effect on metal tolerance based on the assumption that higher levels of GSH and PCs will lead to more efficient metal sequestration[32]. *Bacopa monnieri,* a wetland

macrophyte is well known for its accumulation potential of metals and metal tolerance and thus is suitable in phytoremediation. Aquatic plants respond to metal stress by increased production of PCs as well as other antioxidants. *B. monnieri* is wellknown for the accumulation potential of various heavy metals and warrants its evaluation for metal tolerance and detoxification mechanism for its suitability in phytoremediation[9]. *Arabidopsis thaliana* showed that Cd is immediately scavenged by thiols in root cells, in particular PCs, at the expense of GSH. At the same time, a redox signal is suggested to be generated by a decreased GSH pool in combination with an altered GSH:GSSG ratio in order to increase the antioxidant capacity[24]. Overexpression of PCs synthase in Arabidopsis led to 20-100 times more biomass on 250 and 300 μM arsenate than in the wild type. Also, the accumulation of thiol-peptides was 10 times higher when after the exposure to Cd and arsenic, compared to the wild type. Gamma-glutamyl cysteine, which is a substrate for PC synthesis, increased rapidly, after arsenate or cadmium exposure. Overexpression of this gene can be useful for phytoremediating[33]. Also, Legumes are also capable for synthesising homophytochelatins in response to heavy metal stress[32]. Citrus plants were able to synthesize PCs in response to heavy metal intoxication[26]. In wheat, PCs–heavy metal complexes have been reported to accumulate in the vacuole. Retention of Cd in the root cell vacuoles might influence the symplastic radial Cd transport to the xylem and further transport to the shoot, resulting in genotypic differences in grain Cd accumulation[34].

## 4. Phytochelatins in animals

PCs proteins have been broadly described and characterized in plants, yeasts, algae, fungi and bacteria, as well as nematodes and trematodes[35]. PC synthase genes are also present in animal species from several different phyla. PCs synthesis appears not to be transcriptionally regulated in animals[36]. Originally it is thought to be found only in plants and yeast, but PC synthase genes have been found in species that span almost the whole animal tree of life.

### 4.1 Fuctionsl of PCs in animals

Biochemical studies have also shown that these PCS genes are functional: the *Caenorhabditis elegans* PC synthase produces PCs when it is expressed in an appropriate host, and knocking out the gene increases the sensitivity of *C. elegans* to cadmium[37]. In several studies PCs have been measured by direct biochemical analysis of *C. elegans* tissue extracts, and found that cadmium exposure did indeed increase PCs levels in *C. elegans*. $PC_2$, $PC_3$, and $PC_4$ have all been found, with PC2 the highest concentration[6, 38, 39]. Therefore, these studies showed conclude that PCs production plays a major role in protecting *C. elegans* against cadmium toxicity. $PC_2$ and $PC_3$ were increased in autochthonous *Lumbricus rubellus* populations sampled from contaminated sites[36].

The yeast (i.e. *S. pombe*) possesses an ATP-binding cassette (ABC) transporter, Hmt1, which was originally thought to play a possible role in translocation of PCs–metal complexes to the vacuole. However, while knocking out the *C. elegans* HMT-1 (CeHMT-1) the sensitivity toward cadmium does increase, and the increase is greater than could be explained by a lack of PCS alone[40].

It is important to say that MTs are widely established as a key metal detoxification system in animals, even though they certainly have many other biological functions as well. Until now, there is very little known about how MTs and PCs may complement each other for dealing with toxic metals[36].

## 5. Methods for phytochelatins determination

Recently, *Wood et al.*, showed the analytical methodology for quantification of PCs and their metal(loid) complexes[41]. The classical approach to the analysis of PCs is by reversed phase HPLC with post-column derivatization of the sulfhydryl groups and spectrophotometric detection (but this is not specific to PCs). Independent studies showed a sensitive method for determination of PCs by HPLC with fluorescence detection[42, 43]. A simple sensitive method for the identification, sequencing and quantitative determination of PCs in plants by electrospray

tandem mass spectrometry (ESI MS-MS) was showed in different studies[44, 45]. Other study showed the combination of three process for identification PCs: (1) simple sample preparation including thiol reduction, (2) rapid and high resolution separation using ultra-performance liquid chromatography (UPLC), and (3) specific and sensitive ESI-MS/MS detection using multi-reaction mode (MRM) transitions in alga's extract[46]. Zitka et al., optimized high performance liquid chromatography coupled with electrochemical detector for determination of PC2[47].

## 6. Methods for phytochelatin synthase determination

High performance liquid chromatography coupled with electrochemical detector was to suggest as a new tool for determination of the PCs synthesis activity[48]. The optimized procedure was subsequently used for studying PC synthase activity in the tobacco BY-2 cells treated with different concentrations of Cd (II) ions and the results were in good agreement with Nakazawa et al.,[49]. Another study in animals showed that HPLC-LC system coupled to a single quadrupole LC–mass spectrometer equipped with electrospray ionization was sensitive method for determination of PCs synthesis activity [35]. A high sensitive assay for PCS activity was devised, in which the dequenching of Cu(I)-bathocuproinedisulfonate complexes was used in the detection system of a reversed-phase high-performance liquid chromatography. The present assay method is a sensitive tool that can be used to investigate this issue and would allow determination of PCS activity using 10–100-fold less protein[50].

*This work was supported by CZ.1.07/2.3.00/30.0017.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements" for biomedical papers.

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ARTICLE

# Paramagnetic particles for immobilization of metallothionein – promising biomarker of head and neck cancer

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**Paramagnetic particles for immobilization of metallothionein – promising biomarker of head and neck cancer**

Currently, metallothioneins (MTs) are investigated as the biomarkers of various pathological states. High positive association of MT amount was determined particularly in head and neck cancer (comparison with healthy tissue). Thus we suggested paramagnetic particles, based on nanomaghemite core, modified with polyvinylpyrrolidone and gold nanoparticles. Utilizing the natural affinity of thiol, contained in MT cysteines and gold, the MT was immobilized with approximately 25% recovery, when using the ideal conditions 100 mM borate buffer, pH 6 and incubation temperature 37 °C. Our particles may be helpful in isolation of MT as biomarker for subsequent analyses as well as for MT immobilization via external magnetic field on electrode surface for development of heavy metal biosensors.

**Accepted:** 21. 11. 2014
**Keywords:** Biomarker; Brdicka reaction; Cancer; Chromatography; MALDI-TOF/TOF; Metallothionein

## 1. Introduction

Metallothioneins (MTs) are a group of low-molecular weight (~6 -10kDa), cysteine-rich, evolutionarily conserved proteins, found in generally all life forms. The MTs are multi-functional and play crucial roles in the transport of essential trace elements as well as in the detoxification of harmful metallic elements[1]. Four major MT isoforms, MT-1, MT-2, MT-3 and MT-4, have been identified in mammals[2]. Specifically, MT-3 has been reported to be secreted mainly in neural cells. Recent reports established that this isoform play an important protective role in brain injury and metal-linked neurodegenerative diseases[3]. In general, MT is known to modulate three basic processes: the release of hydroxyl radical or nitric oxide[4], apoptosis[5] and the binding and exchange of heavy metals such as zinc[6], cadmium[7] or copper[8]. Previous studies have shown a positive correlation between the expression of MT with invasion, metastasis and poor prognosis in various cancers, including head and neck cancers[9], where increasing levels of MT concentration in tumor (vs. healthy tissues) were observed[10]. Moreover; due to high affinity towards heavy metals, MT can be employed for modification of electrodes in development of electrochemical biosensors[11]. In view of these facts, immobilization of MT on paramagnetic particles (PMPs) may provide many possibilites, such as simplification of biosensing of low levels of MT, through its pre-concentration. The present study demonstrates immobilization of MT on a surface of paramagnetic particles (gold modified). For confirmation of MT presence two detection techniques were employed: matrix-assisted laser desorption/ionization - time of flight mass spectrometry and differential pulse voltammetry with adsorptive transfer technique utilizing Brdicka solution as supporting electrolyte. For binding capacity evaluation MT isolated from rabbit liver was employed and ideal particles for its immobilization were chosen, simultaneously with optimization of immobilization conditions (pH and temperature).

## 2. Materials and Methods

### 2.1 Chemicals

$HAuCl_4$, $NaBH_4$, 2,5-dihydroxybenzoic acid (DHB) and/or α-cyano-4-hydroxycinnamic acid (HCCA) were obtained from (Sigma-Aldrich, St. Louis, MO, USA) in ACS purity. Furthermore, we used sodium citrate, trifluoroacetic acid, Brdicka supporting electrolyte containing 1 mM $Co(NH_3).6Cl_3$ and 1 M ammonia buffer ($NH_3$(aq) $NH_4Cl$, pH = 9.6). All buffer solutions were prepared with ACS $H_2O$ from (Sigma-Aldrich, USA).

### 2.2 Preparation of rabbit liver for MT isolation by using FPLC and confirmation of purity

MT was isolated from rabbit liver and purified by using fast-protein liquid chromatography (FPLC) according to our previous study[12].

The purity was evaluated by using MALDI-TOF/TOF (Bruker ultrafleXtreme, Bruker Daltonik GmbH, Germany). The matrix used in the MALDI method was 2,5-dihydroxybenzoic acid (DHB) and/or α-cyan-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich). The saturated matrix solution was prepared in 30% acetonitrile and 0.1% trifluoroacetic acid (TFA). Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Germany) for 2 minutes at 50% of intensity at laboratory temperature. Sample preparation crystallization method for MALDI-TOF was *dried-droplet* method (DD) – the sample solutions for analysis were mixed with matrix solution in volume ratio of 1:1. After obtaining a homogeneous solution, 1 µL was applied on the MTP 384 polished steel target plate (Bruker) and dried under atmospheric pressure at laboratory temperature. The preparation yielded relatively large crystals on the target surface as well as regions without matrix or analyte. All measurements were performed in linear positive mode in the *m/z* range 4-17 kDa. The mass spectra were typically acquired by averaging 500 sub spectra from a total of 500 shots of the laser with laser power set 5-10% above the threshold.

### 2.3 Preparation of paramagnetic core-shell particles

Nanometric maghemite core was prepared by $NaBH_4$ reduction of $FeCl_3.6H_2O$, according to protocol, as follows: 1 g of $FeCl_3.6H_2O$ was dissolved in 80 mL of MilliQ water and a solution of 0.2 g of $NaBH_4$ in ammonia (3.5%, 10 mL) was poured into the first solution under vigorous stirring. The obtained solution was boiled for 2 h. After cooling down the magnetic nanoparticles were separated by external magnetic field and washed with water for several times. Resulting mixture was stirred overnight, separated using an external magnetic force field and dried at 40 °C. To form a shell structure on a nanoscaled maghemite several various modification processes were employed: in case of MAN18 100 mL of nanomaghemite solution was mixed with 20 mL of $Ti(isopropox)_4$. MAN32 were formed by bare nanomaghemite without shell structure. MAN38 formed addition (3.33 mL) of 3-amiopropyl triethoxysilan (APTES). MAN53 constituted 1.5 g of polyvinylpyrrolidone (PVP-40k) with reduced $HAuCl_4$ (1 mM). MAN57 were modified with 10 mL of 1% hyaluronic acid (HA), MAN59 with 2 mL of 18% poly(4-styrenesulfonic acid, 75k), MAN63 with 1 g of glucose and 100 mg of grafen oxide, MAN65 with 3 g of PVP (10k) with addition of 7.48 g $Fe(NO_3)_3.9H_2O$ and finally MAN70 with 0.5 g of PVP (10k), 1.5 g of glucose and 30 mL of 1 mM $HAuCl_4$. All complexes were washed with water three times and dried at 40 °C prior to use.

### 2.4 Preparation of Sample PMPs for MT Isolation

For isolation was employed 50 µL of dispersion, comprising 40 $mg.mL^{-1}$ of each type of PMPs in 1 mL PBS (pH 7). After incubation in thermomixer (37°C, 60 min, 800 rpm) (Eppendorf, Hamburg, Germany), the sample was dissolved in 3 M hydrochloric acid (250 µL) and evaporated using nitrogen evaporator Ultravap RC (Porvair Sciences, Leatherhead, UK). Finally, the evaporated sample was resuspended with ACS $H_2O$ (250 µL) and analysed using Differential pulse voltammetry with adsorptive transfer technique (AdT DPV) and MALDI-TOF/TOF.

### 2.5 Differential pulse voltammetry with adsorptive transfer technique

Quantification of MT bound on the surface of paramagnetic nanoparticles was carried out using differential pulse voltammetry (DPV), using parameters according to[13]. The recovery of MT binding was calculated as difference between applied and analysed concentration of MT.

### 2.6 Descriptive statistics

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®.

## 3. Results and Discussion

For all experiments MT was isolated from rabbit liver (MT was induced by intraperitoneal injection of 10 mg of $CdCl_2$.kg$^{-1}$ of weight) by using fast-protein liquid chromatography from liver homogenates. After isolation, MT was lyophilized and after resuspendation in water subsequently analyzed by using MALDI-TOF/TOF for its purity. In **Fig. 1** are shown MALDI-TOF/TOF mass spectra obtained in two types of matrixes (**Fig. 1A**) HCCA and (**Fig. 1B**) DHB. In both conditions, monomers of MT were identified (major peaks ~6 kDa), however in DHB matrix ionization was carried out more readily (intensity about 4400 a. u.), and thus it was applied in subsequent measurements. Moreover, it was shown that purity of our isolated protein is very high, hence its applicability for further isolation experiments was confirmed. The iron oxide nanoparticles ($\gamma$-$Fe_2O_3$ and $Fe_3O_4$) are versatile materials, exploitable for magnetic separation of various types of analytes[14,15], in particular due to possibility of surface modifications, which leads to introduction of broad spectrum of binding sites. In our study we employed broad spectrum of various surface modifications, including metals, polymers, sugars, or carbon nanoparticles (for details see chapter **2.3 Preparation of paramagnetic core-shell particles**).

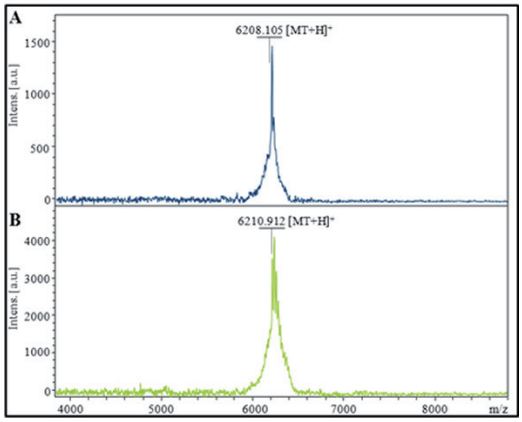


**Figure 1.** MALDI-TOF/TOF mass spectra of MT fractions after the fast protein liquid chromatography purification (concentration of MT - 2.0 µM). In analyses the linear positive mode, (**A**) HCCA and (**B**) DHB matrixes were employed.

The resulting paramagnetic particles were employed for screening of their binding capacity towards MT (1 µM) and by using MALDI-TOF/TOF it was shown that only paramagnetic particles with acronym MAN53, composed of $\gamma$-$Fe_2O_3$ covered with PVP layer with gold nanoparticles resulted from reduction of $HAuCl_4$ with citric acid[16], are able to bind MT (**Fig. 2**). The bond in other types of particles was likely to weak to withstand the washing steps or was not present at all. In this case MALDI-TOF/TOF was shown as a perfect tool for rapid screening tests of protein binding with very low requirements for sample volume (1 µL).

Since MT is constituted of more than 30% of cysteine residues[17], containing sulfhydryl moieties with high affinity towards metals, surface modification with gold nanoparticles seems to be the most suitable for MT binding (it was described that affinity of MT towards Au is much higher than towards Zn or Cd[18]). Moreover, the thiol/gold bond is strong enough to withstand washing steps, involved to remove the undesired impurities.

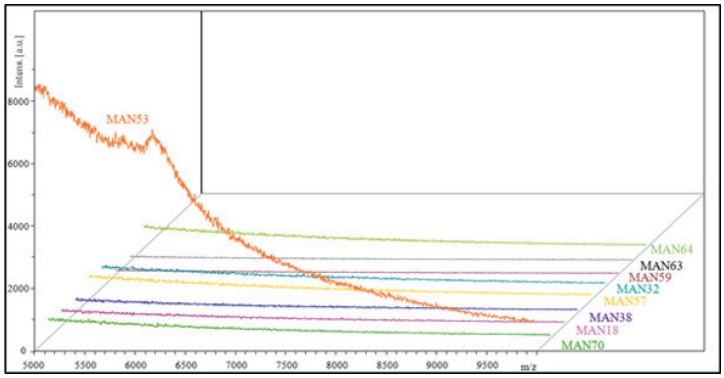


**Figure 2.** MALDI-TOF/TOF mass spectra showing the results of screening of various types of paramagnetic particles (MANX) for their ability to bind MT (1 µM) on their surface. MS measurements were carried out in linear positive ion mode, with DHB matrix and laser power established to 80%.

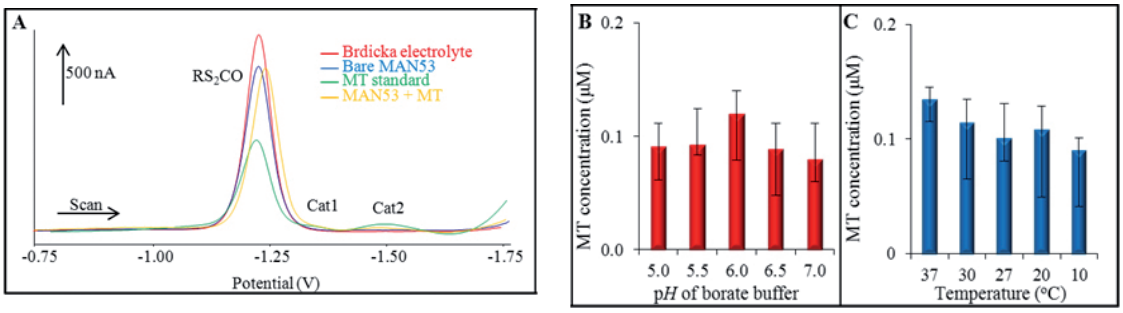


**Figure 3.** (**A**) Typical voltammogram, obtained by using AdT DPV with Brdicka electrolyte, where (**a**) stays for Brdicka electrolyte (1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3$(aq) and $NH_4Cl$, pH = 9.6), (**b**) for bare MAN53, (**c**) for MT standard (500 nM) and (**d**) for MAN53 + MT conjugate. (**B**) Optimization of ideal binding conditions – pH of binding borate buffer (5.0 - 7.0). (**C**) Influence of temperature (10 – 37 °C) on binding of MT on MAN53. All optimization conditions were evaluated by using AdT DPV. Values are means of three independent replicates (n = 3). Vertical bars indicate standard error.

Since electrochemistry offers many advantages in determination of thiol compounds[13], we employed AdT DPV for quantification of MT immobilized on MAN53 surface. In **Fig. 3A** are depicted typical electrochemical voltammograms for PMPs, MT and their interleaving records (labelled PMPs + MT). Voltammograms obtained in Brdicka solution show three characteristic peaks ($RS_2Co$, Cat1 and Cat2). $RS_2Co$ is around -1.25 ± 0.05 V and is associated with the reduction of protein thiol moieties[19]. Cat1 (around -1.30 ± 0.05 V) and Cat2 (around -1.50 ± 0.05 V) are related to the reduction of hydrogen developed from electrolyte catalyzed by thiol groups on the mercury electrode[19] and the height of Cat2 peak is related to the amount of MT in sample[20]. Further in **Fig. 3A** is obvious the absence

Although it was shown that MAN53 particles immobilize MT, we further optimized the conditions of incubation. In particular, pH of buffer in which binding is carried out and incubation temperature can significantly influence the immobilization efficiency[15].

As it is represented in **Fig. 3B** the largest immobilization yields were achieved in 100 mM borate buffer with pH 6.0 (0.12 µM MT). With both - decreasing and increasing pH the amount of electrochemically determi-

ned MT on particles was reduced (0.09 μM in pH 5.0 or 0.08 μM in pH 7.0). Thus, further optimization of incubation temperature was carried out with 100 mM borate buffer, pH 6. In case of temperature of incubation (10 - 37°C), the highest yields were observed when using 37°C, while lowering of temperature lead to decrease of MT amount. After application of ideal optimized conditions the recovery of MT immobilization was established to be about approximately 25%. Paramagnetic particles modified with gold nanostructures can thus serve for immobilization of MT through strong bonds. This phenomenon can be employed for isolation of MT from biological matrixes and moreover for immobilization of MT on electrodes surface to enhance the sensitivity and specifity towards heavy metal detection.

## 4. Conclusion

In particular, because of low fabrication cost and easy manipulation paramagnetic particles have currently attracted much attention. In this present study nanomaghemite core was synthesized and subsequently functionalized with shell, composed of PVP and gold nanoparticles evolving from reduction of $HAuCl_4$ with sodium citrate. Resulting structure binds MT with approximately 25% recovery, when using optimized conditions of incubation - 100 mM borate buffer, pH 6 and incubation temperature 37°C. Such paramagnetic particles can be applicable in isolation of MT from various biological matrixes for its subsequent determination as a biomarker of various pathological states, including head and neck cancer. Moreover, particles can be used for immobilization of MT in separation part of heavy metal biosensor.

*This work was financially supported by project SPINCANCER NT/14337.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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ARTICLE

# Delivery of doxorubicin using protein nanocarriers

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**Delivery of doxorubicin using protein nanocarriers**

In this study, we compared two types of protein nanocarriers, bacteriophage λ and apoferritin, by their ability to encapsulate the chemotherapeutic drug doxorubicin. The successful encapsulation was proven by absorbance and fluorescence measurement of the nanocarrier after the removal of free doxorubicin by dialysis. Phage λ was able to encapsulate much higher (4 times) amount of doxorubicin than apoferritin. Also, more doxorubicin was released during the washing from apoferritin, which is undesired. Based on the obtained data, phage λ seems to be a better protein nanocarrier than apoferritin.

**Accepted:** 9. 12. 2014
**Keywords:** apoferritin; doxorubicin; phage λ; protein nanocarriers; theranostics

## 1. Introduction

Anthracycline antibiotic drugs are widely used in treatment of many patients with cancer. First anthracycline drug, daunomycin (or daunorubicin) was found in a number of different wild type strains of Streptomyces. However, in cancer treatment the most commonly used anthracycline drug is doxorubicin (DOX) or one of its 2000 known analogs[1].

DOX is used in treatment of many different types of cancer such as neuroblastomas, leukaemia, lymphomas or breast, testicle, ovarian, lung, bladder, thyroid gland or head and neck carcinomas. Although it is so widely used, many side effects have been observed in patients such as sores in mouth and on lips, darkening of palms and nails, unusual bleeding and bruising, nausea and vomiting, and life-threatening cardiotoxicity[2].

To eliminate the negative side effects of cancer treatment, researchers are trying to find either new analogs of DOX which are non-toxic for healthy cells or new way to deliver DOX directly into the cancer cells. For targeted delivery, it is possible to administer the drug directly into solid tumor. However, non-solid tumors or tumors with unknown location in patient's body require encapsulation of DOX in suitable nanocarrier. Liposomal form of DOX is already being sold under the trade name Myocet[3]. For enhanced biocompatibility, the liposomes were modified with polyethylene glycol under the trade name Doxil[4].

Protein based natural nanocarriers in comparison with artificial nanocarriers seem to be more suitable for delivery of the drugs in patient's body because of their lower immune response. The protein nanocarriers are usually self-assembled and can either be protein cages, viral capsids or virus-like particles[5].

In this work, we compared two types of protein nanocarriers – phage λ and apoferritin, by their ability to encapsulate anthracycline drug doxorubicine. The encapsulation was verified by fluorescence of doxorubicin after the removal of free, non-encapsulated doxorubicin by dialysis.

## 2. Materials and Methods

### 2.1 Cultivation and purification of phage λ

Phage λ-producing strain of *Escherichia coli* was cultivated in Luria-Bertani broth (1 % tryptone, 0.05 % yeast extract and 1 % sodium chloride) with 0.2 % maltose for 24 h at 37 °C and 600 rpm. After cultivation, the culture was mixed with chloroform in 6:1 ratio and incubated for 1 h at 25 °C to kill the growing

*E. coli*. The samples were centrifuged at 5200 g and 4 °C for 10 min to remove *E. coli* and then at 10000 g and 4 °C for 6 min to remove remaining contaminants. Next, the supernatant containing phage was ultracentrifuged at 130000 g and 4 °C for 3 h. The pellet containing phage was resuspended in PBS at a concentration of 15µg/ml for protein and stored at 4 °C.

### 2.2 Encapsulation of doxorubicin in phage λ

The drug, doxorubicin was encapsulated in purified phage by infusion method. 80 µl of phage was mixed with 80 µl of doxorubicin at different concentrations (200; 100; 50; 25; 12.5 and 0 µg/ml). Incubation was conducted for 2 h at 25 °C in dark. Free doxorubicin was subsequently dialyzed using Amicon 3K (Merck-Millipore, MA, USA) for 15 min at 6000 g and 20 °C and the phage was rinsed twice with water. The volume was then made to original volume (160 µl).

### 2.3 Encapsulation of doxorubicin in apoferritin

Doxorubicin was encapsulated in apoferritin by opening and closing in various pH. 80 µl of apoferritin (15 µg/ml) was mixed with 80 µl of doxorubicin at different concentrations (200, 100, 50, 25, 12.5 and 0 µg/ml). 1 µl of 1M hydrochloric acid was added to sample to make the pH lower to 2 and open the apoferritin. The samples were mixed by centrifugation at 25 °C and 600 rpm for 15 min. 1 µl of 1M sodium hydroxide was added to make the pH higher to 7 and encapsulate doxorubicin in apoferritin. Free doxorubicin was subsequently filtered using Amicon 3K (Merck-Millipore, MA, USA) and centrifuged for 15 min at 6000 g and 20 °C and the phage was rinsed twice with water. The volume was then made to original volume (160 µl).

### 2.4 Verification of doxorubicin encapsulation in nanocarriers

Absorption spectra of the nanocarriers with encapsulated doxorubicin were measured from 200 to 800 nm. Emission spectra of the samples were measured with excitation at 480 nm (absorption maximum of doxorubicin) and emission from 515 to 815 nm.

## 3. Results and Discussion

### 3.1 Encapsulation of doxorubicin in phage λ

We used infusion method for encapsulation of cytostatic drug in apoferritin. This method does not require any surface modification of phage λ proteins, which can change its properties. Many drugs are able to enter the phage capsid using the pores between individual copies of major capsid protein E and they can bind either to viral nucleic acid or its proteins[6,7].

In present experiment, the phage λ protein (concentration 20 µg/ml) was mixed with doxorubicin of various concentrations (0, 12.5, 25, 50, 100 and 200 µg/ml) and incubated in dark to avoid quenching of doxorubicin. The phage was then dialyzed and washed to remove the excess molecules of doxorubicin.

**Fig. 1A** shows the absorbance spectrum of phage with encapsulated doxorubicin. The control sample with no addition of doxorubicin shows absorbance maxima at 260 and 280 nm, corresponding to the viral nucleic acid and its proteins. With higher applied concentrations of doxorubicin, we can see new absorbance maxima at 480 nm, which corresponds to the absorbance maximum of free doxorubicin. The absorbance at this maxima is linearly (determination coefficient 0.8013) increasing with the increasing applied concentration of doxorubicin.

**Fig. 1B** shows the measured fluorescent spectrum of these samples. The control sample exhibited no fluorescence under the conditions used. However, with the increasing concentration of applied doxorubicin, the fluorescence of whole phage was linearly (0.8855) increasing.

### 3.2 Encapsulation of doxorubicin in apoferritin

The apoferritin structure is dependent on the surrounding pH. In low pH, the apoferritin subunits are disassociated. After mixing with the desired drug, the pH can be made back to neutral, the subunits will associate again and the drug will be encapsulated in the nanocarrier[8,9].

The apoferritin was prepared with the same concentration as phage λ (20 µg/ml). The doxorubicin with various concentrations (0, 12.5, 25, 50, 100 and 200 µg/ml) was encapsulated in apoferritin by pH-changing method. The free doxorubicin was then removed by dialysis.

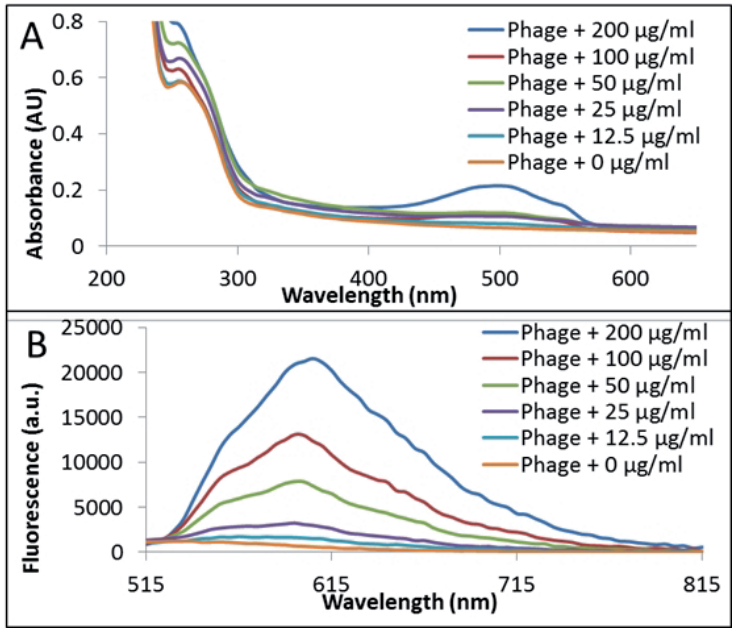


**Figure 1.** The influence of doxorubicin encapsulation on absorbance (1A) and emission (1B) spectra of phage λ.

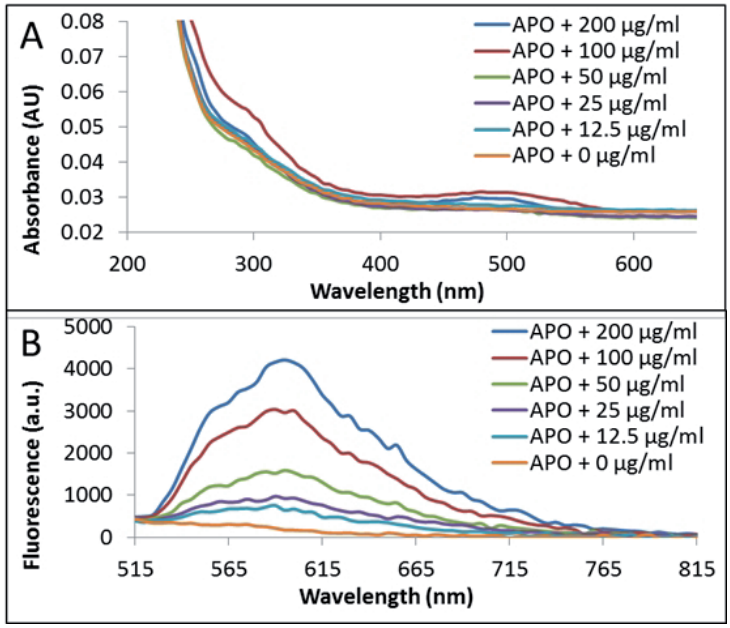


**Figure 2.** The influence of doxorubicin encapsulation on absorbance (2A) and emission (2B) spectra of apoferritin.

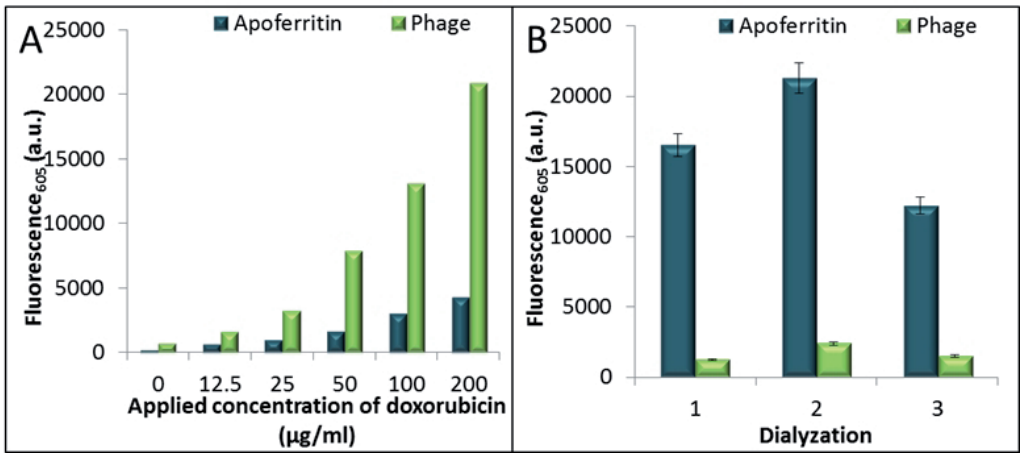


**Figure 3.** The comparison of phage and apoferritin as nanocarriers. Fluorescence of doxorubicin encapsulated in phage and apoferritin (3A) and undesired release of doxorubicin from nanocarriers during dialyzation measured by fluorescence (3B).

**Fig. 2A** shows the absorbance spectrum of apoferritin with encapsulated doxorubicin. Control apoferritin with no added doxorubicin shows only the absorbance maximum at 280 nm, corresponding to absorbance maximum of proteins. Similar as with phage λ, the increasing concentration of doxorubicin caused linearly (determination coefficient 0.788) increasing absorbance at 480 nm.

We also measured a fluorescent spectrum of the apoferritin with encapsulated doxorubicin (**Fig. 2B**). The fluorescence was also linearly (determination coefficient 0.925) dependent on the applied concentration of doxorubicin.

However, the fluorescence was 4 times higher in the case of encapsulation in phage λ than in apoferritin (**Fig 3A**). Also, we studied the undesired release of doxorubicin from both nanocarriers during dialysis. The doxorubicin was 10 times more released from apoferritin than from phage λ (**Fig. 3B**).

## 4. Conclusion

In this work, well-studied coliphage λ was used as a nanocarrier for doxorubicin, using the infusion method of encapsulation. The successful encapsulation was proven by absorbance and fluorescence measurement of the whole phage. The absorbance of phage λ at 480 nm increased from 0.06 to 0.22 after encapsulation of 200 µg/ml doxorubicin. The fluorescence of phage λ at 600 nm (the emission maximum of doxorubicin) increased from 1000 to 22000 after encapsulation of 200 µg/ml doxorubicin.

Encapsulation by phage λ was compared with protein nanocarrier apoferritin. At the same concentration, apoferritin was able to encapsulate 4 times lower amount of doxorubicin than phage. Undesired release of doxorubicin from apoferritin was 10 times higher in comparison with phage. Based on the obtained data, phage λ seems to be a better protein nanocarrier than apoferritin.

*The study was financially supported by Grant Agency of the Czech Republic (NANOCHEMO GA CR 14-18344S).*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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# MIR-150 electrochemical detection connected with specific isolation based on magnetic particles

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**MIR-150 electrochemical detection connected with specific isolation based on magnetic particles**

Since the establishment of association between microRNA (miRNA) abnormal expression profiles and diseases development the miRNAs become a very significant group of molecules. Due to their potential in diagnostics and therapy the considerable effort to develop sensitive, simple and low-cost detection method exists. For these reason was the sensitive electrochemical detection combined with selective magnetic particle-based separation.

In this work, the square wave voltammetric detection (SWV) of miRNA was optimized. The limit of detection of miR-150 by SWV was 1 nM. This detection method was subsequently connected with magnetic separation when the magnetic particles (MPs) were modified by probe specific to miR-150. The sensitivity of coupled SWV with MPs-based separation was established by nanomolar concentration (9 nM) limit of detection.

**Accepted:** 9. 12. 2014
**Keywords:** isolation, magnetic particles, miR-150, voltammetry

## 1. Introduction

The microRNA (miRNA) belongs to short noncoding RNA molecules (generally 20-25 nt in length) which are able of specific gene regulation expression[1]. These regulatory molecules have significant influence on different biological processes like cell proliferation, development, differentiation, apoptosis and metabolism[2].

The aberrant expression of various miRNAs is involved in the cell pathological status formation. The abnormal miRNA expression levels were found at number diseases like inflammatory and autoimmune diseases, diabetes, cardiovascular and neurodegenerative diseases and cancer [3-6]. Specific miRNAs expression profiles were identified at various tumors so miRNA are interesting diagnostic biomarkers. Moreover their detection might be helpful to find more about cancer staging, prognosis and/or response to treatment [7]. Different studies have shown that miR-150 is upregulated in lung cancer tissue and lupus nephritis, downregulated at patients with heart failure, acute myeloblastic leukemia, colorectal cancer and systemic sclerosis[8-14].

Because of miRNAs importance the simple and fast detection method is needed. Therefore, the sensitive electrochemical analysis was combined with specific magnetic particles--based isolation.

## 2. Material and Methods

### 2.1 Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The 1× binding and washing (BW) buffer (5 mM Tris-HCl, 0.5 mM EDTA and 1.0 M NaCl, pH 7.5), solution A (0.1 M NaOH and 0.05 M NaCl) and solution B (0.1 M NaCl) were employed for MPs washing. For biotinyled anti-miR-124 immobilization the 2× BW buffer (10 mM Tris-HCl, 1 mM EDTA and 2.0 M NaCl, pH 7.5) was utilized. The phosphate buffer I for washing MPs with immobilized oligonucleotide was composed of 0.1 M NaCl, 0.05 M $Na_2HPO_4$

and 0.05 M $NaH_2PO_4$, pH 7.8. All solutions were treated with DEPC or prepared using DEPC treated water.

The composition of hybridization solution was as follows: 0.1 M $Na_2HPO_4$, 0.1 M $NaH_2PO_4$, 0.6 M guanidinium thiocyanate (Amresco, Solon, OH, USA), 0.15 M Tris-HCl and 0.5 M NaCl (pH 7.5). The elution solution composition was as follows: 0.2 M NaCl, 0.1 M $Na_2HPO_4$ and 0.1 M $NaH_2PO_4$. Acetate buffer (0.2 M $CH_3COOH$ and 0.2 M $CH_3COONa$, pH 5) was used for electrochemical analysis.

The hsa-miR-150-5p (5´-UCUCCCAACCCUUGUACCAGUG-3´) and complementary biotinylated oligonucleotide (ODN) antisense miR-150 (5´-Btn- CACTGGTACAAGGGTTGGGAGA-3´), both synthesized by Sigma-Aldrich (St. Louis, MO, USA), were used for magnetic separation optimization.

### 2.2 Electrochemical analysis

Electrochemical measurements were performed with AUTOLAB PGS30 Analyzer (EcoChemie, Utrecht, Netherlands) connected to VA-Stand 663 (Metrohm, Zofingen Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 $mm^2$ was employed at the working electrode. An Ag/AgCl/3M KCl electrode served as the reference electrode. Pt electrode was used as the auxiliary electrode.

Adsorptive transfer technique was used for the RNA electrochemical determination. The adsorptive transfer technique is based on the sample (5 µl) accumulation (120 s) onto the working electrode surface and consequently on the electrode washing and square wave voltammetric (SWV) measurement. All experiments were carried out at room temperature (21ºC). SWV measurements were carried out in the presence of acetate buffer pH 5.0. SWV parameters: start potential 0 V, end potential -1.8 V, potential step 5 mV, frequency 280 Hz, and amplitude 25.05 mV. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed.

Optimized parameters were accumulation time (0, 30, 60, 90, 120, 150, 180, 210 s); purge time (0, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540 s) and pH of acetate buffer (3, 4, 5, 6, 7). 25 nM miR-150 was used for the optimization steps.

### 2.3 The miR-150 isolation by magnetic particles

The magnetic microparticles Dynabeads M-270 Streptavidin (Life Technologies, Invitrogen, Oslo, Norway) and magnetic separation rack MagnaRack (Life Technologies, Invitrogen, Oslo, Norway) were used for miRNA isolation. The miRNA experiments were performed in RNA/DNA UV-cleaner box UVT-S-AR (Biosan, Riga, Latvia).

The biotinylated antisense miR-150 immobilization on MPs surface was done according to the manufacturer‘s recommendations. A microcentrifuge tube with 50 µl of resuspended MPs was placed on the magnetic rack and the supernatant (storage solution) was removed. The MPs were 3 times washed by 50 µl of 1× BW buffer. Subsequently the MPs were washed twice by 50 µl of the solution A and once with the solution B (50 µl). Thus, washed MPs were ready for immobilization of biotinylated ODN. MPs were resuspended in 100 µl of 2× BW buffer and the 1.02 µg of biotinylated probe in water (final volume 100 µl) was added. The mixture with MPs and biotinylated ODN was incubated for 10 minutes on rotator-mixer (multi RS-60, Biosan, Riga, Latvia) at 60 rpm at the room temperature. After incubation, probe-coated MPs were separated on the magnetic rack and twice washed with 50 µl of 1× BW buffer and once with 100 µl of phosphate buffer I.

The hybridization step was performed according to Huska et al.[15]. 50 µl of hybridization solution and 50 µl of sample (miR-150 diluted in water) were added to the probe-coated MPs. The hybridization process took placed on rotator-mixer at 60 rpm for 40 minutes at the room temperature. After incubation the MPs with coupled miR-150 were three times washed with 100 µl of phosphate buffer.

In the next step the MPs were resuspended in 50 µl of the elution solution. The miR-150 elution was done in Thermomixer 5355 Comfort/Compact (Eppendorf, Hamburg, Germany) for 5 minutes at 70 ºC and 350 rpm. The tube was

placed on magnetic stand after heating-up. The solution with eluted miR-150 was pipetted to a new tube. The miR-150 amount was determined by electrochemistry.

## 3. Results and Discussion

### 3.1 The electrochemical detection optimization

For the maximal miRNA electrochemical signal achievement was the SWV method optimized. At first the time required for miRNA accumulation on HMDE surface was determined (**Fig. 1A**). The tested times were 0 – 210 s. With increasing accumulation time the signal height significantly grew to the value 120 s. Then the signal increase was not so significant and for shortening the analysis time the accumulation for 120 s was selected.

Next, the influence of electrolyte (acetate buffer) pH on electrochemical signal was investigated. The tested pH range was 3 – 7. From the Fig. 1B is clear that pH 5 is optimal for miRNA SWV detection.

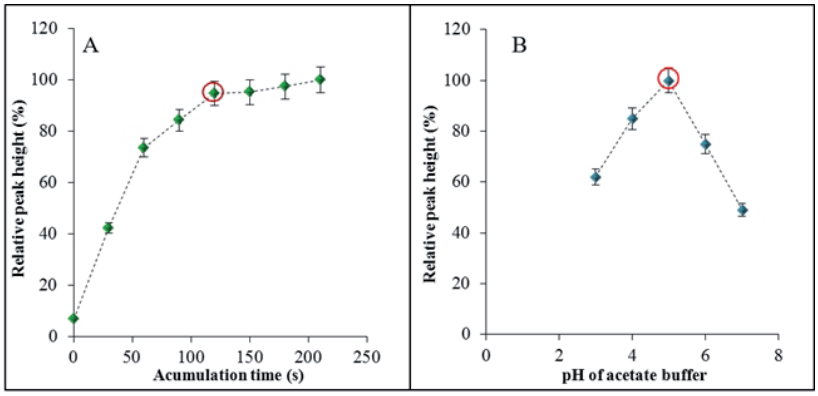


**Figure 1.** Electrochemical detection parameters optimization. (A) Optimization of accumulation time. (B) Optimization of acetate buffer pH. Selected parameters were highlighted. Results are expressed as average ± standard deviation (number of measurement n = 3).

### 3.2 MIR-150 and antisense miR-150 calibration curves

With optimized parameters of AdTS SWV method was detected influence of miRNA and biotinylated probe concentration on peak height and calibration curves were set down. In the **Fig. 2A** the dependence of peak height on miR-150 concentration is shown. The limit of detection was calculated as 1 nM and limit of quantification as 5 nM. The dependence of antisense miR-150 peak height on its concentration is shown in the **Fig. 2B**. The limit of detection was established as 0.6 nM and limit of quantification was determined as 2 nM.

### 3.3 The electrochemical detection of magnetic separation yield

For the specific miRNA isolation the magnetic separation was used. The targeted miRNA was miR-150 and for specific isolation streptavidin-coated MPs modified with antisense probe complementary to targeted miR-150 sequence were used. The magnetic separation process was performed according to Smerkova et al. [16]. The calibration curve for the specific isolation connected with optimized electrochemical detection was obtained (**Fig. 3**). The SWV response is in that range linear with determination coefficient $R^2$ = 0.9958. The voltammograms of miR-150 separated by MPs are shown in inset of **Fig.3**. The limit of detection was determined as 9 nM and limit of quantification was established as 30 nM. The average isolation yield of miR-150 by magnetic separation was 17 %.

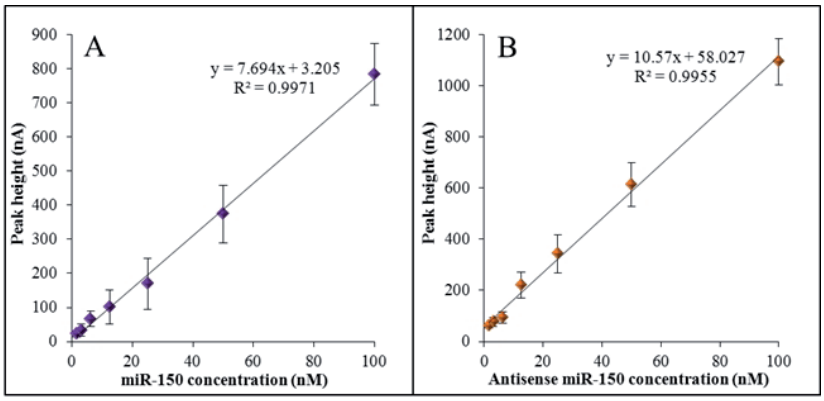


**Figure 2.** SWV peak height dependence on concentration of (A) miR-150 and (B) antisense miR-150. SWV parameters were as follows: time of accumulation 120 s, start potential 0 V, end potential -1.8 V, potential step 5 mV, frequency 280 Hz, amplitude 25.05 mV. Results are expressed as average ± standard deviation (number of measurement n = 3).

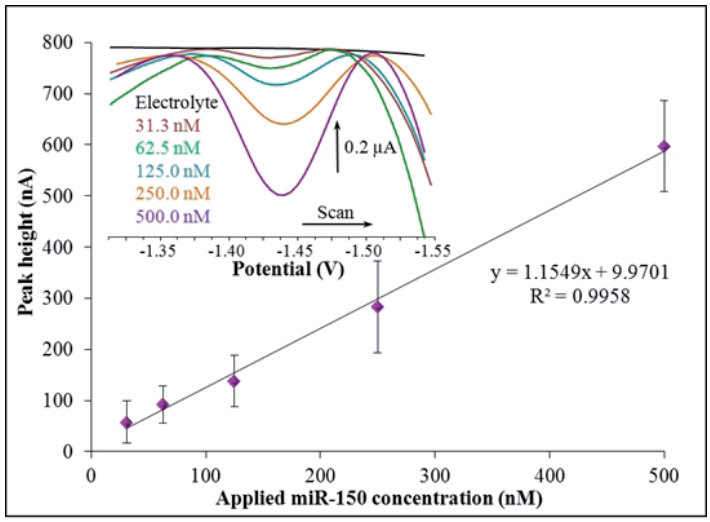


**Figure 3.** (A) Dependence of the peak height on applied miR-150 concentration using the MPs-based isolation. Inset: the isolated miR-124 voltammograms (SWV conditions as in Fig. 2). Results are expressed as average ± standard deviation (number of measurement n = 3).

## 4. Conclusion

The miRNAs are very important diagnostic and prognostic biomarkers at various diseases. Therefore, the easy and sensitive detection method is required. The combination of magnetic separation with electrochemical detection is very advantageous. Because of modifiable surface of magnetic particles by probe the specificity is guaranteed and the electrochemical method contributes by the sensitivity. In this work, the possibility of connection the magnetic particles-based separation with sensitive square wave voltammetric detection was demonstrated.

## Acknowledgement

*Financial support from GACR P102/11/1068 NanoBioTeCell is highly acknowledged.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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ARTICLE

# Nanomaghemite core functionalized with ion-exchange resins for isolation of biogennic amines

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**Paramagnetic particles composed of nanomaghemite core suitable for isolation of biogennic amines**

Herein, we describe a synthesis of nanomaghemite core, functionalized with two types of ion-exchange resins - Dowex and sulfoxyethyl cellulose, and their utilization for evaluation of isolation potential towards a chemical group of biogenic amines (BAs). Isolation was carried out after charging of resins with Britton-Robinson buffer (pH 2) and the binding attributes were characterized by using ion-exchange chromatography with Vis detection (440 nm) of complexes, resulting from post-column derivatization with ninhydrin. Based on recovery calculations, Dowex-based particles were able to bind most of BAs with relatively good recovery (36% for tyramine; 33% for cadaverine), while sulfoxyethyl cellulose particles were more specific towards spermidine (21%), but not to other BAs. Our suggested particles are suitable for BAs isolation, and thus may serve as a first isolation step of BAs prior analysis. Moreover; due to their perfect paramagnetic properties may be applied in development of sensors.

**Accepted:** 1. 12. 2014
**Keywords:** Biogenic Amines; Dowex; Iron Oxide Nanoparticles; Isolation; Ion-exchange Chromatography; Sulfoxyethyl cellulose

## 1. Introduction

Biogenic amines (BAs) are basic nitrogenous low mass compounds with aliphatic (spermine, spermidine, putrescine, cadaverine), heterocyclic (e.g. tryptamine, histamine) or aromatic (e.g. tyramine) structure derived mainly from the decarboxylation of amino acids[1]. They may be formed by the action of yeast, lactic acid bacteria or other microorganisms during alcoholic and malolactic fermentation[2].

Most of BAs have strong physiological effects and play important biological role as source of nitrogen and precursors for synthesis of broad spectrum of biomolecules, such as hormones or nucleic acids[3]. On the other hand BAs have been widely studied as potentially toxic substances, since excessive intake of BAs manifests as food poisoning[4]. Moreover, BAs are potential precursors for the formation of carcinogenic N-nitroso compounds[5].

In order to determine the concentrations of biogenic amines in biological matrices, techniques providing high resolution and sensitivity are demanded. To determine BAs is challenging because of strong polarity and no natural UV absorption nor fluorescence. Thus BAs must be pre or post-column derivatized before detection[6]. Magnetic separation may be employed for isolation of a sample from complicated biological matrixes (food, body fluids) and may thus form the first separation and pre-concetration step prior to analysis to enhance an applied methodological approach[7].

The main aim of the present study is synthesis of nanomaghemite core and its functionalization with ion-exchange resins (Dowex and sulfoxyethyl cellulose), which can provide binding sites for chosen BAs (Tyramine-Tyr; Putrescine-Put; Histamine-His; Cadaverine-Cad, Spermine-Spm and Spermidine-Spd respectively).

Synthetic particles were finally employed for isolation and subsequent analysis by using ion-exchange chromatography.

## 2. Materials and Methods

### 2.1 Chemicals and pH measurements

Standards of biogenic amines (Tyr, Put, His, Cad, Spm, Spd) in purity of 99 % were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solutions of biogenic amines were prepared with dilution buffer called sodium cycle composed of 1.5 mM $N_3Na$, 197 mM NaCl and 73 mM $C_6H_8O_7$ in MilliQ $H_2O$. Further, we used citric acid, sodium citrate, isopropanol, potassium hydroxide, potassium bromide, hydrochloric acid and ninhydrine all purchased from Sigma-Aldrich. Methyl cellosolve was purchased from Ingos (Prague, Czech Republic), as well as tin chloride. All buffer solutions were prepared with deionized water obtained using a reverse osmosis equipment Aqual 25 (Aqual s.r.o., Brno, Czech Republic).

### 2.2 Synthesis of Paramagnetic Microparticles

Two types of paramagnetic microparticles were employed in this study. Both were based on nanometric maghemite core, synthesized according to protocol[8].

Nanomaghemite obtained in this way was further modified. (I) In the case of paramagnetic microparticles MAN1, 20 mL of nanomaghemite solution was mixed with 0.5 g sulfoxyethyl cellulose (Sigma Aldrich) (m/v) or (II) with 0.5 g of Dowex 50WX4-400 (Sigma Aldrich) (m/v). Mixtures were stirred at Biosan OS-10 (Biosan, Riga, Latvia) overnight. Resulting products were separated using external magnet and washed with water. Finally the product was dried at 40 °C.

### 2.3 Sample Preparation

To obtain information about behaviour of our own prepared PMPs, biogenic amines (volume 250 µL) in concentrations of 100 $\mu g.mL^{-1}$ were bound to them according to isolation conditions optimized in our preliminary study dealing with amino acid[9]. For isolation 250 µL of suspension of paramagnetic microparticles in PBS (10 $mg.mL^{-1}$) was employed. After isolation, sample was dissolved in 3 M hydrochloric acid (250 µL) and evaporated using nitrogen evaporator Ultravap RC (Porvair Sciences, Leatherhead, UK). Finally evaporated sample was resuspended with dilution buffer (250 µL) and analysed using ion-exchange liquid chromatography. In addition, real urinary samples (250 µL) were prepared similarly to be ready to be analysed by paramagnetic microparticles.

### 2.4 Ion-Exchange Liquid Chromatography

As a second part of our 2D separation approach an AAA 400 (Ingos, Prague, Czech Republic) ion-exchange liquid chromatography (IEC) apparatus was used. The system consisted of a glassy filling chromatographic column and steel precolumn, two chromatographic pumps for transport of elution buffers and derivatization reagent, cooled carousel for 25 eppendorf tubes of, dosing valve, heat reactor, Vis detector, and cooled chamber for derivatization reagent according to[9].

### 2.5 Descriptive Statistics

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise. The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner[10], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

## 3. Results and Discussion

BAs analyses are commonly challenging due to nature of target analytes and the undesired effects of matrix which complicates the accuracy and sensitivity of analyses. Since magnetic separation offers few advantageous attributes such as simple operation, ability to pre-concentrate a sample for further analyses or low production costs, we attempted to combine the magnetic separation with subsequent off-line analysis by using IEC.

IEC separation is commonly employed for separation of BAs, based on their pI[11] and we uti-

lized this technique for monitoring the binding capacity/recovery of paramagnetic particles. The conditions for separation and detection of BAs were optimized in our previous study[12]. The separation and elution was based on gradient elution using buffers with different ionic strength and pH together with temperature gradient. By using this method, we were able to discriminate all of six chosen biogenic amines (Tyr, Put, His, Cad, Spm, Spd) as is shown in **Fig. 1** with LoD between 59 - 110 ng.mL$^{-1}$.

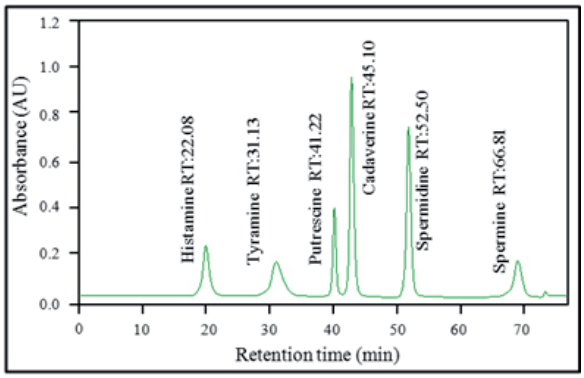


**Figure 1.** Chromatogram of mixture of six biogenic amines (His, Tyr, Put, Cad, Spm, Spd) in final concentration of 100 µg.mL$^{-1}$, obtained by using ion-exchange liquid chromatography with Vis detection in wavelength of 570 nm.

Magnetic separation by using nanomaghemite-based materials can be easily used for isolation and preconcentration of many different molecules, such as sarcosine[13], viral particles [14] and many others. Nanomaghemite can be modified with various chemical moieties, forming both - sites for covalent or non-covalent bonds. Thus we employed nanomaghemite to constitute a core for further modification with shell formed of ion-exchange resins: *i*) Dowex in case of paramagnetic particles, hereinafter named MAN21 and *ii*) sulfoxyethyl cellulose in paramagnetic particles, hereinafter named MAN1. Both resins are insoluble support structures, providing high surface area, where the trapping of ions occurs with concomitant releasing of other ions[15]. To remove the undesired impurities, paramagnetic particles were washed with Britton-Robinson buffer (pH 2), which simultaneously introduction of $SO_3^-$ of both ion-exchange resins. After washing, standards of BAs (100 µg.mL$^{-1}$) were isolated following optimized incubation protocol[9] and finally BAs immobilized by using external magnet were dissolved in HCl (3 M). The evaporated samples were then diluted in dilution buffer for subsequent IEC analyses. Chromatograms of determined biogenic amines with corresponding formula are shown in **Fig. 2A-F**. It is obvious that paramagnetic particles MAN21 shows bigger affinity towards most of BAs (Spm, Tyr, Put, His and Cad), whereas MAN1 were evaluated as more suitable only for isolation of Spd. To reveal a real binding attributes of both types of paramagnetic particles, recoveries of isolation were calculated as (1):

$$cBAdetermined / cBAapplied * 100 \quad (1)$$

Recovery describes the real binding capacity of certain material and thus it is highly important for its characterization.

In **Fig. 3** there are summarized recoveries for both particles

From **Fig. 3A** it is obvious that Dowex functionalization (MAN21) lead to much higher binding of BAs (Tyr - 36%; Cad – 33%; His - 30%; Put - 22%; Spm - 12% and Spd - 5%, respectively), when compared to sulfoxyethyl cellulose (MAN1) (Spd - 21%; Cad - 11%; Tyr - 8%; Spm - 6%; Put - 5%; His - 3%). Thus, it can be concluded that MAN21 offer lower specifity towards chosen BAs, however they are able to isolate practically all of them instead of Spd with relatively good yields. On the other hand MAN1 exhibit lower isolation yields; nevertheless they bind more specifically Spd. This phenomenon is tightly connected with core functionalization and the principle of increased/decreased specifity is hard-to-describe, although it is probably linked with steric arrangement of functional moieties and their charge-charge interaction with target BA.

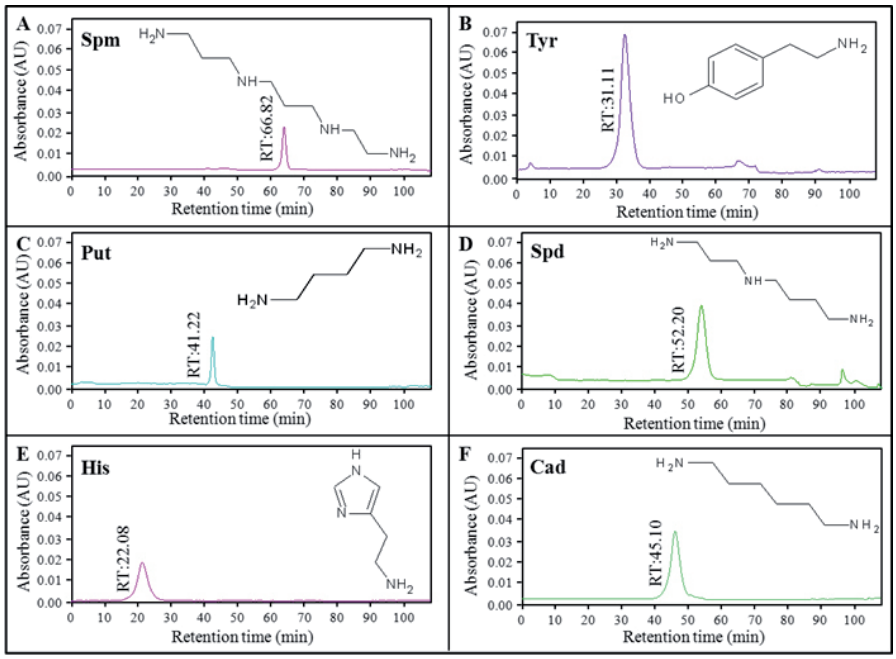


**Figure 2.** The chromatogramms of biogenic amines (100 μg.mL$^{-1}$) corresponding to (**A**) spermine isolated by using MAN21, (**B**) tyramine isolated by using MAN21, (**C**) putrescine isolated by using MAN21, (**D**) spermidine isolated by using MAN1, (**E**) histamine isolated by using MAN21 and (**F**) cadaverine isolated by using MAN21, with expression of their retention times. All records were obtained by using ion-exchange liquid chromatography with Vis detector (440 nm).

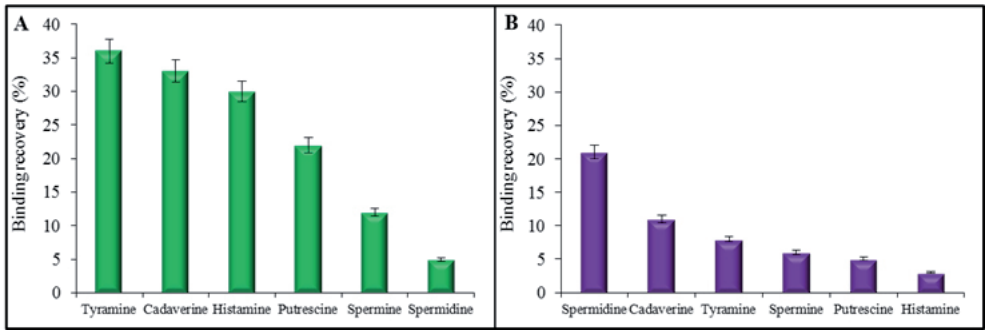


**Figure 3.** Expression of binding recovery of all of six biogenic amines (His, Tyr, Put, Cad, Spm, Spd) after isolation on paramagnetic particles (**A**) MAN21 and (**B**) MAN1. Biogenic amines (100 μg.mL$^{-1}$) were isolated following optimized conditions and recovery was calculated from quantification of biogenic amines carried out on ion-exchange liquid chromatography.

## 4. Conclusion

We succesfully suggested and synthesized paramagnetic particles composed of nanomaghemite core functionalized with two types of ion-exchange resins - Dowex and sulfoxyethyl cellulose. Both paramagnetic particles were able to bind biogenic amines, particularly spermidine, tyramine, cadaverine and histamine, and thus may be employed as a first isolation step of these compounds from various biological matrixes with subsequent separation on ion-exchange liquid chromatography or other analytical approaches. Moreover, paramagnetic particles are well suitable for utilization in fluidic devices or biosensors combined with the power of magnetic field.

*This work was financially supported by project MAG-AMINO (TR9140018).*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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# Modern techniques of increase the antibacterial properties of the instruments

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**Modern techniques of increase the antibacterial properties of the instruments**

Complication caused by resistant bacteria require qualified technical and technological measures since bacteria proliferation can occur in operations where aseptic environment is required. Constituents used as decontaminating compositions can be either bactericidal inorganic or organic substances like chemotherapeutics and antibiotics. In research special attention is paid to the problem of bacterial resistence toward antibiotics. A possible solution to issue consists in the employment of ionts and metal complexes such as silver nitrate or new nanoparticles (silver, cooper), whose antibacterial properties are known over centuries. In the focus of this research is the preparation of a composite material, consisiting of graphene oxide and nanoparticles of zinc oxide (ZnO), silver phhosphate ($Ag_3PO_4$) and silver. Graphene oxide was prepared by Hummers method, involving the oxidation of graphite flake in sulfuric acid with permanganate with subsequent addition of the necessary components for the formation of metal nanoparticles. Physicochemical methods are considered for their characterisation. The size of nanoparticles ranges from 50 – 200 nm. Subsequently, the antibacterial effect of composites was tested by disk method on bacterial cultures of S.aureus, E.coli, methicilin-resistant S. aureus (MRSA). Selenium nanoparticles exhibit the highest antibacterial activity from selected nanoparticles containing graphene oxide composite with an inhibitory zone with the size 5 ± 1 mm. Silver nanoparticles display also a distinguishable antibacterial effect with inhibition zones of 2 ± 1 mm. Graphene oxide which is modified by zinc oxide nanoparticles shows no inhibitory effect. The obtained results show suitability of the prepared composite materials as candidates for alternative antimicrobialmaterials.

**Accepted:** 9. 12. 2014
**Keywords:** antibacterial, resistance, nanoparticles of metals, graphene oxide

## 1. Introduction

Nowadays food and textile industries as well as health centers have employed advanced technologies and precaution measures which prevent bacteria proliferation and distribution among staff, consumers and patients.[1-3] These technologies and measures aren't always effective to fulfill the aseptic requirements. One of the main objectives to aseptic conditions is related to the necessity for the treatment of surfaces, tools and working materials by decontaminants. The application of chemotherapeutics or antibiotics isn't always a good choice since they may be in direct contact with the product, or may migrate and cause the transfer of contaminants into the product [3]. Some organic compounds used for desinfection purposes pose some disadvantages, including their toxicity for the human body [4]. Thereofore there is an increasing of interest in using of inoarganic disenfectants, such as metal nanoparticles (NPs) [5]. Furthermore, a crucial factor related to the use of chemotherapeutics and antibiotics is the enhancement of resistant bacteria toward these decontaminant agents [6]. Therefore it's necessary to develop new compounds wich would

show baktericide properties also toward bacterial species immuned with special resistance or multiresistance to antibiotics [6,7]. Bacteria with enhanced resistance are often found in hospital operations as a consequence of long-term antibiotics administration to patients, leading to genetic mutations and formation of various defense systems against damaging mechanism of antibiotics [8]. The most resistant form which evolved from the bacteria *S. aureus* is methicilin-resistant *S. aureus* (MRSA). Enhanced resistance to antibiotics or other bactericidal agents in staphylococ is present due to the variable genetic element such as pathogenicity islands genome, plasmids, transpons and others. The responsible gene for resistance enhancement in MRSA if found in the staphyloccocal chromosomal cassette mec (SCCmec) [9].

The integration of SCCmec into the genome of *S. aureus* leads to methicillin-resistant strain (MRSA) which is resistant to nearly all β-lactam antibiotics. The resistance toward methicillin is dedicated to the presence of penicillin-binding protein 2a (PBP2a) and the β-lactamase enzyme that cleaves β-lactam antibiotics (penicillin-based antibiotics), and is encoded by the mecA gene[10]. The PBP2a is an analogue of PBP transpeptidases whose presence is essential for normal cell, as they play an important role in cell-wall synthesis [11]. The presence of β-lactam antibiotics inhibit their function, and the cell loses its ability to produce cell wall and consequently dies. However PBP2a exhibits a low affinity toward β-lactam antibiotics, therefore is not subject to their inhibition[9]. All of this lead to cost uneffective and time consuming therapy for hospitalized patients with MRSA [12].The treatment of surfaces, tools, fabrics or packages with antibacterial agents in the above mentioned areas is an absolute necessity, and it is therefore it is necessary to ensure an effective treating agent with pronounced antibacterial effect. Such a property is prone of metal nanoparticles [13], graphene oxide (GO) or biofunctionalized graphene [14]. Graphene with antimicrobial effect is used in containers for food packaging or for the coating of biomedical devices, wherein the bacterial colonization on the surface are unwanted [15]. Modification of graphene metal nanoparticles with antibacterial effect streamlines this effect.

## 2. Material and Methods

### 2.1 Preparation of graphene oxide and metal nanoparticles

**Preparation of graphene oxide (GO)**

Graphene oxide was prepared by Hummers method using potassium permanganate. This occurred by transferring 46 ml of concentrated $H_2SO_4$ into an ice bathed beaker under continuous stirring of 2 g of graphite, 1 g $NaNO_3$ and 6 g $KMnO_4$. After it, the beaker was removed from the ice bath and placed at at room temperature. Later on, 92 ml of water were added into the resulting solution , followed by 15 minutes of still standing, and the addition of 280 ml of warm water. Finally a 3% $H_2O_2$ was transferred into the prepared solution until until it exhibits a bright yellow color.

**Preparation of zinc oxide nanoparticles (ZnO)**

Zinc acetate dihydrate (assay ≥ 98%) was used as the raw material to synthesize ZnO nanostructures. According to this method 1 g of zinc acetate dihydrate was placed in an oven and then it was heated up for 2 h at 300 °C. The heating rate seemed to be a very important parameter in the amount of the product and the undesired phases which might appear during the heating. So the heating rate was set to 10 °C/min and the samples were annealed at 300 °C for 2 h, and then cooled to room temperature for 12 h. It was observed that two different kinds of products were obtained after sample annealing. A white porous powder covering the crucible walls was denoted as sample No. 1 a gray black powder stucking to the bottom of the crucible denoted as sample No. 2. After microwaving and naturally cooling down, the samples were transferred in eppendorf tubes and washed with ethanol. After that the precipitate at the bottom of the eppendorf tube was washed 3 times with sufficient amounts of MiliQ water. The obtained sample was kept at ambient conditions 12 hours drying, yielding thereafter a total amount of 72 mg.

**Preparation of silver nanoparticles (SPNPs)**

1 ml of $AgNO_3$ (850 mg of $AgNO_3$ in 10 ml of MiliQ) was mixed with 25 ml of MiliQ and separately 1 ml of $Na_2HPO_4$ (850 mg of $Na_2HPO_4$ in 10 ml of MiliQ) was mixed with 25 ml of MiliQ. Immediately after preparation, these two solutions were mixed together and mixed a few minutes on magnetic stirrer until its color turned to light yellow. Then, 500 mg of cekol was added into this complex under continuous mixing of 2 hours.

**Preparation of selenium nanoparticles (SeNPs)**

5 ml of $Na_2SeO_3$ ($Na_2SeO_3$ 263 mg/ 50 ml of MiliQ) was dissolved in 45 ml water followed by adding 80 µl of 3-mercaptopropionic acid (MPA). Its pH was adjusted to 9 by the addition of 1 M NaOH (0.2 ml). The reaction mixture was stirred for 2 hours yielding to a red color. Then these two solutions were mixed under magnetic stirring for 30 minutes. The color of solution was black and its pH was 6. Finally, 1 g of cekol was added into the solution under continuous mixing for two hours[6].

### 2.2 Cultivation of *S. aureus*, methicillin-resistant *S. aureus* and *E. coli*

*S. aureus* (NCTC 8511), methicillin-resistant *S. aureus* (7111 2/A8) and *E. coli* were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. Cultivation media (LB = Luria Bertani) were inoculated with bacterial culture for 24 hours on a shaker at 130 rpm and 37°C. The bacterial culture was diluted by cultivation medium to $OD_{600}$ = 0.1 for the following experiments.

### 2.3 Determination of antimicrobial properties

Inhibition zones and growth curves

Agar surface in a Petri dish was covered with a mixture of 100 ml of 24 hour grown culture of *S. aureus* or *E. coli* and 3 ml of LB medium (Luria Bertani medium). The circles with a diameter of 1 cm were delivered by the factory (Vyzkumny ustav pletarsky in Brno) and mixed with SPNPs with a concentration of 300 µM in complexes with hyaluronic acid (8.3 mM) or chitosan (9.7 mM). Petri dishes were incubated in a thermostat at 37 °C for 24 hours. The antimicrobial effect of tested compounds was measured by measuring the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Germany). The culture was diluted with LB medium to obtain absorbance 0.1 AU at 600 nm. In the microtitration plate S. aureus or E. coli culture was mixed with various concentrations of SPNPs (0, 10, 25, 50, 75, 150, 225 and 300 µM) and constant concentration of hyaluronic acid (8.3 mM) and chitosan (9.7 mM). Total volume in the microtitration plate wells was always 300 µl.

## 3. Results and Discussion

### 3.1 Characterization of particles

Average size of nanoparticle and distribution curves were determined by a dynamic light scattering with a device Zetasizer Nano (Malvern) (**Fig. 1**). The average size of the nanoparticles was GO 200 nm, ZnO 50 nm, SPNPs 100 nm and SeNPs 15 nm. The composition of the nanoparticles was also demonstrated by using X-ray fluorescence spectrometry (XRF) instrument Spectro XEPOS. In the spektra were found peaks corresponding to lines of zinc, silver, phosphorus and selenium.

For using graphene oxide modified by nanoparticles of zinc oxide to creating to inhibition zones around the diffusion dishes in any of the tested cultres. A similiar study Leung (2012) dealt with the GO modified by ZnO nanoparticles, when the researchers demonstrated the antibacterial effect of this composite applications to surface materials. Diferent results are caused by the different preparation, size and surface finish of GO and ZnO nanoparticles[16]. Graphene oxid is modified with silver nanoparticles and it was used as the second potential antibacterial components. For these particles were give 2 mm of inhibition zone during incubation with *E. coli, S. aureus* was inhibited by

formation of 3 mm inhibiton zone and MRSA was inhibited by formation only 1 mm inhibition zone. Method of measuring the inhibition zones is useful as a primary test the inhibitory effect of substances [17]. In the third composite with GO were chosen selenium nanoparticles (SeNPs). Selenium nanoparticles are new alternative growth inhibition of bacterial cultures, the effect of which dealt with the study [6], when were compared the effect SeNPs and SPNPs. For SPNPs were measured inhibition zones of 3 mm, while SeNPs inhibition zone of size 7 mm. Graphene oxide modified by SeNPs worked on bacterial cultures in the most efficient inhibition effect of the test substances. For bacterial *E. coli* culture was made 4 mm inhibition zone from the diffusion disk with inhibitory substance. In *S. aureus* was measured 6 mm inhibition zone and in MRSA was measured 5 mm zone of inhibition. Graphene oxid was modified with selenium nanoparticles and it can be classified as an effective inhibitory components on $G^+$, $G^-$ and resistant bacteria.

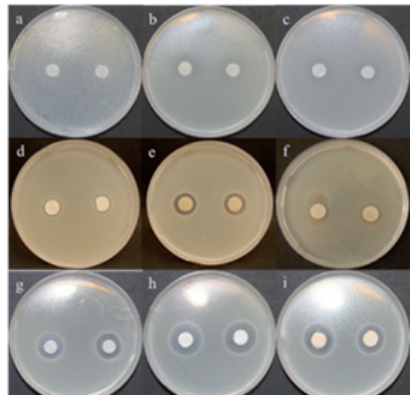


**Figure 2.** Testing the antibacterial action by method of inhibition zones. While we tested GO modified by ZnO avoid to the formation of inhibition zones around any of the test bacterial cultures *E. coli* (**a**), *S. aureus* (**b**) a MRSA (**c**). In GO modified by SPNPs have been created 2 mm inhibition zone in *E. coli* (**d**), 3 mm in *S. aureus* (**e**) a 1 mm in MRSA (**f**). In modification GO by SeNPs particles leads to the formation of 4 mm inhibition in *E. coli* (**g**), 6 mm in *S. aureus* (**h**) and 5 mm in MRSA (**i**).

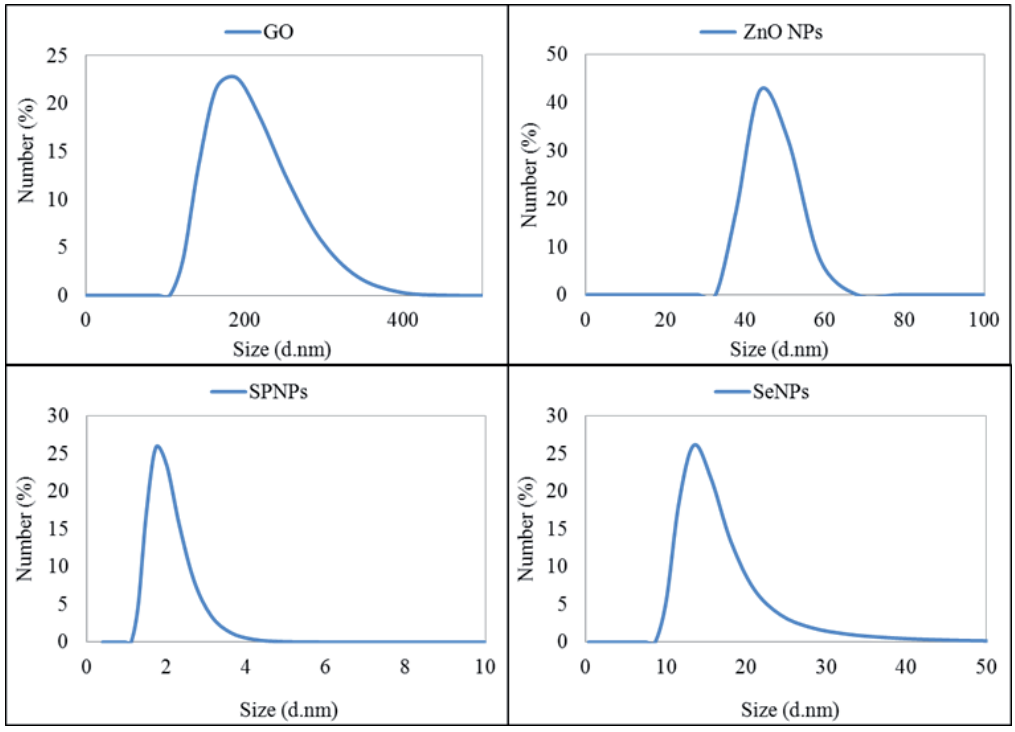


**Figure 1.** Results of measurment of particle size by using dynamic light scattering.

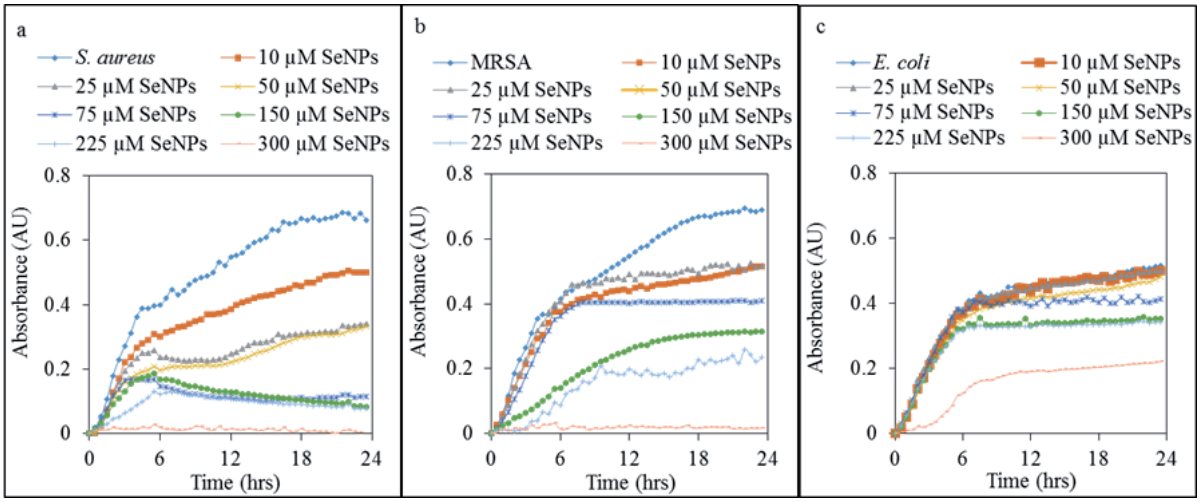


**Figure 3.** Growth curves of bacterial cultures after application grephene oxide with different concentrations of selenium nanoparticles. Using concentration of grephene oxide was stacionary for all different concentrations of selenium nanoparticles. *S. aureus* (**a**) and *MRSA* (**b**) growth in a similar downward trend with increasing concentrations of selenium nanoparticles in complex with graphene oxide (1 mg/ml). For *E. coli* was not too significant downward trend (**c**).

An inhibition effect was different for diversity of the selected bacteria. Gram negative *E. coli* has a stronger cell wall due to the presence of the outer membrane resulting in a poor transport of nanoparticles inside cells [18]. High inhibition effect on S. aureus is due to absence of lipopolysaccharide and outer membrane layer in cell wall [19]. The mechanism causing resistence to β-lactam antibiotics fails to protect the resistant bacteria to antibiotics against complex of graphene oxide and selenium nanoparticles [20].

From the above results we can conclude that the GO modified by SeNPs act by inhibitory effect in all the cultures, while the GO modified by ZnO doesn't provide an inhibitory effect on any of these cultures. A similiar Leung study (2012) dealt with the GO modified by ZnO nanoparticles, when researchers proved antibacterial effect of this composite applications on surface materials. Different results may be caused by different prepare GO and ZnO nanoparticles [16].

## Acknowledgements

*Financial support from the project ANTIBAKT TR9140017 is highly acknowledged*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements" for biomedical papers.

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# Detection of sentinel lymph node using magnetic nanoparticles

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There was developed further application of nanotechnology- detection of sentinel lymph node by magnetic nanoparticles. Sentinel lymph node biopsy is the standard surgical procedure for the axilla in early node-negative breast cancer. If no cancer is detected in the sentinel lymph node, there is no need for an axillary lymph node dissection in which usually 30 or so lymph nodes are removed. Axillary node dissection is often complicated by arm lymphedema. Moreover sentinel lymph node biopsy procedure results in shorter breast cancer operations and better patient recovery compared to node dissection.

To date is used radioistope Tc99 and/or blue day to localize the sentinel lymph node. Sienna+® is a tracer capable of an induced magnetic response and was developed for use with the SentiMag® instrument. Instrument generates alternating magnetic field which temporarily magnetizes iron oxide in Sienna+® particles and the magnetic signature generated by those particles is then detected by the probe. Sienna+® is an aqueous suspension of organically coated, superparamagnetic iron oxide particles. It is injected subcutaneously where the natural physical action of the lymph nodes filters out the particles, enabling the sentinel nodes to be located with the SentiMag®. The system was developed by Endomagnetics Limited, Cambridge, UK.

Optimum localisation agents should remain fixed at the sentinel nodes and do not travel to other nodes. This depends on particle size, for ideal localisation, agents should have a particle diameter 20 - 100 nm. Routinely used Tc99 Nanocoll has particles 4-100 nm. Sienna+® was designed with a tight distribution around 60 nm.

Clinical prospective, multicentric and multinational study which included 150 breast cancer patients per arm showed that magnetic sentinel lymph node localization can be performed easily, safely and equivalently well in comparison to the radiotracer method.

Advantages of Sienna+® are: simple storage and handling procedure, and significantly improved workflow compared with radioactive tracers, localisation can start after only 20 mins following injection, dark colour eliminates requirement for separate dye injections, and long shelf life.

*Th is work was fi nancially supported by project NANOLABSYS CZ.1.07/2.3.00/20.0148 is highly acknowledged.*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

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LABORATORY REPORT

# Opening of working and meeting room for nanobiometalnet project

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**Accepted:** 2.12. 2014

## Introduction

Thanks to the work of our technical stuff we can now introduce the new open-office which was built due to the Reconstruction project in Laboratory of Metallomics and Nanotechnologies. There is 10 working places an each workstation is equipped with modern computer with 24" screen, barcode reader and desktop LED lighting. All workstations are connected in network with central print server. Room is also equipped by multimedia TV with controlling computer for organizing workshops and meetings. The entire space is air-conditioned. The space is designed preferably for workers as postdocs, Ph.D. and pregradual students.

*This work was supported by project „Rekonstrukce v laboratorním provozu LMN ID110".*

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

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# New directions of electrochemistry, bioelectrochemistry, nanoelectrochemistry and bioengineering

René Kizek[a], Zbyněk Heger[a], Petr Michálek[a], Ondřej Zítka[a]

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**Accepted:** 28. 10. 2014

Working seminar „New directions of electrochemistry, bioelectrochemistry, nanoelectrochemistry and bioengineering“under the CEITEC project, whose main mission was to build an important European center of science and education with excellent facilities and conditions for the best scientists in Brno was held on the premises of biotechnology center (INBIT), Kamenice 771/34, Brno, September 29, 2014. The seminar was attended by both undergraduate and graduate students as well as experienced researchers from Mendel University, University of Technology and Masaryk University.

The event in the area the Masaryk University campus was backed by the Vice-Rector for Science and Research, Mendel University in Brno[1] . Workshop at the end of September (29th September 2014, 10:00 a.m. to 2:00 p.m.), which was held in the meeting room INBIT Kamenice was attended by fifty participants including foreign presenters from India, Vietnam and Spain. It is necessary to recall the integral share of support from the Eppendorf company, carried by Dr. Milan Řezka. The plenary lectures led to productive discussions of workers of Laboratories of metallomics and nanotechnology, Laboratories of sensors, and the Department of biomedical engineering, primarily on new directions in the field of electrochemistry, bioelectrochemistry, nanoelectrochemistry and bioengineering and gave a new impulse for further cooperation and planning of shared project and outcomes. An important part and also pleasant duty of all involved was to commemorate the birthday of Mrs. Libuše Trnková, Assist. Prof., personality respected in the field of an electrochemical research. According to the program of the workshop the meeting was started with a lecture by Professor René Kizek, followed by Assist. Prof. Vojtěch Adam with presentation with the headline - Electro-Metalomics, were new findings in the field of thiol compounds, especially metallothionein, were presented. Research in the Laboratory of metallomics and nanotechnology has been focused in the long term on this particular thiol compound. Another presentation with the title „Modern micro- and nanotechnologies for bioapplications“ was presented by associate professor Jaromir Hubálek. The presentation summarizes and discusses new technologies suitable for the study of biomolecules, wherein the attention has been focused on quantum dots and a new generation of microelectrodes. The possibilities of using electrochemical methods for studying biological processes and structural changes of nucleic acids were presented by Dr. Michal Masařík in a lecture titled „Electrooncology“. The voltage of cell membranes is a parameter that significantly affects the contraction and the relaxation of muscle cells. Micro- and possibly nanoelectrodes can be used to study these processes. These options with illustrative examples of measurement of myocardial cells was demonstrated in a lecture by Professor Ivo Provazník. An interesting direction seems to be also fluorescent labeling of these cells and monitoring the changes in emission maximums. In the next block of the seminar lectures of foreign participants were presented. The block started with the lecture by Miguel Angel Merlos Rodrigo about new chip techniques for monitoring of the expression profiles not only in cancer cells, but also for identifying viruses. These technologies work with different detection methods, especially fluorescent labels, but also electrochemistry as in

Journal of Metallomics and Nanotechnologies **2014**, 4, 57—58

the case of ElectraSense device, which has been detailed described in the lecture in the lecture. Hoai Viet Nguyen presented a presentation entitled „Electroanalysis of etoposide“ about the potential of electrochemical methods serving as a sensitive tool for monitoring the interaction of anticancer drugs with biomolecules on a specially modified electrode. The glassy carbon electrode is cleaned by ultrasound and alumina and then polished on velvet. The electrode is then modified by multiple-walled carbon nanotubes (MWCNT). After drying, thus prepared surface is covered with the anticancer drug which is thus prepared for monitoring interactions with biopolymers such as DNA. The entire block was concluded by Dr. Amitava Moulick with a lecture „Synthesis of peptides and nucleic acids“ where the technology of preparing synthetic oligonucleotides and linear peptides were described in detail. Discussion was held on the use of these molecules in nanomedicine, as well as individual parts of synthesis, wherein the functional groups for binding the individual building blocks of peptides or oligonucleotides are deprotected.

We can conclude that workshop with its outputs contributed to improving communication between the various partners and brought new methodological information for use in all partner groups. All presentations were summarized in a special electronic output, which is available in an internal information system[2].

Group photo of participants of the seminar: „New directions of electrochemistry, bioelectrochemistry, nanoelectrochemistry and bioengineering“. INBIT, 29th September 2014, 12:00 pm

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

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MY. VY. **SPOLEČNĚ.**

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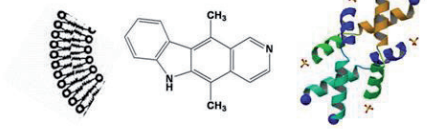

NANOCHEMO

Vývoj nanočástic obsahujících cytostatika a enzymy pro zlepšení chemoterapie lidských neuroblastomů a studium mechanismu jejich působení
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