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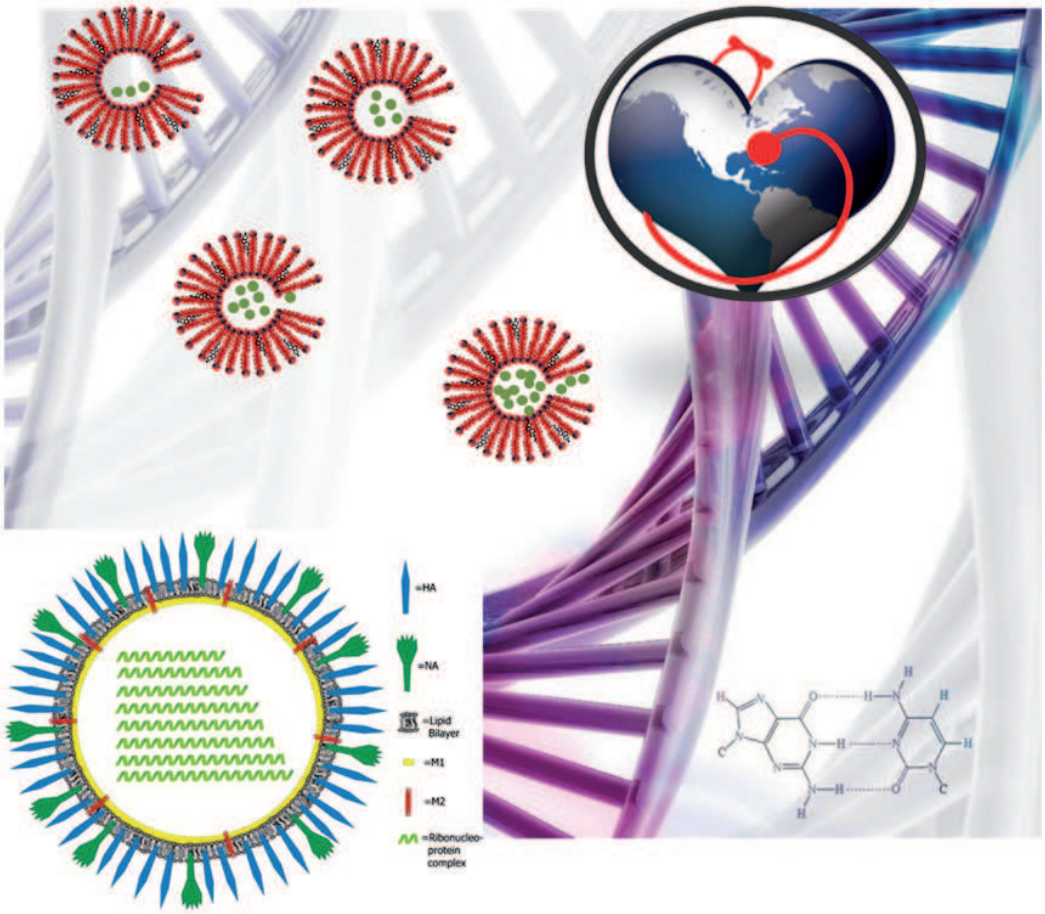
Moderní nástroje pro zobrazování biologicky významných molekul pro zajištění zdraví



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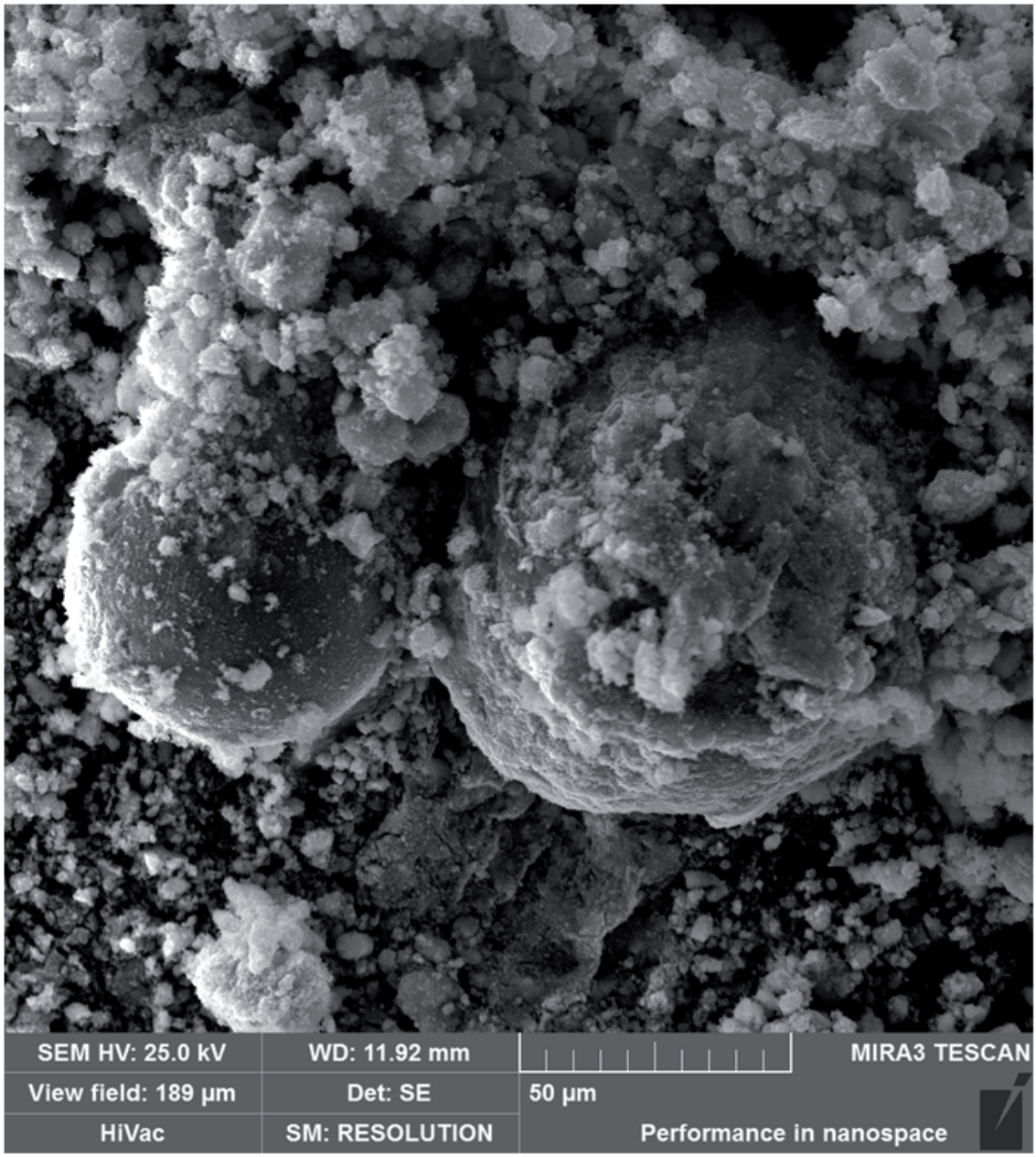

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Na obrázku na první straně je SEM fotografie polymerních mikročástic pokrytých feromagnetickým oxidem železa a funkčními skupinami pro izolaci nízkomolekulárních sloučenin (fotografie: Doc. Kynický).

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EE2.3.20.0148 - Mezinárodní spolupráce v oblasti „in vivo“ zobrazovacích technik (2012-2014, MSM/EE)

Hlavním cílem projektu je vytvoření výzkumné sítě mezi pěti subjekty v rámci EU (ČR, UK, SK, Španělsko a Bulharsko) v oblasti zavedení elektronických laboratorních systémů s ohledem na praktické využití v in vivo nabiotechnologických aplikacích. Výzkumné skupiny spolupracují na vybraných dílčích úkolech v oblasti nanobiotechnologií, nicméně potenciál spolupráce zůstává nevyužit z důvodu administrativních komplikací v oblasti koordinace výzkumných aktivit, nedostatečného zázemí pro sdílení dosažených výsledků a nedostatku zdrojů pro mobilitu výzkumných pracovníků.

Dílčí cíle projektu jsou:
a) propojení jednotlivých výzkumných skupin;
b) pilotní vytvoření elektronických laboratorních deníků;
c) zvýšení a posílení technologického a organizačního zázemí.

Mezinárodní spolupráce v oblasti „in vivo“ zobrazovacích technik

CZ.1.07/2.3.00/20.0148

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Obsah

PŘEDMLUVA

Vážení čtenáři,

Dostává se vám do rukou publikace zaměřená na problematiku moderních nástrojů a technologogií aplikovatelných pro zvládání závažných onemocnění. Kniha je převážně fokusována na problematiku virových onemocnění. Je velmi dobře zámo, že virová onemocnění představují velmi závažný zdravotnický a celospolečenský problém. V případě závažné pandemie (nejpravděpodobněji chřipky) bude ve velmi krátké době zasažena celá planeta a při smrtnosti mezi 70 - 80 % vyvolá globální zhroucení správní struktury. Z dosposud známých informací je naprosto zřejmé, že taková smrtící epidemie nastane (je jen otázkou času kdy se to stane). Vyhledání a navržení rychlých, efektivních způsobů identifikace takové hrozby je jednou z prioritních záležitostí výzkumu mnoha týmů po celé plantě. Je to jeden z hlavních cílů výzkumné skupiny CEITEC Chytré nanosoučástky. Navržené postupy se soustřeďují na využití různých typů nanotechnologických nástrojů. V našem zorném poli jsou především různé přístupy v přípravě magnetizovatelných částic (mikro i nano) a jejich následné modifikace. Povrch těchto částic je výhodné modifikovat nukleovými kyselinami, ty vytváří základy pro vývoj unikátních senzorů detekující nukleovou kyselinu (genom) viru. Rozpoznání přítomnosti této nukleové kyseliny je možné několika rozličnými cestami a způsoby (detekce nukleové kyseliny přímo spektrofotometricky, elektrochemicky). Mezi další náročnější způsoby můžeme využít různých typů fluorescenčních značek, barevných i elektricky aktivnách značek a také zářících nanočástic. Mezi další způsoby rozpoznání přítomného patogenu je rozpoznání jeho strukturních proteinů. Jako nejvhodnější se pro takové účely používají specifické protilátky. Modifikace protilátek různými typy značených biomolekul (fluorescenční, enzymatické, radioaktivní, kvantové tečky, barevné a elektrické značky). Jejich detekce tak umožní určit přítomnost patogenu v prostředí.

Navíce je nezbytné připomenout, že množství patogenů v prostředí je na vlastním počátku extrémně nízké a pro jeho zviditelnění je potřebné využít výrazných aplifikátorů signálu. Také aplifikátory jsou na prvním místě různé katalytické reakce (včetně enzymatických), ale také různé transportéry vyplněné aplifikátorem (apoferitin, liposom).

Závažný problém však dále představuje zachycení patogena z prostředí. Pro takový účel je nezbytné využít co nejmodernějších způsobů izolování z prostředí. Výhodu nám v této oblasti přinášejí mikrofluidní systémy kombinující nástroje průtoku kapalin řadou kanálků. Některé z nich obsahují rezervoáry se speciálně upravenými magnetizovatelnými částicemi. Průběh celé reakce je v řádu desítek minut a jednoduchá elektrochemická detekce poskytne výsledek prakticky ihned. Je pravdou, že i tyto systémy nedosahují potřebné rychlosti analýzy, tak jak by bylo potřebné pro monitorování velkých prostor jako jsou letiště, nádraží, přístavy, velké správní budovy a podobně.

Autoři shrunuli doposud známé hlavní směry v oblasti nanotechnologií detekující viry. Jsou ukázána a kriticky zhodnocena úskalí a postupy doposud známé vědy.

Brno, 1. dubna 2013, René Kizek

NANOTECHNOLOGY AS A POWERFUL TOOL FOR DETECTION OF VIRUSES

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Abstract

It is imperative to develop logical solutions for early diagnosis and containment of contagious diseases. Traditional techniques suffer from limitations, including laborious sample preparation, bulky instrumentation and slow data readout. In view of the urgency for sensitive, specific, robust and rapid diagnostics, numerous advancements have been made in the area of diagnostics. Broadly, most of these innovative approaches, have utilized the unique properties of nanomaterials in order to achieve detection of infectious agents, even in complex media like blood, urine.

WHD_02_2013

Introduction to Wireless Sensors

Various activities including politicians have led to an increase in threat of potential biological weapons endangering the survival of mankind. Broadly, these bio-chemical agents, Bacillus anthracis (anthrax), Variola major virus (small pox), Botulinum neurotoxins, Yersinia pestis, Francisella tularensis, Burkholderia pseudomellei, Burkholderia mallei, Rickettsia spp, Coxiella burnetti, Venezuelan equine encephalitis virus, Marburg and Ebola viruses and influenza viruses are considered to have the great potential for mass causalities and civil disruption [1, 2]. Besides these, there are several emerging infectious diseases with the potential for significant public health consequences, including malaria, HIV virus related complications, Dengue fever,West Nile fever, and Rift Valley fever as well as tuberculosis [2]. As with biowarfare agents, emerging infectious disease agents may be directly transmissible or vector borne. Further, infectious diseases prevalent in the developing world result in increased rates of morbidity and mortality. While infectious diseases can initiate in a localized region, they can spread rapidly at any moment due to the ease of traveling from one part of the world to the next.

Because of the threat posed by both biowarfare agents and emerging or reemerging infectious disease agents, there is a need to develop diagnostics for rapid identification of such agents in clinical setting in order to treat the individuals at risk and to improve public health surveillance and epidemiology [3].

Potential of Nanotechnology

Conventional molecular diagnostic techniques are widely used in laboratories throughout the world to identify pathogenic agents with high degree of sensitivity and reproducibility. However, most of these techniques cannot be utilized in the field (e.g. airports and food distribution centres) or in developing countries where resources are scarce, because they often require sophisticated, expensive instrumentation that needs to be used by trained personnel. Additionally, the high cost and short shelve half-life of some reagents, such as enzymes and DNA primers, limit the application of most conventional pathogen detection techniques in rural areas of developing nations. Furthermore, despite their sensitivity, current technologies, like ELISA and PCR, require extensive sample preparation and have long readout times, which delay prompt response and disease containment. Hence, taking advantage of the unique electrical, magnetic, luminescent, and catalytic properties of nanomaterials, faster, sensitive and more economical diagnostic assays can be developed that can assist in the battle against microbial pathogenesis. Apart from striving for sensitivity and speed, nanotechnologists have geared their efforts towards the development of nanotechnology-based systems that are affordable, robust and reproducible, making them suitable for applications even in rural areas of developing nations. Moreover, using innovative approaches, nanotechnology has the potential to build assays that can be performed in opaque media, like blood and milk, without any sample preparation, providing fast and reliable results in simple and user-friendly formats [4].

Liposomes

Liposomes are small vesicles consisting of one or more concentric lipid bilayers surrounding aqueous compartments. Particle size and physicochemical characteristics of liposomes can be manipulated for specific applications [5]. Gangliosides are expressed on cell surface and serve as natural receptors

for bacterial and viral toxins. Therefore, for detection of toxins, methods have been developed based on gangliosides targeting. Singh et al. [6] engineered and fabricated GT1b or GM1 ganglioside bearing liposomes (~120–130 nm) for detecting three bacterial toxins tetanus, botulinumand cholera. Their study demonstrated that these engineered liposomes were able to mimic cells and recognize target toxins, thus forming a basis for toxin detection. These liposomes were labeled with fluorescent markers (rhodamine dyes) followed by a sandwich fluoroimmunoassay on antibody coated microtiter plates. Through this assay, they reported detection of toxins as low as 1 nM concentration.

Carbon nanotubes

Carbon nanotubes are considered as a sheet of graphite rolled into a tube with bonds at the end of the sheet that close the tube. A single walled nanotube (SWNT) can have a diameter of 2 nm and a length of 100 μm, making it effectively a one dimensional structure called as nanowire. The Multiwalled nanotubes (MWNT) can be considered as SWNTs kept one inside of another. These nanotubes lend themselves for biofunctionaliztion with multiple copies of biomolecules (e.g. carbohydrates and antibodies) for an enhanced detection of the antigen in the analyte [3].

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Dendrimers

Dendrimers are hyperbranched, tree-like rigid structures and have compartmentalized chemical polymers with unique structural and topological features. Dendrimers generally have a uniform molecular weight with no specific molecular weight distribution. Dendrimers contain far more surface groups capable of being functionalized compared with proteins of similar size. In contrast to other linear, crosslinked, and branched polymers, the three-dimensional structure of dendrimers gives them a variety of unique properties such as low polydispersity and high functionality, and thus a wide range of potential applications using dendrimers as nanoscopic objects have been explored [7].

During the past two decades a great number of dendrimer structures have been developed and investigated based on inspiration from biological systems. Among them, polyamidoamine (PAMAM) dendrimers, polypropyleneimine (PPI) dendrimers, and biomolecules derived dendrimers such as amino acid dendrimers, carbohydratemodified dendrimers, nucleic acids–nucleobases dendrimers and polyester dendrimers are notable.

Gold nanoparticles (AuNPs)

The unique physical, chemical properties and high surface to volume ratio enable AuNPs as an ideal material for adsorption of biomolecules without compromising their biological activities. Antigens or antibodies functionalized AuNPs can serve as optical labels, electrochemical markers, surface plasmonic amplifiers or signal transfer mediator for the quantitative analysis of ligands, AuNPs in combination of other signal generators or other nanoparticles could doubly amplify the signal. They can be prepared with different geometries (size b50 nm) and a range of shapes are available such as nanospheres, nanoshells, nanorods or nanocages. The amenability of AuNPs for signal amplification makes it a versatilemetal nanoparticle for diagnosis [4, 8].

Iron oxide nanoparticles

Superparamagnetic nanoparticles are being widely used in magnetic immunoassay diagnosis techniques. This approach for magnetic detection is inspired by the binding effect of the substrates on the superparamagnetic nanoparticles that can align under an applied magnetic field so that uniformly aligned particles are detected by magnetic detectors; others that are randomly oriented are ignored. As a result, this technique does not require a washing step before imaging, because other non-specific moieties inside the same such as buffer or sample will not bind to these particles and thus will not affect the imaging. Bound to a suitable antibody, they are used to label specific molecules, cell populations and structures or microorganisms. Binding of antibody to target molecules or disease-causing organism is used for the immunomagnetic separation of nucleic acids, proteins, viruses, bacteria and cells and it forms the basis of several tests [9].

Quantum dots (QDs)

QDs have become one of the most promising and interesting materials for diagnostic applications of bioimaging, labeling, and sensing, due to their exceptional optical properties. They are nanocrystals composed of a core of a semiconductor material generally of atoms from groups II and VI (i.e. CdSe, CdS, and CdTe etc.) or III and V (i.e. such as InP) of the periodic table, enclosed within a shell of another semiconductor that has a larger spectral band gap (such as ZnS and CdS). The shell prevents the surface quenching of excitons in the emissive core and hence, increase the photostability and quantum yield for emission. They are neither atomic nor bulk semiconductors, since their properties originate from their physical size, which ranges from 10 to 100 Å in radius. They have high sensitivity, broad excitation spectra, stable-bright fluorescence with simple excitation and no need for lasers. These characteristics make them suitable for various biomedical applications such as sensing and detection of biomarkers including antigens and pathogens, immunolabeling of cells and tissues. Also their strong light absorbance makes them to be used as fluorescent labels for biomolecules [4, 7, 10, 11].

Bacteriophage/virus particles

Though many methods are available, the low cost and ready production of large numbers of bacteriophage, along with their specificity for the target bacterial species make them ideal for detecting bacteria. The advantage of detection with bacteriophage/virus particles is that they usually detect living bacteria thereby avoiding the false positives that often arise from the use of approaches such as PCR [12, 13].

In phage amplification assays, bacterium is infected with the phage which results in the release of many phage particles that can be detected by a second (nonpathogenic), sensitive bacterial strain. Phage based detection systems use engineered recombinant phages, which upon infection of their hosts, these engineered phage deliver reporter genes (such as luxAB genes from Vibrio harveyi) [14]. Replication of the viral genome results in many copies of the viral genome being produced and subsequent expression of these genes ensures the amplification of the initial phage–bacterium interaction into a signal that can be readily detected (bioluminescence in the case of luxAB) [15].

Conclusion

Cell-based sensors are an emerging frontier in the area of nanodiagnostics. The use of cells as sensors is a very attractive way to devise sensitive biochemical detectors. With their highly selective and sensitive receptors, channels and enzymes, intact cells are very attractive candidates for the development of biosensors. The main advantages of the cells as biosensors are that cells have built-in natural selectivity to biologically active chemicals and they can react to analytes in a physiologically relevant mode.

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MIKROFLUIDNÍ NÁSTROJE V IDENTIFIKACI VIROVÝCH INFEKCÍ

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Abstract

Poměrně nový, rychle se rozvíjející obor v analytické chemii, mikrofluidika, je univerzálním nástrojem pro analýzu kovů, malých organických molekul nebo biomolekul. Z hlediska analýzy biomolekul je velmi perspektivní oblastí analýza nukleových kyselin se zaměřením na stanovení virů. V současnosti mikrofluidika prochází vývojem nejen technickým, jako je nalézání vhodných materiálů a vyvíjení způsobů výroby, ale nachází uplatnění v analýze virových onemocnění, kde vyniká nízkou spotřebou vzorků a díky miniaturizaci také možností použití přímo v terénu a za nízkou pořizovací a provozní cenu.

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Úvod

Potřeba miniaturizace instrumentace v oboru analytické chemie se stala jedním z iniciátorů vzniku nového oboru zvaného mikrofluidika. Mikrofluidní nástroje využívají proudění tekutin ve velmi malých zařízeních od jednotek centimetrů až po milimetry. Použití mikrofluidních systémů má značné výhody a to díky nízké spotřebě vzorku, rychlejší analýze, jednoduché instrumentaci, snadné automatizaci a relativně dobré citlivosti. Tyto výhody předurčují mikrofluidní systémy pro použití v oblasti biosenzorů nebo v tzv. „laboratoři na čipu", nebo „laboratoři na ventilu" a tak se tyto technologie staly novým zájmem pro řadu oborů jako je biomedicína, diagnostika, enviromentální chemie, farmacie a další.

Instrumentace mikrofluidních nástrojů

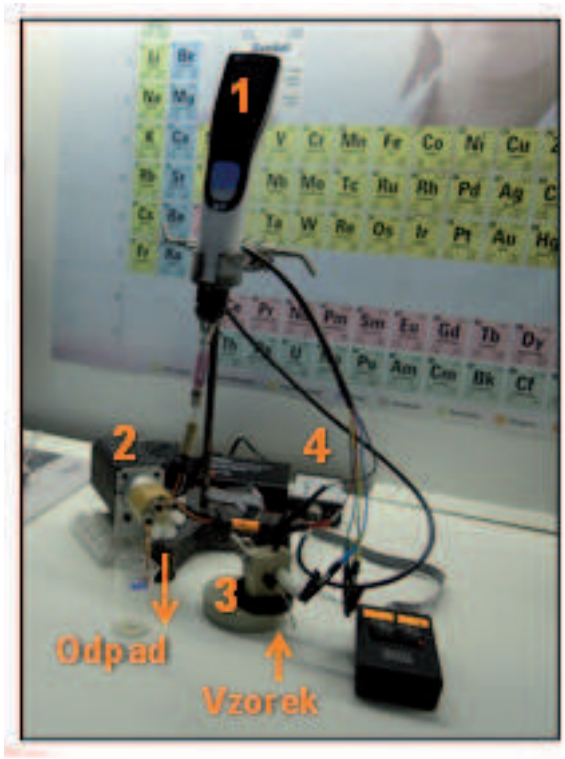
Mikrofluidní systémy jsou definovány jako zařízení, které pro dopravu reagencií a vzorků používají proudění kapilárami menšími než 1 mm. V těchto systémech se uplatňují fyzikální jevy jako difúze, laminární proudění, fluidní odpor a povrchové napětí [1]. V současnosti se vývoj a aplikace mikroprůtokových zařízení soustřeďuje na miniaturizaci přístrojů pro elektroforézu, isoelektrickou fokusaci, imunoanalýzu, PCR amplifikaci, analýzu DNA, manipulaci s buňkami, průtokovou cytometrii a kapalinovou chromatografii [2]. Uplatnění zde nachází čipy, mikroprůtokové cely, digitální stopy či mikrosenzory. Použití materiálu a výrobní techniky se především řídí potřebami analýzy. V uplynulém desetiletí byly testovány nové materiály pro výrobu těchto zařízení, avšak nejvíce se osvědčilo použití skla nebo taveného křemenu a to především díky výborným elektroosmotickým vlastnostem a možností využití pro optickou detekci. Také polymerní materiály jako polydimetylsiloxan, poly(etylen tetraftalát), poly(-metlymetakrylát) a polykarbonát jsou pro tyto účely využívány díky snadné výrobě. Nevýhodou ovšem zůstává možnost adsorpce některých proteinů na povrch materiálu nebo interference surfaktantů s analyty [3]. Pro výrobu mikrofluidních zařízení bylo publikováno mnoho způsobů, z nichž nejvíce využívanými jsou mikroobrábění, lehká litografie, embosování, konstrukce in situ, vstřikování a laserová ablace [3].

Dosud jsou dostupné komerční zařízení, které již mnohé firmy nabízí a jsou plně využívány v bioanalytických analýzách. Avšak díky neustálému vědeckému pokroku je také třeba tyto systémy zlepšovat a vyvíjet nové materiály a postupy výroby tak, aby bylo co nejvíce zamezeno interferencím mikroprůtokového zařízení s analyty a tím dosaženo vysoké senzitivity a robustnosti tak malého zařízení.

Identifikace virových onemocnění

Viry jsou nebuněčné organismy, které obsahují pouze jediný typ nukleové kyseliny a to buď DNA, nebo RNA. Nejsou schopny se sami rozmnožovat, avšak pomocí proteosyntézy hostitelských buněk, které napadají, se mohou nekontrolovatelně rozšířit do celého organismu. Infekčnost virů spočívá v míře vyjádřit svoji genetickou informaci do hostitelské buňky [4]. V posledních letech byl zaznamenán nárůst virových onemocnění, které mohou být smrtelné pro děti, seniory či pro lidi s oslabeným organismem. Nebezpečí virových onemocnění spočívá v rychlosti množení a snadnou cestou přenosu mezi jedinci téhož druhu, ale i mezi jinými organismy. Proto ve vědecké sféře narůstá počet studií, které se zabývají kinetiku jejich aktivace [5] nebo rychlou detekcí jejich přítomnosti v organismu. V této souvislosti jsou vyvíjeny nové nástroje, které by tyto analýzy umožňovaly pokud možno v co nejkratší době a s nízkou spotřebou vzorků. Zmíněné nároky plně splňují mikrofluidní nástroje, které mohou být využity pro sledování exprese proteinů a které také umožňují řídit koncentraci virových částic působících na buňky a to za pomocí laminárního průtoku a difúze [6]. Pro separaci a detekci RNA virů byl využit plně integrovaný čip zahrnující zařízení pro kapilární elektroforézu, PCR s on-line detekcí poháněný peristaltickou mikropneumatickou pumpou. Díky propojení technologií bylo umožněno detekovat virus na základě PCR umístěného do čipu, díky čemuž byla umožněna kvantifikace virových částic [7]. V další studii byl mikročip pro analýzu DNA virů ze vzorků moči korelován s běžně dostupnými klinickými metodami [8]. Průtoková cytometrie je běžně využívanou metodou v klinické praxi pro detekci buněk nebo jiných biologických částic. Mikroprůtoková cytometrie, která umožňuje purifikaci a detekci virových částic byla spojena s imunoanalýzou využívající

magnetické částice. Do konstrukce čipu byl také vložen inkubační modul s pneumatickými mikropumpami, mikroprůtokový cytometrický modul a modul pro optickou detekci. Limit detekce metody byl stanoven na 10^{-3} PFU/ml [9]. Využití paramagnetických částic, na které se virus naváže, se v mirkofluidice stále více rozšiřuje a umožňuje detekovat virus v řádech pikogramů [10]. Mimo spektrofotometrické stanovení mohou být DNA čipy pro analýzu virů spojeny s elektrochemickou či fluorescenční detekcí [11]. Nejen nižší spotřebu chemikálií, ale také i rychlejší analýza byla dosažena pomocí mikrofluidního zařízení pro imunoanalýzu oproti běžné ELISE. Ze získaných výsledků bylo patrné, že výsledky klasické ELISY a mikrofluidního nástroje pro imunoanalýzu se shodují a jsou vhodné pro běžnou diagnostiku virových onemocnění [12]. Využití mikrofluidních systémů pro detekci virů jako je chřipka nebo ebola může probíhat také nepřímo, kvantifikací adeninu z přesně definoavné sekvence jejich genomu. K tomuto účelu může být využit mikroprůtokový systém injekční analýzy v zastaveném toku (Obr. 1) s odifikovanou pracovní elektrodou CuS nanočásticemi. Díky modifikaci, a využití malých objemů vzorku se tak jedná o velmi perspektivní metodu.



Obrázek 1: Mikroprůtokový systém SFIA. 1) nástřiková pumpa, 2) nástřikový ventil, 3) elektrochemická cela, 4) minipotenciostat

Závěr

Budoucnost a uplatnění mikroprůtokových zařízení je nyní velmi perspektivní oblastí výzkumu analytických a biologických oborů s cílem vyvinout tzv. laboratoř na čipu. Tyto systémy vynikají snadnou manipulací, nízkou spotřebou vzorků a rychlostí analýzy. Uvedené vlastnosti jsou velmi výhodné pro analýzu virových onemocnění, kde se předpokládá využití v komerční sféře.

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CHŘIPKA JAKO POTENCIONÁLNÍ PŮVODCE PANDEMIE, PREVENTIVNÍ OPATŘENÍ, DIAGNOSTIKA A LÉČBA

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Abstrakt

Vznik pandemie je jednou z největších světových hrozeb 21.století. Rostoucí mezinárodní obchod, letecká doprava a růst populace představují neustále se zvyšující riziko rozšíření jakékoliv infekční choroby.

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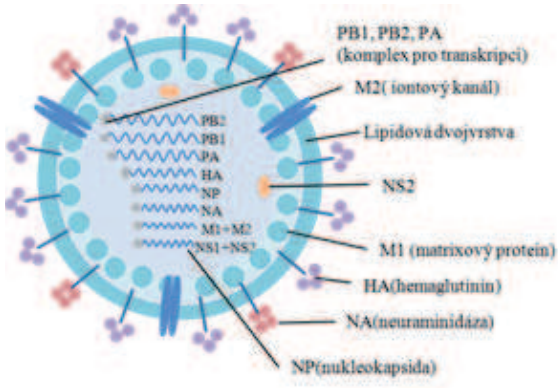
Úvod

Vznik pandemie je jednou z největších světových hrozeb 21.století. Rostoucí mezinárodní obchod, letecká doprava a růst populace představují neustále se zvyšující riziko rozšíření jakékoliv infekční choroby.

Běžná chřipka se vyznačuje poměrně vysokou nemocností a nízkou úmrtnostív běžné populaci. Ohrožené jsou rizikové skupiny, které jsou tvořeny malými dětmi, starými lidmi a pacienty s oslabenou imunitou[1]. Na druhou stranu je chřipka považována za jednoho z nejsilnějších členů skupiny potencionálních původců pandemie, díky konstantním mutačním změnám ve struktuře chřipkového viru, které jsou až stokrát intenzivnější než u ostatních virů (např.: Eboly). A právě rychlost a konstantnost mutačních má za následek to, že je chřipka považována za nejpravděpodobnějšího původce případné pandemie[2], která by mohla mít obrovské ekonomické i ekologické důsledky.



Obrázek 1: Schema chřipkového virionu

Chřipkové viry- taxonomie, struktura virionu

Chřipkové viry patří do čeledi Orthomyxoviridae, která se dále dělí na tři rody: chřipka typu A, B a C. Velikost chřipkového virionu (80- 120 nm) a jeho struktura (Obr 1) je u všech tří rodů téměř shodná, vzájemně se od sebe rody liší v počtu genomových segmentů, specifickém nukleoproteinu, hostitelské specifitě a virulenci (schopnost vyvolat onemocnění). Nejvíce virulentní je typ A, který se dále dělí na subtypy a to na základě různých kombinací dvou povrchových antigenů, kterými jsou hemaglutinin (HA) a neuraminidáza (NA)[3]. Oba hrají klíčovou roli v životním cyklu viru. HA je zodpovědný za vazbu na receptor hostitelské buňky (který je tvořený sialovou kyselinou), za fůzi membrán a průnik viru do hostitelské buňky. Neuraminidáza ma povahu enzymu, který štěpí sialovou kyselinu a účastní se uvolnění nově replikovaných virionů z hostitelské buňky[4]. Oba antigeny se vyskytují ve více subtypech HA (1-17) a NA (1-9). HA 17 byl objeven teprve v roce 2012 u kaloňovitých.

Historie chřipkových pandemií

V průběhu minulého století byla chřipková pandemie zaznamenána celkem třikát. V roce 1918 to byla Španělská chřipka, na kterou během pár měsíců zemřelo téměř 50 miliónů lidí (více obětí než si vyžádala AIDS v průběhu posledních dvaceti let)[5]. Dalšími dvěma pandemiemi byly Hong Kongská chřipka a Asijská chřipka, které vyžádaly asi milion obětí. Doba mezi jednotlivými pandemiemi se postupně zkracuje, z tohto důvodu můžeme další možnou pandemii očekávat téměř kdykoliv. V průběhu tohoto století byla možnost vzniku pandemie mediálně diskutována celkem dvakrát a to ve spojení s ptačí (2005) a prasečí (2009) chřipkou.V roce 2005 se poprvé objevil vysoce patogenní subtyp ptačí chřipky (HPAI), který je přenosný na člověka[6, 7]. Tento subtyp je přenosný pouze ze zvířete na člověka a vyznačuje se tudíž poměrně nízkou nemocností (nakaženo bylo asi 500 lidí), ale vysokou úmrtností téměř 60%[8]. U tohoto subtypu zatím nebyl prokázán přenos mezi lidmi[7]. Pokud by tento subtyp zmutoval a získal tuto vlastnost, mohli bychom se ocitnout na prahu nové pandemie. Možnou hrozbu vzniku pandemie opravdu nelze podceňovat.Dokonce i běžná chřipka se vyskytuje v pravidelných epidemiích, které mají sezóní charakter a propukají především v zimních měsících a to vždy s šestiměsíčním odstupem mezi severní a jižní polokoulí.

Protiepidemická opatření

Vakcinace, rychlá diagnostika a účinná léčba jsou základními podmínkami k tomu, abychom byly schopni předcházet vzniku a šíření epidemií.

Z pohledu kontroly vzniku onemocnění je nejúčinnějším opatřením vakcinace[9]. Vakcína proti chřipce existuje, ale je použitelná pouze jeden rok a to z důvodu mutačních změn, které změní v průběhu roku strukturu chřipkového virionu tak, že by opětovné použití stejné vakcíny i následující rok nebylo účinné.

K léčbě chřipky se používají dva typy antivirotik. Inhybitory neuraminidázy (NI - Zanamivir, Oseltamivir) a inhybitory M2 iontového kanálu (M2I – Amantadine, Rimantadine). Inhybitory neuraminidázy blokují uvolnění zreplikovaných virionů z hostitelské buňky a další šíření viru v organismu, inhybitor M2 kanálu stéricky bolukje iontový kanál a zabraňuje průniku protonů, čímž brání rozpadu virionu. Byla prokázána rezistence k oběma typům antivirotik [10]. Rezistence k Amantadinu dosahuje u některých subtypů až 90%.

Navzdory preventivním opatřením onemocní chřipkou miliony lidí ročně, z toho 250 - 500 000 pacientů zemře. Rychlost mutačních změn, rezistence k antivirotikům a neustálé riziko vytvoření nových a více nebezpečných subtypů s sebou přináší požadavky na rychlejší a citlivější metody detekce. Běžné metody, jako kultivace, RT-PCR a imunologické metody jsou často zdlouhavé, náročné na čas a vyžadují školený personál. V současné době se objevuje stále více metod, které jsou založené na principu biosensorů. Elektrochemické biosensory významně ovlivňují vývoj nových metod detekce infekčních agens. Jejich vysoká citlivosts možností miniaturizace, kompatibilita smoderními technologiemi, nízká cenaa možnost in situaplikacepředstavujívynikající vlastnostipro diagnostikupatogennícha geneticky vázaných nemocí[11].

Poděkování

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MICRO FLOW ANALYSIS OF INFLUENZA PROTEIN

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Abstract

We report a new three-dimensional (3D), bead-based microfluidic chip developed for rapid, sensitive and specific detection of influenza hemagglutinin. The principle of microfluidic chip is based on implementation of two-step procedure that includes isolation based on paramagnetic beads and electrochemical detection. As a platform for isolation process, streptavidin-modified MPs, x, which was conjugated via biotinylated glycan (by streptavidin-biotin affinity) followed by linkage of hemagglutinin to glycan, were used. Vaccine hemagglutinin (HA vaxi) was labelled by CdS quantum dots (QDs) at first. QDs-labelled HA can be specifically recognized and bound on the surface of the MPS modified with glycan. Detection of the isolation product by voltammetry on mercury and glassy carbon electrodes was the end point of the procedure. Proposed and developed method can be used also for detection of other specific substances that are important for control, diagnosis or therapy of infectious diseases..

Introduction

Potential pandemic viruses could have a great impact on a large amount of people [1]. Influenza is probably the most powerful member of the group of potential pandemic agents, because of the constant mutational changes in its surface antigens, hemagglutinin (HA) and neuraminidase (NA). They play opposite roles in the mechanism of their interaction with sialic acid (SA) receptors on a host cell [2].

HA is a trimeric glycoprotein expressed on the membrane of influenza virus [3]. It binds to SA receptors on the surface of the host cell and subsequently mediates fusion of the viral and target membranes [4]. Tropism and adaptation of influenza viruses to new hosts dependent partly on the distribution of the sialic acid receptors, which bind the viral HA [5]. Commonly, the human influenza virus prefers the SA-α-2,6-Gal terminal glycan, whereas the avian influenza virus prefer SA-α-2,3-Gal one [6]. Sialic acids (SAs) are located on the terminal positions of the glycan on a cell surface. These acids play important role in the spreading of infection and metastasizing of cancer [7].

HA is considered to be the main target for antibodies upon vaccination as well as native infection [8, 9]. Vaccination is the most effective prophylactic method against influenza [10]. Quantitative and qualitative analysis of vaccine antigens is a turning point before the vaccine is placed on the market and used for immunization [11].

Material and Methods

As the standard of Influenza A and B HA, was used influenza vaccine Vaxigrip (Sanofi Pasteur, France), which contains inactivated and split virions of the following strands: A/California/7/2009 (H1N1) – derived strain used NYMC X-179A, A/Perth/16/2009 (H3N2)-like strain used NYMC X-187 derived from A/Victoria/210/2009 and B/Brisbane/60/2008. Strains was propagated in fertilised hens´eggs from healthy chicken flocks. Vaxigrip contain 15 micrograms of all of three HA per 0.5ml.

CdS quantum dots (QDs) were prepared using a slightly modified version of a published method [12]. Cadmium nitrate tetrahydrate $Cd(NO_3)_2 \bullet 4H_2O$ (0.1 mM) was dissolved in ACS water (25 ml). 3-mercaptopropionic acid (35 µl, 0.4 mM) was slowly added to the stirred solution. Afterwards, the pH was adjusted to 9.11 with 1 M NH_3 (1.5 ml). Sodium sulphide monohydrate $Na_2S \bullet 9H_2O$ (0.1 mM) in ACS water was poured into the solution while vigorously stirring. The acquired yellow solution was stirred for 1 h. Prepared CdS-quantum dots were stored in the dark at 4 °C.

Vaccine was mixed with a solution of QDs (100 µl). This mixture was shaken for 24 h at room temperature (Vortex Genie2 (Scientific Industries, USA)). The volume of solution was reduced to 100 µl on an Amicon Ultra 3k centrifugal filter device (Millipore, Massachusetts, USA). Centrifuge 5417R (Eppendorf, Hamburg, Germany) was performed under the following parameters 15 min, 6000 rpm, 20 °C. The obtained concentrate was diluted with 400 µl of ACS water and reduced on centrifuge to 100 µl. The process was repeated 5 times. The washed sample was diluted to 300 µl and used for succeeding measurements

Results and Discussion

This work is focused on the designs of microfluidic systems that allow separation, elution and electrochemical detection. Four microfluidic chips for electrochemical analysis were designed (Figure 1D). The chip contained a conventional three-electrode detector. As a working electrode was used commercially produced carbon tip, the reference electrode was made of graphite filling (pencil leads) 0.5 mm in diameter. The auxiliary electrode was made of platinum wire. The differential pulse voltammetry (DPV) was used as the measuring method with the following parameters: initial potential -1.3 V, end potential -0.1 V, deposition potential -1.3 V, deposition time 35 s, modulation amplitude 0.1 V, step potential 0.005 V, scan rate 0.05 V/S. Acetate buffer (0.2 M, pH 5) was used as the supporting electrolyte. The experiments were carried out at 20 °C. The electrochemical signal was detected PGSTAT101 Autolab potentiostat (Metrohm, The Netherlands) and evaluated by the Software NOVA 1.8 (Metrohm, The Netherlands).

Procedure include two steps: isolation and electrochemical detection. 10 µl of Mps was dosed by the peristaltic pump (Pump Amersham Biosciences, Sweden). Using an external magnet was anchored in the reaction chamber and subsequently washed by 2.0 ml of (PBI). MPs were modified by biotinyled Glycan (50 µg/ml), folowed by washing

by 2.0 ml of (PBI). Last part of isolation was binding of HA-CdS on glycan modified MPs, again followed by washing with 2 ml of PBI, after that was chip with conjugated MPs immersed in an ultrasonic bath, where links between MPs and HA-CdS was disconected. Complex HA-CdS was detected electrochemically by Cd signal (figure 1C-a) .

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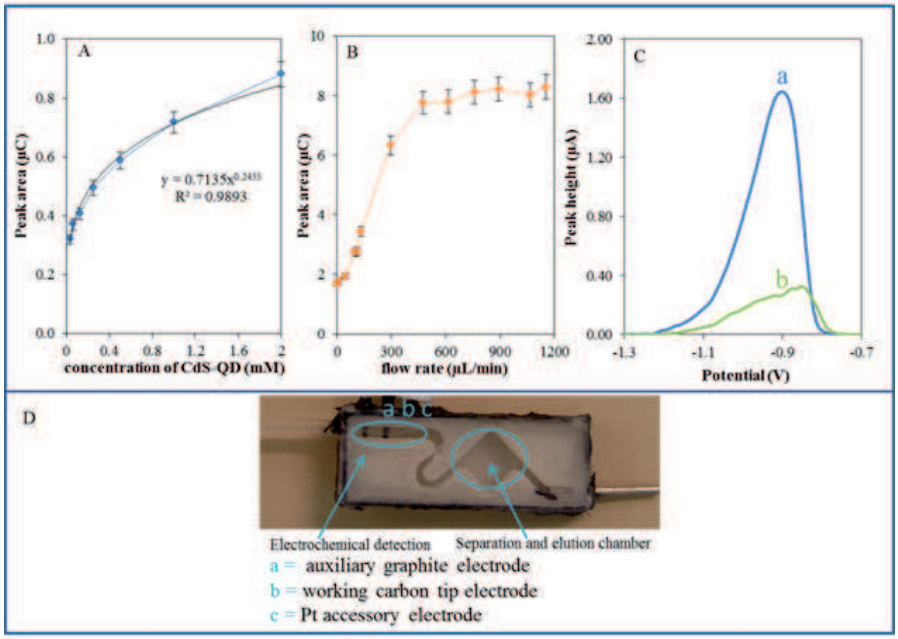

FIGURE 1: A) calibration curve CdS-QD (0.01- 2 mM). B) Optimization of the flow rate by a peristaltic pump (0 – 1150 µl/min). C) Voltammograms captured after elution haemagglutinin, a = CdS-HA (0.5 mM), b = 0.2 M acetate buffer pH . D) The microfluidic chip with electrochemical detection and separation - elution chamber.

Conclusion

In this work we managed to design and print three microfluidic 3D chips, that were equipped with electrochemical three-electrode detector. Thanks to 3D printing technology different shapes of microfluidic chips could be created, that may be used in various applications. Here presented chip was successfully tested in the analysis of influenza proteins.

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Spectrophotometric and fluorescence quantum dots interaction studies with viral protein

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Abstract

Present globalized world is full of dangerous. The result of population migration is increasing danger of flu pandemics. We could say with certainty that flu pandemic will come, but question is when? So we need to find faster and cheaper options for detection of virus in human population. In this study we have proof that flu protein hemaglutinin (HA) could create complex with CdS quantum dot (QD). This complex does the change of absorption (300-600 nm) and fluorescent (500-900 nm) spectra in time from 0 to 90 min of interaction. Thanks to QD could be HA detected by electrochemistry or some kinds of fluorescein analytical methods.

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Introduction

Growing globalization of the world, which has its share of increasing airplane transport of materials, food and people, brings with it a risk of spreading disease. The possibility of a global pandemic is thanks to airplane transport multiplied many times. Influenza is one of the most powerful potential epidemic agents, due to constant mutational changes in surface antigens hemaglutinin (HA) and neuraminidase (NA), which participate in life cycle of virus and have impact on virulence of the subtype of virus. The fluorescence labeling of viruses is also useful technology for virus detection and imaging [1]. A promising is usage the nanoparticles [5]. The nanoparticles with good fluorescence properties are quantum dots (QD) [2-6]. The attachment of influenza virus to susceptible cells is mediated by viral protein HA, which recognizes cell surface glycoconjugates that terminate in α-sialosides [7]. Thus formed complex (QD with HA) may be used for various applications. In this experiment, we focused on UV / VIS spectrophotometry and fluorescence monitoring interactions with HA-CdS QD.

Material and Methods

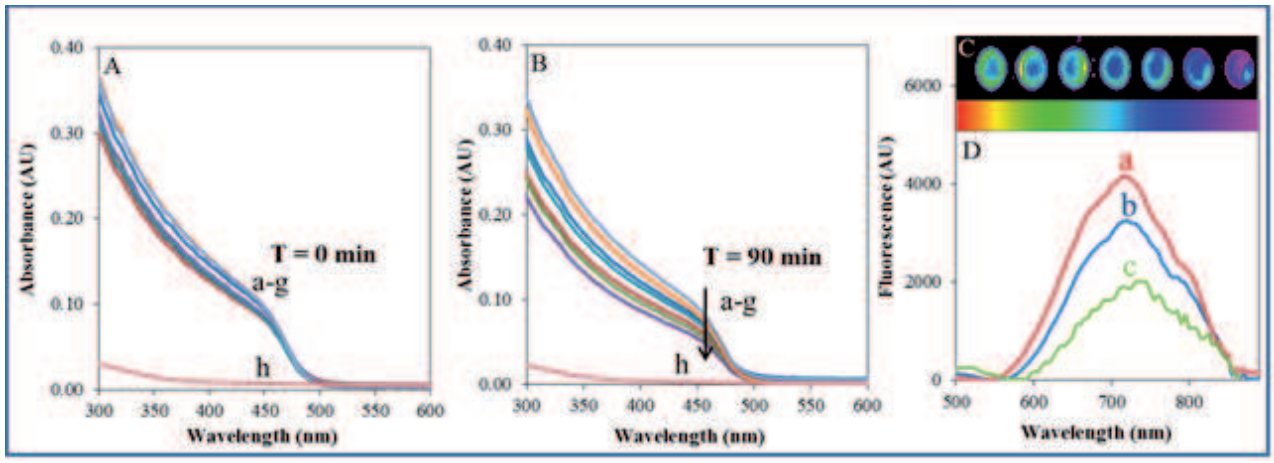
CdS quantum dots (QDs) were prepared using a slightly modified version of a published method [8]. Cadmium nitrate tetrahydrate Cd(NO3)2•4H2O (0.1 mM) was dissolved in ACS water (25 ml). 3-mercaptopropionic acid (35 µl, 0.4 mM) was slowly added to the stirred solution. Afterwards, the pH was adjusted to 9.11 with 1 M NH3 (1.5 ml). Sodium sulphide monohydrate Na2S•9H2O (0.1 mM) in ACS water was poured into the solution while vigorously stirring. The acquired yellow solution was stirred for 1 h. Prepared CdS-quantum dots were stored in the dark at 4 °C.

As the standard of Influenza A and B HA, was used influenza vaccine Vaxigrip (Sanofi Pasteur, France), which contains inactivated and split virions of the following strands: A/California/7/2009 (H1N1) – derived strain used NYMC X-179A, A/Perth/16/2009 (H3N2)-like strain used NYMC X-187 derived from A/Victoria/210/2009 and B/Brisbane/60/2008. Strains was propagated in fertilised hens´eggs from healthy chicken flocks. Vaxigrip contain 15 micrograms of all of three HA per 0.5ml.

Fluorescence and absorption spectrum were acquired by multifunctional microplate reader Tecan Infinite 200 PRO (TECAN, Switzerland). Absorption spectra were recorded in the range 300-600 nm. Fluorescence scan was measured within the range from 500 to 900 nm per 2-nm steps. 440 nm was used as an excitation wavelength. Each intensity value is an average of three measurements. The detector gain was set to 100. The sample (50 µl) was placed in transparent 96 well microplate with flat bottom by Nunc (Thermo Scientific, USA). All measurements were performed at 25 C °C controlled by Tecan Infinite 200 PRO (TECAN, Switzerland).

Results and Discussion

In this experiment we were focused on interaction of CdS-QD with HA and thanks to UV/VIS spectrofotometry (changes in absorption and fluorescent spectrum). First were the flu protein (HA) mixed with cadmium quantum dots (CdS-QD). By titration were prepared seven concentrations which included 1 mM CdS-QD with HA (0, 0.27, 0.54, 2.7, 5.4, 8.1, 10.8 and 13.5 mg/ml). Immediately after the mixing we do the measurement of absorption spectra in range from 300-600 nm (picture 1A) and fluorescent spectra in range from 500-900 nm. In 30 minutes intervals were measurement repeated. After this experiment we could say that CdS-QD and HA together creating a complex which causes decreasing the fluorescein spectra (300-600 nm) with higher application of HA (picture 1 B). Interaction of QD-CdS with HA causes also decreasing of fluorescein spectra how could we see on picture 1 D, a = 1mM CdS-QD, b = 1mM CdS-QD with 13.5 mg/ml of hemaglutinin in T = 0 min and c = 1mM CdS-QD with 13.5 mg/ml of hemaglutinin in T = 90 min.



Obrázek 1: A) Spectrofotometric record in range from 300 – 600 nm, a-g = 1 mM CdS-QD with 0-13.5 mg/ml hemaglutinin, h = 13.5 mg/ml hemaglutininu. Record were made immediately after mixing CdS with HA (T=0 min). B) Concentrations of reagents and conditions of measurement are same like in Fig A. Record were made after 90 minutes of interactions CdS-QD with HA (T=90 min). C) Intensity of fluorescence of 1mM CdS-QD with 0-13.5 mg/ml hemaglutinin were made by Carestream In-vivo Xtreme Imaging System. D) Fluorescent record CdS-QD with HA in range from 500 – 900 nm, a = 1mM CdS-QD s 13.5 mg/ml of hemaglutinin in time T = 0 min and c = 1mM CdS-QD s 13.5 mg/ml of hemaglutinin in T = 90 min.

Conclusion

In this experiment we were able to demonstrate that HA react with CdS-QD and create complex. Created complex we were able to configured the complex with absorption spectra (300-600 nm) and change of intenzity fluorescence (500-900 nm. This complex could be used for electrochemistry or fluorescein detection of HA with CdS-QD.

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Apoferritin and gold clusters formation

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Abstract

Apoferritin is a protein with cage like structure, which serves in most of living organisms for binding of excess iron ions. The protein is composed of 24 polypeptide subunits that form structure with cavity about 8 nm in diameter. However, it was found that the cavity can be utilized for storage of other ions or molecules and can be utilized for synthesis of nanoparticles with defined size. Moreover, the surface of apoferritin can be modified. This offers further possibility of delivering encapsulated drug to a target cell in more effective way and minimalizing thus side effects particularly toxicity to nontarget organs by drugs. In this work we report on current state of art in preparation and study of gold nanoparticles in apoferritin structure and their application in medicine and imaging.

WHD_07_2013

Introduction

Apoferritin is composed of 24 subunits of two types called H as heavy or heart of molecular weight 22 kDa and L subunits also as light or liver with molecular weight of 19 kDa.[1] These subunits assemble into a protein with outer diameter of 12 nm and inner sphere of 8 nm. The formation of apoferritin molecule is pH dependent. When pH is lower than 3, molecule disassembles and reassembles again when pH is above 5. There are 14 channels in the structure of apoferritin, among them six are hydrophobic and eight are hydrophilic. The flow of ions into the hollow core takes place through these specific channels at the interface of the apoferritin protein subunits. There are a large number of aspartate and glutamate residues on the inner surface of the apoferritin which probably cause bounding and the formation of metallic compounds in the cage.[2]

As gold nanoparticles show biological activity, there are papers dealing with their preparation and characterization with apoferritin combination. Dmochowski et al. used reaction between either $AuCl_4$- or neutral $AuCl_3$ and apoferritin, but they observed gold deposition on the outside of the protein only. They used $NaBH_4$ or 3-(N--morpholino)propanesulfonic acid as the reducing agents. $AuCl_4$- reduction by $NaBH_4$ was complete within a few seconds, whereas reduction by the second agent was much slower. Transmission electron microscopy (TEM) showed that the gold nanoparticles were associated with the outer surface of the protein. The average particle size diameters were 3.6 and 15.4 nm for $NaBH_4$ and 3-(N-morpholino) propanesulfonic acid reduced gold, respectively.[3]

Similarly, human apoferritin was modified by removing gold-binding amino acids, such as cysteine and histidine, from the outer surface and by lining the interior surface with cysteine residues. Gold nanoparticles were also incorporated inside the cavity of this modified apoferritin by the addition of $AuCl_3$ reduced by 3-(N-morpholino)propanesulfonic acid.[4] Except of horse and human apoferritin, hyperthermophile Archaeoglobus fulgidus apoferritin with gold nanoparticles was obtained by the same group of authors. They report on negatively charged colloidal gold in the cavity of apoferritin proved by transmission electron microscopy.[5]

Novel strategy to prepare gold nanoparticles inside apoferritin was described by Orner et. al.[6] Gold ions have a poor natural preference for the apoferritin interior over that of the exterior, which thereby favors nanoparticle formation on the outside of the cage. Therefore, to encourage formation of particles only on the inside, they designed a strategy to trap a small number of gold ions inside the apoferritin cavity and then isolate the encapsulated gold away from solution gold, which would have had the potential of mineralizing on the outside of the protein. Subsequently the entrapped gold ions could be rapidly reduced by $NaBH_4$ to form gold nanoparticles. Then in a second reduction step, additional gold ions could be added along with a weak reductant such as ascorbic acid that preferentially permits mineralization on the nanocluster seed. The nanoparticle grows until it matches the size of the inner cavity.

A strategy to assemble two gold nanoclusters at the ferroxidase active sites of apoferritin was designed. (Scheme 1.)The studies showed that the resulting nanostructures retain not only the intrinsic fluorescence properties of noble metal, but gain enhanced intensity, show a red-shift, and exhibit tunable emissions due to the coupling interaction between the paired Au clusters. Furthermore, nanostructure showed organ-specific targeting ability, high biocompatibility, and low cytotoxicity. The study shows that an integrated multimodal assembly strategy is able to generate stable and effective biomolecule-noble metal complexes of controllable size and with desirable fluorescence emission characteristics. Such agents are ideal for targeted in vitro and in vivo imaging with great potential for biomedical applications.[7]

Scheme 1: Preparation of gold clusters in apoferritin cavity

Results and Discussion

Preparation of gold clusters. All the reagents were supplied by Sigma-Aldrich. Horse spleen apoferritin 20 µl (1 mg) was diluted with water (300 µl). $HAuCl_4$ (1 mM, 100 µl) was added (pH = 5). 1 µl 1M NaOH was added with the raise of pH to 9.36. The mixture was shaken on Vortex for 10 min. After that 4 µl of 1 M NaOH was added to raise pH to 12. The solution was heated at 40 °C for 48 h. After cooling, the solution was washed several times with water on Amicon 3k. The final volume of sample was 1 ml. Content of gold in apoferritin was proved by measurement on AAS. It was found that all gold was in apoferritin cavity. The emission spectra show bands at 310 and 700 nm, respectively. The fluorescence intensity and far red or near infrared emissions are perfect for tissue imaging applications where low background autofluorescence and enhanced tissue penetration are desirable characteristics.

Conclusion

Apoferritin can be used as a nanoreactor for preparation of gold clusters that are biocompatible and thus can be exploited in medicine and imaging. Their kidney specific targeting was proved. In the future cytotoxicity of the clusters could be also used for targeting of other organs.

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Quantum dots and the possibilities of analysis by atomic absorption spectrometry

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Abstract

Quantum dots containing different elements are succesfully applied in current research for labelling of biomolecules. The fluorescence properties of these bioconjugates are studied and the results contribute to better understanding of different biological processes. Atomic absorption spectrometry provides determination of element, especially metal included in the bioconjugates therefore it is used for analysis of various conjugates of quantum dots with biomolecules.

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Introduction

Semiconductor nanoparticles (also called quantum dots, QDs) have been extensively studied considering the application as fluorescent probes in biological staining and diagnostics. Nanoparticles are usually conjugated to highly specific biomolecules like antibodies, oligonucleotides, enzymes or aptamers to improve assay selectivity. Compared with conventional fluorophores, the nanocrystals have a narrow, tunable symmetric emission spectrum and are photochemically stable [1, 2]. QDs still present better photostability than organic dyes in the application of fluorescence analysis, so that they can be exposed to many cycles of excitation but still maintain an excellent fluorescence signal. QDs have a longer emission lifetime and more robust signal intensity than organic dyes [3]. The typical diameter of QDs is in the 1–20 nm range and they can contain from 100 to 100,000 atoms per nanoparticle. QDs can be synthesised with binary alloys of atoms from 12–16 (ZnS, CdS, CdSe, HgS), 13–15 (GaAs, InP, InAs, GaN) or 14–16 (PbTe, PbSe) groups. Ternary alloys of CdZnS, CdSSe, InNP or InGaAs have been also synthesised with analogous properties [4]. The advantageous properties of QDs have led them becoming of great interest for biological labeling. QDs are used in various areas of biological research, such as targeted drug delivery systems, cellular labelling, detection of site-directed mutagenesis, and molecular therapeutics. Bioconjugating with various biomolecules provides hybrid materials that combine the unique optical and magnetic properties of nanoparticles with the specific and selective binding behavior of biomolecules. Bioconjugated QDs could be powerful tool for biosensor, bioanalytical methods and for sensitive detection in relation to health, food, water and environmental safety measures [5].

Results and Discussion

Due to the presence of metal in the structure of QDs the total content of this metal in the sample is possible to determine by several analytical methods. Electrochemical methods were successfully applied for metal included in the structure of conjugated QDs with biomolecules [6-8]. For metal determination different methods of atomic spectroscopy can be used. One of them is atomic absorption spectrometry (AAS), the method based on the measurement of the energy absorbed by free atoms during the excitation process. As the radiation source the hollow cathode lamp is used, which produces the monochromatic light of specified wavelength for each element. It allows the specific determination of individual elements in the presence of others. Two systems are commonly used to produce atoms from the sample: flame and electrothermal atomization. Flame AAS is intended for analyte in the concentration of tenths to tens mg/l, electrothermal AAS tens ng/l to tens µg/l. Atomic absorption spectrometry can be used for determination of elements in the aqueous solution of QDs after the synthesis. After preparation of the solution analyte can be determined directly or the solution must be purified by dialysis or precipitation. The amount of cadmium was determined by flame AAS in the aqueous solution of CdS capped with 3-mercaptopropionic acid or glutathione. In the case of conjugated QDs with biomolecules, where the small amount of the sample is available, it is advantageous to use electrothermal AAS for total content of the element determination. This method requires only 10-20 µl. The sensitive method of electrothermal AAS provides for example determination of Cd, Zn, Pb or Au in the samples of labeled biomolecules (DNA, proteins).

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Interakce kvantových teček s metalothionem

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Abstrakt

Interakce anorganických nanočástic s biomolekulami (proteiny, DNA) může být potenciálně využita pro diagnostiku v medicíně a návrh následné léčby. Z tohoto důvodu se tato studie zabývá interakcí nanočástic s proteinem a jsou zkoumány vzniklé komplexy MT- QDs. Konkrétně byly vybrány kvantové tečky CuS a protein metalothinein. Interakce těchto dvou komponentů byla studována v čase 2 – 6 hodin a hodnota signálu vzniklých komplexů (Cat2, Cat1, RS2Co, Y, X) byla odečtena.

WHD_09_2013

Úvod

Metalothionein (MT) je nízkomolekulární protein bohatý na cysteiny. MT zajišťuje ochranu proti působení těžkých kovů (Hg, Cd, Pb, Cu) a účastní se udržování intracelulární homeostáze zinku [1]. MT má antioxidační účinky, vstupuje do oxidačně-redukční rovnováhy uvnitř buněk, působí na transport kovů iontů a reguluje expresi řady významných genů. Metalothionein hraje důležitou roli při nádorovém bujení [2] a znečištění životního prostředí [3].

Kvantové tečky (QDs) jsou částice o velikosti 3-11 nm^2 [4] složené z kovové části (Cu, Cd, Pb, Zn) a nekovové části (S, Te, Se). Tyto částice jsou vodivé a mají fluorescenční vlastnosti; jsou používány v aplikacích od bio-imagingu po chemické sensory [5].

V této práci byla elektrochemicky studována interakce CuS s metalothionei-nem pomocí diferečí pulzní voltametrie v Brdičkově elektrolytu.

Chemikálie a metody

Metalotionen byl připraven izolací z 2 g rozmělněných králičích jater, která byla dále na ledu homogenizována pomocí Ultra-Turrax T8 (Scholler instruments) v 8 mL 10 mM Tris–HCl pufru (pH 8.6). Tato směs byla poté vortexována (Vortex Genuie, Germany) a centrifugována (Universal 320, Hettich Zentrifugen, Germany) při 5 000 rpm po dobu 30 min při 4 °C. Supernatant byl zcentrifugován (Eppendorf centrifuga 5417R) v mikro-zkumavce při 4 °C (25 000 rpm, 30 min). Supernatant byl zahřát v termomixéru (Eppendorf thermomixer comfort, Germany) při 99 °C (10 min) a zcentri-fugován (Eppendorf centrifuga 5417R, Germany) v 1.5-mL microzkumavkách při 4 °C (25 000 rpm, 30 min). Takto připravený vzorek byl použit při studiu interakce MT s kvantovými tečkami.

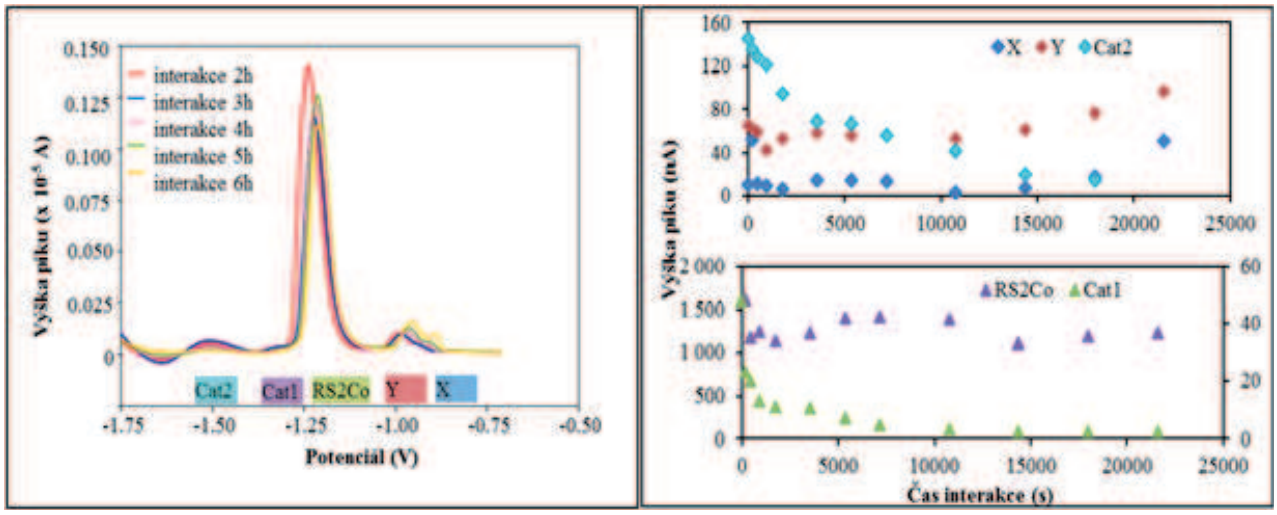
CuS kvantové tečky (CuS QDs) pokrytých merkaptosukcinovou kyselinou (MSA) byly připraveny reakcí $Cu(OAc)_2 \bullet H_2O$ (0,02 g, 0,1 mM) rozpuštěného v ACS vodě (25 ml) s merkaptosukcinovou kyselinou (0,08 g, 0,53 mM). 0,5 ml 1M NH_4OH byl přidáván do získání žlutého roztoku. Následným přidáním $Na_2S \bullet 9H_2O$ (0,012 g, 0,05 mM) v 24,5 ml of ACS vodě dojde ke změně barvy na světle hnědou.

Ke stanovení interakce CuS QDs s MT bylo využito elektrochemické stanovení pomocí Brdičkova roztoku diferenční pulzní voltametrií. Stanovení metalothio-neinu probíhalo pomocí přístroje 747 VA Stand ve spojení s 693 VA procesorem a 695 autosamplerem (Metrohm, Švýcar-sko). Při měření bylo použito standardní tříelektrodové zapojení spolu s chlazením vzorků na 4°C (Julabo F25, JulaboDE). Jako pracovní elektroda byla zvolena rtuťová kapková elektroda s povrchem kapky 0,4 mm^2, argentchloridová (Ag/AgCl/KCl 3 M) elektroda sloužila jako referentní, platinová elektroda byla elektrodou pomocnou. Parametry stanovení jsou následující: čas interakce 2, 3, 4, 5 a 6 hodin, počáteční potenciál -0,7 V, koncový potenciál -1,75 V, čas modulace 0,057 s, časový interval 0,2 s, krokový potenciál 2 mV, amplituda -250 mV, čas probublávání 99,9% argonem 120 s, objem vzorku 20 µl (MT : QDs 1:1), objem měřící nádobky 2 ml (20 µl vzorku + 1980 µl Brdičkova roztoku). Naměřená data byla vyhodno-cena v programu GPES 4.9.

Výsledky a diskuze

Interakce mezi CuS QDs a MT byla studována v interakčním case 2, 3, 4, 5 a 6 hodin DPV Brdičkovou reakcí. Tato reakce má katalytický mechanismus, kdy získaný signál, resp. píky, odpovídají vytvořeným komplexům při interakci Co (III) v elektrolytu s HS- skupinou v MT [6]. Naměřené voltamogramy z interakce MT s CuS QDs jsou zobrazeny v **Obr. A**. Voltamogramy obsahují pět characteristic-kých signálů. RS2Co (-1,24 V), Cat1 (-1,35 V) a Cat2 (-1,52 V) jsou signály spojené se samotným MT.

Vzniklé píky X (-0.94 V) a Y (-0.99 V) souvisí pravděpodobně s interakcí CuS QDs s metalothioneinem. Je zřejmé, že s prodloužením času interakce klesá pík Cat2, jehož výška odpovídá koncentraci MT, a naopka stoupá pík X a Y (**Obr. B, C**). Pravděpodobně dva poslední píky souvisí s kovouvou částí v QDs.



Obrázek A, B, C: A – DP voltamogram interakce QDs s MT při různých interakčních časech. B- Odečtena výška píku při dané interakci pro pík X, Y a Cat2. C- Odečtena výška píku při dané interakci pro pík RS2Co a Cat1.

Závěr

Cílem této práce bylo charakterizovat jednoduchý a levný systém pro identifikaci nádorového onemocnění. K tomuto jsou v posledních letech využívány různé nanočástice (kvantové tečky, nanotrubice, atd.). V nasí práci jsem se zabývali charakteristikou interakcí za vzniku komplexů CuS QDs-metalothionein. Výsledky získané při interakci dalších QDs budou prezentovány v budoucnosti.

Poděkování

Práce na projektu byla podpořena projekty NANOLABSYS CZ.1.07/2.3.00/20.0148 a CEITEC CZ.1.05/1.1.00/02.0068.

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MALDI-TOF-MS analysis in influenza viral proteins

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Abstract

The timely and accurate diagnosis of specific influenza virus strains is crucial to effective prophylaxis, vaccine preparation and early antiviral therapy. The influenza A genome contains 11 genes on eight pieces of RNA, encoding for 11 proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2 (NEP: nuclear export protein), PA, PB1 (polymerase basic 1), PB1-F2 and PB. Each seasonal influenza vaccine contains peptides or proteins representing three or four influenza virus strains. In this study, we show that Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) is a valuable tool for the analysis of peptides and proteins in influenza vaccine.

WHD_10_2013

Introduction

Mass spectrometry has become a fundamental technology for virology and an important tool in probing the structure and function of viruses. It has been used to identify viral capsid proteins, detect viral mutants, characterize post-translational modifications, and measure intact viruses [1]. Mass spectrometry has been highly successful for identifying all classes of proteins including those originating from viral capsids and membrane proteins from enveloped viruses using existing genomic databases. Protein identification generally involves purification (commonly performing using gel electrophoresis), proteolytic digestion, and mass analysis. The development of soft ionization and desorption of biological macromolecules, electrospray, and MALDI-TOF-MS was the most important step for using MS in biology and medicine [2, 3]. The influenza A virion, which is a typical representative of the family Orthomixoviridae, includes eight segments of single stranded negative sense RNA in a complex with nucleoprotein (NP) and polymerase complex proteins (PB1, PB2, PA). The virion envelope includes two glycoproteins: the major hemagglutinin (HA) responsible for the virus entry into the cell and the enzyme neuraminidase (NA), as well as the minor protein M2, which is an ion channel. Under the lipid membrane there is a layer of molecules of the main structural protein, matrix protein M1. Some copies of protein NS2 (NEP) are also found in virions [4]. We describe in this short communication the method for determination and/or identification hemagglutinin in vaccine by MALDI-TOF.

Material and Methods

The HA proteins of H5 type were prepared using the vaccine, which contains protein or fragments of different influenza viruses HA. The sample of vaccine was mixed with fresh sinapinic acid (10 g/L) in a 1:1 (v/v) ratio. The complex was directly deposited onto the sample plate, dried in air, and then subjected to analysis on a MALDI--TOF/TOF (Chou, Hsu et al. 2011). To obtain a stable signal, a typical mass spectrum was constructed by averaging 1000 laser shots. The MALDI-TOF MS analysis was acquired with an laser (355 nm) operating at a repetition rate of 200 Hz. The spectra were recorded in

the linear mode using an accelerating voltage of 20 kV. Spectra were analyzed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitine, and cytochrome c.

Results and Discussion

The MALDI-TOF spectra showed three distinct peaks corresponding to the different fragments of HA vaccine (**Figure 1**). MALDI-TOF/TOF was used to mass spectra characterization of fragments or total protein of different influenza viruses HA in vaccine. The Figure 1. represents the spectra of each protein in vaccine. The signals shown in Figure 1.A. were assigned as follows: $[A]^+$ (m/z 27686.3), $[B]^+$ (m/z 47958.36) and $[C]^+$ (m/z 55763.27). These results were compared with the results obtained in SDS-PAGE gel, showing the similar results.

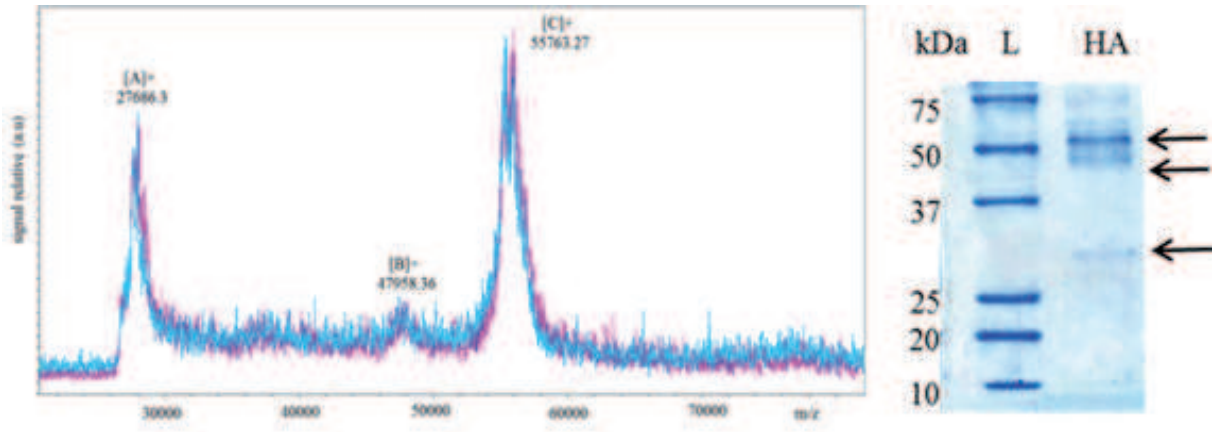

Figure 1: A) Spectra HA protein in influenza vaccine by MALDI--TOF-MS. B) Separate proteins according to their size in influenza vaccine by SDS-PAGE

Conclusion

Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) is a valuable tool for the analysis of peptides and proteins. Particularly useful features include high sensitivity, fast data acquisition, ease of use, and robust instruments for detection of protein in virus.

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Cloning in viral research

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Abstract

Due to the great advances in molecular biology, any virus can now be recovered from the nucleotide sequence and cloned in plasmid vectors. Viral generation requires in-depth knowledge in a wide variety of fields like molecular biology, cell biology, virology and biochemistry.Breakthroughs in the area the cloning of human viruses are helping in the development of antivirals and vaccines for life-threatening diseases.In the past, studies concerning human viruses have been hard to conduct as it is a very difficult to sustain the virus outside of the human body. Thus, being able to clone a virus will help scientists to work towards creating antivirals and vaccines.The complete genomes of many DNA viruses, including polyomaviruses, papillomaviruses, and adenoviruses, are sufficiently small to be carried in plasmid vectors. However, conventional plasmid vectors cannot accommodate the larger DNA genomes of herpes or poxviruses; therefore cosmids and bacterial artificial chromosome vectors, which can accept larger inserts, have been used. Such vectors have also been used to carry DNA copies of the largest RNA genomes, those of members of the Nidovirales.

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Introduction

A virus is a small infectious agent that can replicate only inside the living cells of an organism. Viruses can infect all types of organisms, from animals and plants (Eukaryota) to Bacteria and Archaea, indicating an origin very early in the evolution (Peters, Jabra-Rizk et al. 2012; Nishida 2013).

Viruses are found in almost every ecosystem on Earth. The origins of viruses in the evolutionary history of life are unclear: some may have evolved from plasmids pieces of DNA that can move between cells while others may have evolved from bacteria. In evolution, viruses are an important means of horizontal gene transfer, which increases genetic diversity (Nishida 2013).

Currently, ICTV (International Committee on Taxonomy of Viruses) divides viruses in 6 orders, 87 families, 349 genera and over 2284 species according to the Baltimore system or other criteria. Viruses are classified on the basis of morphology, chemical composition, and mode of replication (Kuhn and Jahrling 2010).

Viruses consist of a single long molecule of RNA or DNA, which carries the genetic information, a protein coat that protects these genes and in some cases an envelope of lipids that surrounds the protein coat when the viruses are outside a cell (Stagno, Ma et al. 2012; Terry, Marine et al. 2012). The shapes of viruses range from simple helical and icosahedral forms to more complex structures (Massiah, Starich et al. 1994; Kononchik, Nelson et al. 2009).

For propagation viruses depend on specialized host cells supplying the complex metabolic and biosynthetic machinery of eukaryotic or prokaryotic cells (Iwasaki 2012). A complete virus particle is called a virion. The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be expressed (transcribed and translated) by the host cell (Alizon and Magnus 2012; Tandon and Mocarski 2012). The most important methods of detection are Immunodetection, ELISA, PCR, electron microscopy, and serological methods (Guesdon 1992; Goldsmith and Miller 2009; Guy, Gold et al. 2009).

Some viruses are closely related to oncogenic processes. Most studied viruses can modify the programmed cell death (apoptosis) (Banerjee, Ray et al. 2010; Ewald and Ewald 2012).

Viral infections in animals provoke an immune response that usually eliminates the infecting virus. Immune responses can also be produced by vaccines, which confer an artificially acquired immunity to the specific viral infection (Moyle and Toth 2013; Nabel 2013). However, some viruses including those that cause AIDS and viral hepatitis evade these immune responses and result in chronic infections. Antibiotics have no effect on viruses, but several antiviral drugs have been developed (Fashner, Ericson et al. 2012).

The great advances in molecular biology make it possible that any virus can be recovered from its nucleotide sequence and cloned in plasmid vectors (Bedoya and Daros 2010; Wasala, Shin et al. 2011).

Digital genetic sequences obtained from DNA from virus sequencing may be stored in sequence databases, be analyzed, digitally altered and used as templates for creating new actual DNA using artificial gene synthesis (Gibbs and Ohshima 2010).

The analysis of expressed sequence tags (EST) offers a rapid and cost effective approach to elucidate the transcriptome of an organism, but requires several computational methods for assembly and annotation. Recently, EST Explorer has been developed, a semi-automated computational workflow system, in order to achieve the rapid analysis of EST datasets. In this study, we evaluated EST data analysis for the organism using EST Explorer, compared with database (Nagaraj, Deshpande et al. 2007; Wise, Moscou et al. 2007; De Luca, Salim et al. 2012).

We evaluated the efficacy of EST Explorer in analysing

EST data, and demonstrated that computational tools can be used to accelerate the process of gene discovery in EST sequencing projects. The annotated EST set provides further insight into the molecular biology towards the identification of genes (Nagaraj, Deshpande et al. 2007; Ma, Wang et al. 2011).

Molecular cloning is a set of experimental methods in molecular biology which are used to assemble recombinant DNA molecules and to direct their replication within host organisms (Chao 2007). The cloning refers to the fact that the method involves the replication of a single DNA molecule starting from a single living cell to generate a large population of cells containing identical DNA molecules (Altmiller 1986). Molecular cloning generally uses DNA sequences from two different organisms: the species that is the source of the DNA to be cloned, and the species that will serve as the living host for replication of the recombinant DNA (Chong 2001; Baker, Schountz et al. 2013).

Molecular cloning takes advantage of the fact that the chemical structure of DNA is fundamentally the same in all living organisms.

Therefore, if any segment of DNA from any organism is inserted into a DNA segment containing the molecular sequences required for DNA replication, and the resulting recombinant DNA is introduced into the organism from which the replication sequences were obtained, then the foreign DNA will be replicated along with the host cell's DNA in the transgenic organism (Altmiller 1986; Chong 2001).

In a conventional molecular cloning experiment, the DNA to be cloned is obtained from an organism of interest. Subsequently, these fragments are combined with vector DNA to generate recombinant DNA molecules. The recombinant DNA is then introduced into a host organism (laboratory strain of E. coli bacteria) (Di Napoli, Maltese et al. 2004). This will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. Because they contain foreign DNA fragments, these are transgenic or genetically modified microorganisms (GMO) (Vakhlu, Sudan et al. 2008). This process takes advantage of the fact that a single bacterial cell can be induced to take up and replicate a single recombinant DNA molecule. This single cell can then be propagated exponentially to generate a large amount of bacteria, each of which contains copies of the original recombinant molecule. Thus, both the resulting bacterial population and the recombinant DNA molecule are commonly referred to as "clones" (Altmiller 1986; Balbas and Gosset 2001).

A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes (Balbas and Gosset 2001; Silva, Queiroz et al. 2012).

There are many types of cloning vectors, but the most commonly-used ones are genetically engineered plasmids. Cloning is generally first performed using Escherichia coli, and cloning vectors used in E. coli include plasmids, bacteriophages (such as phage λ), cosmids, and bacterial artificial chromosomes (BACs) (Adler, Messerle et al. 2003). Genetic investigation of cytomegaloviruses (CMV) has improved due to the cloning of CMV genomes into bacterial artificial chromosomes (BAC) (Borst, Hahn et al. 1999).

This technique greatly facilitates genetic manipulation of viral genes in the context of the viral genome, enables amplification of the genome in the absence of selective pressure, and yields clonal viral genomes without the need of plaque purifications (Borst and Messerle 2000).

Some DNA however cannot be stably maintained in E. coli, for example very large DNA fragments, and other organisms, such as yeast, may be used. Cloning vectors in yeast include yeast artificial chromosomes (YACs) (Noskov, Chuang et al. 2011).

All commonly-used cloning vectors in molecular biology have key features necessary for their function such as a suitable cloning site and a selectable marker. Others may have additional features specific to their use. For reasons of ease and convenience, cloning is often performed using E.coli, the cloning vectors used therefore often have elements necessary for their propagation and maintenance in E. coli such as a functional origin of replication (Balbas and Gosset 2001).

All cloning vectors have features that allow a gene to be conveniently inserted into the vector or removed from it. This may be a multiple cloning site (MCS) which contains many unique recognition sites for restriction enzymes (Zaleski, Wawrzyniak et al. 2012). The restriction sites in the MCS are first cleaved by restriction enzymes, and a PCR-amplified target gene, also digested with the same enzymes, is then ligated into the vectors using DNA ligase. The target DNA sequence can be inserted into the vector in a specific direction (Chande, Raina et al. 2013).

Other cloning vectors may use topoisomerase instead of ligase and cloning may be done more rapidly without the need for restriction digest of the vector or insert.

In this TOPO cloning method a linearized vector is activated by attaching topoisomerase I to its ends, and this vector may then accept a PCR product by ligating both the 5 ends of the PCR product, releasing the topoisomerase and forming a circular vector in the process (Xiao, Xin et al. 2007).

In order to facilitate the screening to find a successful clone, some cloning vectors contain features that allow successful clone to be identified. A selectable marker is carried by the vector to allow the selection of positively transformed cells. Antibiotic resistance is often used as marker. Some vectors contain two selectable markers, and a shuttle vector, which is designed to be maintained in two different organisms, would also require two selectable markers (Chong 2001; Xiao, Xin et al. 2007; Goto and Nagano 2013).

Such features present in cloning vectors may be the lacZα fragment for α complementation in blue-white selection, and/or marker gene or reporter genes in frame with and flanking the MCS to facilitate the production of fusion proteins.

Examples of fusion partners that may be used for screening are the green fluorescent protein (GFP) and luciferase (Tanuma and Shiokawa 1999; Chao 2007).

In expression vectors, the target DNA may be inserted into a site that is under the control of a particular promoter necessary for the expression of the target gene in the chosen host. Where the promoter is present, the expression of the gene is preferably tightly controlled and inducible so that proteins are only produced when required. Some commonly used promoters are T7 promoters and lac promoters (Sawers and Jarsch 1996; Ohkouchi, Koshikawa et al. 2000).

The goals of viral research are to identify molecular determinants that affect the transmission or pathogenesis of the virus, and to use this information to create and develop vaccines and antivirals. Advances in molecular genetics, and biotechnology have afforded the development of techniques of DNA recombinant being utilized in virus characterization and in the production of specific viral antigens that can be applied to the production of vaccines (Borst and Messerle 2000; Williams, Carnes et al. 2009).

The large genome size of some viruses has hindered the development of techniques for the production of vaccines this issue has been addressed by a unique cloning strategy that allows for assembly of the genome in an ordered fashion, leading to the generation of an infectious virus. This technology provides a powerful tool in the development of vaccines, antivirals, and biologics for various strains of viruses. Furthermore, this technology can be utilized to assemble any virus with a large RNA genome including SARS CoV, Hepatitis C, and Yellow Fever Virus (Moyle and Toth 2013; Zeltins 2013).

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TRENDS IN MOLECULAR MEDICINE FOR PREPARATION OF BACTERIOPHAGE λ VIRAL TRANSPORTERS

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Abstract

Nanomedicine is an upcoming field of medicine today. An attention is paid towards gene, drug and DNA vaccine delivery. All of these methods require the use of a transporter. Bacteriophages seem as suitable transporters, because they cannot amplify in eukaryotic cells and are metabolically inert. Bacteriophage λ is very stable both to extreme pH and temperatures, co it can be efficiently used also in Developing World.

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Introduction

Bacteriophages are bacteria viruses that require the host bacterium for growth and propagation. Due to the fact that bacteriophages are metabolically inert and cannot replicate in eukaryotic cells, they can serve as transporters for many uses, such as DNA vaccine [1], gene [2], or drug delivery.

Well known is bacteriophage λ whose normal host is intestinal bacterium Escherichia coli. Its phages are exhibiting enhanced stability both in gastrointestinal tract and lymph [1, 3, 4]. Bacteriophage λ also has high thermostability, which makes him suitable for use in Developing World. It is also resistant to desiccation and freeze-thaw cycles [1]. Bacteriophage λ is filamentous phage, it can therefore replicate and assemble without killing the host bacteria [5].

Gene delivery

Gene therapy is efficient targeting of therapeutic gene to specific cell or tissue [2, 6]. It can be used not only as therapeutic method, but also in disease prevention by replacing disease-causing alleles [7]. It is possible to target retroviral vectors [8], adenoviral vectors [9] or non-viral vectors [10]. However, these methods are applicable only with targeting molecules that bind a cell-surface receptor [2]. Thus, new methods were invented using peptides and antibody fragments directly from filamentous phage libraries [2, 11], where displayed targeting ligand determine the tropism for mammalian cells [12]. Bacteriophages have an ability to undergo receptor-mediated endocytosis, so they can be used both for cell binding and internalization into mammalian cells [13]. Though bacteriophages are prokaryotic viruses, it is possible to re-engineer them to infect eukaryotic cells, thus express genes inserted into phage genome [2].

Due to the stability of bacteriophage λ, it can be selected by affinity for a target protein under harsh conditions, such as chaotropic agents, proteases or extreme pH (from 3 to 11 for up to 24 hours) [1, 12]. It also has high serum half-life (especially variants with mutations in their D and E capsid proteins) [14] and it can be stored for up to 6 months at 4 °C in water without losing its infectivity [1].

Bacteriophage λ can hold 53 kb, which is more than most viral vectors (up to 10 kb) [15]. It is one of the most commonly used bacteriophage species for gene delivery and its coat proteins can be engineered to incorporate targeting ligands without affecting the structure [16].

The binding and internalization of bacteriophage λ can be made more efficient by increasing the valency of the targeting ligand [12]. Internalization is usually more efficient (almost 100%) than gene transfer (about 10%) [12].

DNA vaccine delivery

Traditional vaccines require antigenic components or whole pathogenic microbes, but it is also possible to use nucleic acid as the vaccine material. Thus there are no complications with uncontrolled growth of vaccine agent, its reversion to a more pathogenic form or the need of multiple injections. Also, nucleic acid vaccines can produce copies of antigens for a long period of time [17], have fewer side effects and induce not only cellular, but also humoral immune system [18, 19]. The genes used as nucleic acid vaccines are under control of exogenous eukaryotic promoter and they induce the expression of protective antigens within the host organism[19].

One of the possible uses of bacteriophage λ is as cheap, very stable and easy to produce vehicles for DNA vaccine delivery [1, 17, 20, 21], where whole viral particles, rather than purified DNA, are used as the immunogen [21]. The protein coat of the bacteriophage serves as protection from degradation of the DNA vaccines in a host. The DNA vaccines (up to 20 kb) can be cloned into a λ DNA cloning vector with eukaryotic promoter [17, 22]. The stability of phage λ can be negatively influenced by excessively large or small inserts [23]. Phage particles are administrated subcutaneously, intramuscularly or orally [1, 24, 25] into the host, where they are taken up by antigen presenting cells and transferred into spleen and liver [24, 25].

Drug delivery

A huge trend in molecular medicine are drugs selectively delivered only to the diseased cells [26]. With targeted drugs, it is possible to maintain an effective drug concentration at the target tissue without any side effects for other tissues [27]. The basis for development of targeted drugs is the characterization of the disease cell surface and iden-

tification of useful disease markers [26]. Many of the new drugs are targeted intracellularly, but their properties prevent them from being taken up into target cells [27].

Polypeptides or antibodies that bind to disease markers can be displayed on the surface of phage [26, 28], where the phage amplifies gen for desired peptide [5]. This peptide usually origins in another organism and it is fused to the capsid proteins of phage [5].

Phagemid can also be used for protein display. This is a plasmid that carries an antibiotic resistance marker, bacterial and phage origins of replication, the phage gene from which the library is supposed to be displayed and a phage packaging signal [5].

Bacteriophages conjugated with antibodies have high specificity for the target cells or even only for cancer cells. The internalization of phage particles into cells can be observed in 2 h and it is probably occurring through receptor-mediated endocytosis into lysosome [28].

The conjugation of phage particles with drugs (for example doxorubicin or antibacterial drugs) can be done by forming an amide bond between the free primary amine on the drugs and an exposed carboxyl side chains on the phage coat [28].

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Characterization, isolation and amplification of selected bacteriophage genes

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Abstract

Bacteriophage λ is one of the best known phages and therefore it is an excellent model sample for research of cell processes by various molecular biology methods including DNA isolation using magnetic particles.

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Introduction

One of the best characterized phages is bacteriophage λ. The phage particle recognizes and binds to its host, E. coli, causing injection of DNA into the cytoplasm of the bacterial cell through the bacterial tail [1, 2]. The genome of this phage is very well known and therefore it is an excellent model sample for biochemical experiments. It was discovered more than 60 years ago and since then has been intensively studied and has become model organism for its dsDNA. The phage λ genome includes gene xis [3], which encodes excisionase (Xis) protein.

Isolation of nucleic acid is one of the most important approaches for molecular biology [4-7]. There are many methods for separation of DNA, like phenol-chloroform extraction [8] or adsorption on silica in the presence of a chaotropic salt [9]. However, these methods can be time consuming, laborious and cross-contamination can occur. That is the reason why experiments with magnetic particles (MPs) are carried out [10, 11].

Material and Methods

Frozen stock culture of bacteriophages was diluted with water and mixed with fresh grown indicator (host) Escherichia coli and soft agar. The dishes with mixture were incubated at 37 °C for 24 hours. Bacteriophage culture was removed from the dish with sterile handle. For DNA isolation the magnetic microparticles Dynabeads MyOne Silane (Life Technologies) was used. The isolation was employed according to optimized magnetic separation [12]. For xis gene fragment amplification the forward primer (5´-CCTGCTCTGCCGCTTCACGC-3´) and reverse primer (5´-TCCGGATAAAAACGTCGATGACATTTGC-3´) were used. The cycling conditions were: initial denaturation at 95 °C for 120 s; 25 cycles of denaturation at 95 °C for 15 s, annealing at 64 °C 15 s, extension at 72 °C 45 s and a final extension at 72 °C for 5 min. For DNA detection the agarose gel elecctrophoresis and electrochemical measurement was used.

Results and Discussion

Magnetic particles used in this study are silica coated beads with the diameter of 1 μm. The beads are composed of highly cross-linked polystyrene with evenly distributed magnetic material. The beads are further coated, enclosing the iron oxide inside the beads and presenting a bead surface with silica-like chemistry. The micrograph of magnetic particles used for DNA isolation is shown in Fig. 1A. In Fig. 1B the isolated DNA in agarose gel is shown. The genomic DNA stayed in well and the PCR product of xis gene fragment provided sharp band with size of 498 bp as expected. To quantify the isolation efficiency the SWV measurement was performed by adsorptive transfer technique, which is optimal for sensitive and effective DNA determination [13-15]. Nucleic acids can be easily accumulated onto surface of HMDE [16-18]. Therefore, it is possible to use adsorptive transfer technique for their detection. The working electrode is immersed into the very low volume (5 μl) of sample and nucleic acid is adsorbed for the desired time of accumulation [19]. The typical voltammograms of DNA isolated by MPs are shown in Fig. 1C. It was found, that in samples was 69 μg/ml of genomic DNA and 102 μg/ml of PCR product.

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

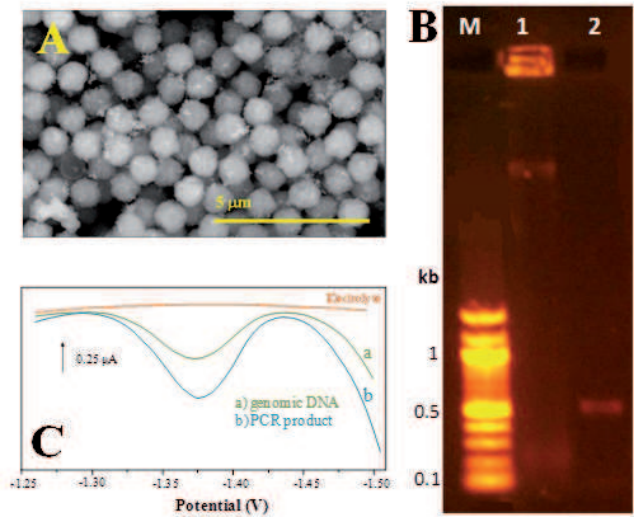

FIGURE 1. A SEM micrograph of Dynabeads MyOne Silane. B Gel electrophoresis of genomic DNA and PCR product in 1% agarose gel, the experimental parameters: 60 min, 100 V. M – mass marker, 1 Genomic DNA isolated by Dynabeads MyOne Silane 2 PCR product (498 bp) amplified from the isolated bacteriophage. C Typical SWV voltammograms. 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.

Conclusion

In this study, the optimized DNA isolation method using MPs was subsequently employed for isolation of bacteriophage λ DNA. The performance of the MPs isolation was monitored by two analytical methods confirming its efficiency and applicability.

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Papillomavirus and cancer

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Abstract

Human papillomavirus (HPV) are small circular; double stranded DNA-viruses that infect epithelial tissues. HPV types are classified as either high risk or low risk. Most of the more than 100 different identified types of HPV are involved in genital tract infections, as well as cancer of the cervix, other anogenital regions, and the head and neck areas. Human papillomaviruses have developed several molecular mechanisms that suppress the ability of virus-infected cells they infect to undergo apoptosis. In this manuscript, we review the current literature regarding cancer of the head and neck by Human papillomavirus (HPV). In particular, we will discuss the epidemiology, risk factors, the modifications in apoptotic pathways caused by proteins encoded by HPV, the molecular mechanisms of carcinogenesis, and the main detection methods of HPV.

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Introduction

Human papillomavirus (HPV) was accepted as the etiologic agent for cervical carcinoma and first reported in association with head and neck cancer in 1985. Almost 650000 patients worldwide are diagnosed with head and neck cancer each year and 350000 patients die from this disease (Badulescu, Crisan et al. 2010). Oral cancer is the sixth most prevalent cancer worldwide. The ratio of males to females who are affected is approximately 2:1(Syrjanen 2005).

The cancer results from the accumulation of specific genetic mutations, many of which have now been identified. These mutations can cause the activation of genes that promote cellular proliferation or inhibit cell death (oncogenes), or they may inactivate genes that inhibit proliferation or promote cell death (tumour suppressor genes) (Rose, Thompson et al. 2000).

When we speak of the term head and neck cancer, we refer to a malignancy area that arises in the skin, oral cavity, salivary glands, lip, pharynx, larynx, nasal cavity, paranasal sinuses and soft tissues of the neck and ear. The majority of these malignancies are squamous cell carcinoma of the mucosal surfaces of the head and neck (HNSCC) (Syrjanen 2005; Forte, Niu et al. 2012; Walden and Aygun 2013).

A recent study suggests a similar role for HPV in all age groups, showing that the incidence of HPV in younger patients is not significantly different from older patients (Sisk, Bradford et al. 2000). HPV is the most common sexually transmitted infection globally. The predominant route of transmission is via sexual contact, although mother-to-child transmission is also possible (Syrjanen and Puranen 2000). HPV infection may exist asymptomatically or may induce the formation of benign or malignant tumours in the genital, oral or conjunctival mucosa.

The association between cigarette smoking and head and neck cancer has been known for over 30 years, and cigarette smoke is a known source rich in chemical carcinogens and reactive oxygen species, many of which likely contribute to the carcinogenic potential (Badaracco, Venuti et al. 2000; Mansour, Ali et al. 2012).

While high-risk HPV appears to be associated with certain sexual behaviors, such as oral sex and increasing numbers of sexual partners, there is still a lack of association with smoking and drinking (D'Souza, Kreimer et al. 2007).

The human body can defend itself against viral attacks by eliminating damaged, virus-infected cells; however, viruses employ a number of different mechanisms to eliminate this defense.

Apoptosis, or programmed cell death, helps to eliminate damaged cells and also contributes to the elimination of virus-infected cells (Stern, van der Burg et al. 2012). A virus employs a number of different mechanisms to avoid the host immune response (Parkin 2006; Wagner, Mayer et al. 2012). Especially the DNA viruses encode a variety of viral proteins that can inhibit or delay these protective actions and affect cellular apoptotic pathways (Garnett and Duerksen-Hughes 2006; Mansfield, Pencavel et al. 2013).

Molecular biology has provided important data on the interaction of the HPV oncoproteins with genes important for cell cycle control. All viral genes are encoded on one strand of the DNA. The L1 and L2 genes encode the capsid proteins which are only expressed in terminally differentiated squamous epithelial cells. The E genes encode non-structural proteins that regulate virus transcription and replication (Chen, Aaltonen et al. 2005) (Fig. 1).

The viral proteins E6 and E7 are implied in tumorigenesis and are known to induce degradation of the p53 and pRb, respectively (Garnett and Duerksen-Hughes 2006). They can suppress apoptosis, alter the function of factors involved in cell-cycle regulation, and thereby facilitate the prolongation of the proliferative stage of keratinocyte differentiation (Strati, Pitot et al. 2006; Dayyani, Etzel et al. 2010).

The majority of cervical carcinomas caused by the two most common HPV types, HPV 16 and 18, contain integrated viral sequences that express E6 and E7 proteins (Smith, Pawlita et al. 2010). E6 and E7 oncogene expression are considered necessary for carcinogenesis.

HPV 16 and 18 are found in over 50% of cervical can-

cers and 90 to 95% of HPV positive HNSCCs (Snijders, Scholes et al. 1996; Badaracco, Venuti et al. 2000; Miller and Johnstone 2001; Ringstrom, Peters et al. 2002). Moreover, HPV 16 and 18 are found in premalignant lesions (Kero, Rautava et al. 2012) whereas HPV types 6 and 11 are classified as low risk types, and infection with these types usually results in the proliferation of epithelial cells and manifests as warts or papillomas on the skin (Fife, Fan et al. 1996; Chang, Su et al. 2002).

There are many techniques for the molecular detection of HPV viruses. Among the most important is the polymerase chain reaction (PCR) amplification of viral RNA which is considered a good approach for the classification of tumors as etiologically associated with HPV (Syrjanen 2005).In Situ Hybridization (ISH) offers the advantage of direct visualization of HPV in tumor nuclei, indicative of HPV integration (Huang, Qiu et al. 1998; Singhi and Westra 2010).

Detection of p16 expression by Immunohistochemistry (IHC) (Shi, Kato et al. 2009) or by measuring HPV16 E6 mRNA using quantitative real-time PCR (qRT-PCR) is a reliable determinant for HPV involvement, since HPV integration induces expression of E6 and E7, thereby providing a more direct evidence of the oncogenic role of HPV (Munoz, Gonzalez et al. 2012; Liu, Liu et al. 2013). Head and neck cancers tissues were found to harbor extrachromosomal HPV-16 plasmid genomes as demonstrated by both Southern blot and viral mRNA sequence analyses (Lace, Anson et al. 2011).

Prophylactic HPV vaccines prevent infection by inducing neutralizing antibodies against HPV capsid proteins L1 and L2 (Rutkowski and Skladowski 2009).

Therapeutic HPV vaccines, however, aim to treat by targeting non-structural early viral antigens of HPV, such as E6 and E7, because HPV-infected basal keratinocytes and HPV-transformed cells generally do not express L1 or L2 (Badaracco, Venuti et al. 2000).

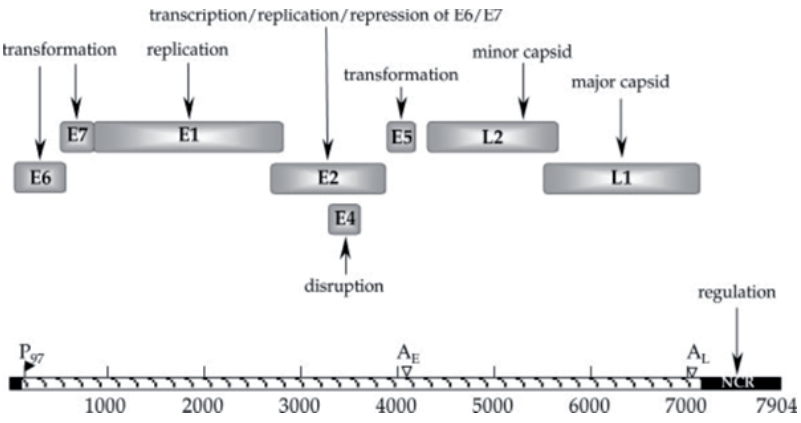

Figure 1. Genetic Map of the Papillomavirus Genome. (Chen, Aaltonen et al. 2005)

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The fluorescence viewing of the virus proteins

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Abstract

Influenza is potential pandemic agent. It should be detect and monitored. The viruses can be detected after the fluorescence labelling. In this work the labelling of the viral protein hemagglutinin was studied. It was proved that CdS quantum dots conjugated with hemagglutinin are good marks for the viruses detection.

WHD_15_2013

Introduction

Influenza, or the flu, is a common respiratory disease caused by influenza virus [1] and is potential pandemic agent [2]. The finding of the methods for rapid and sensitive detection of the virus in the environment, body fluids and tissues is still challenging.

For high-sensitivity analyses of biologically important molecules, such as DNA, proteins, and antibodies, are routinely used fluorescent tags [3]. The fluorescence labeling of viruses is also useful technology for virus detection and imaging [4]. A promising is usage the nanoparticles [5]. The nanoparticles with good fluorescence properties are quantum dots [6-10]. The attachment of influenza virus to susceptible cells is mediated by viral protein hemagglutinin (HA), which recognizes cell surface glycoconjugates that terminate in α-sialosides [11].

The aim of this study was fluorescence detection of the hemagglutinin. The heamgglutinin was labeled by CdS quantum dots. The fluorescence properties were studied in the solution and on the chromatography nitrocelulose membrane.

Material and Methods

As the standard of Influenza A hemagglutinin, was used influenza vaccine Vaxigrip (Sanofi Pasteur, France), which contains inactivated and split virions of the following strands: A/California/7/2009 (H1N1) – derived strain used NYMC X-179A, A/Perth/16/2009 (H3N2)-like strain used NYMC X-187 derived from A/Victoria/210/2009. Strain was propagated in fertilised hens´eggs from healthy chicken flocks. Vaxigrip contain 15 μg of all of three HA per 0.5ml.

CdS quantum dots (QDs) were prepared using a slightly modified version of a published method [12]. Cadmium nitrate tetrahydrate $Cd(NO_3)_2 \cdot 4H_2O$ (0.1 mM) was dissolved in ACS water (25 ml). 3-mercaptopropionic acid (35 μl, 0.4 mM) was slowly added to the stirred solution. Afterwards, the pH was adjusted to 9.11 with 1 M NH_3 (1.5 ml). Sodium sulphide monohydrate $Na_2S \cdot 9H_2O$ (0.1 mM) in ACS water was poured into the solution while vigorously stirring. The acquired yellow solution was stirred for 1 h. Prepared CdS-quantum dots were stored in the dark at 4 °C.

Vaccine (100 μl) was mixed with a solution of QDs (100 μl). This mixture was shaken for 24 h at room temperature (Vortex Genie2 (Scientific Industries, USA). The volume of solution was reduced to 100 μl on an Amicon Ultra 3k centrifugal filter device (Millipore, Massachusetts, USA). Centrifuge 5417R (Eppendorf, Hamburg, Germany) was performed under the following parameters 15 min, 6000 rpm, 20 °C. The obtained concentrate was diluted with 400 μl of ACS water and reduced on centrifuge to 100 μl. The process was repeated 5 times. The washed sample was diluted to 300 μl and used for succeeding measurements.

The Bio-Dot unit (Biorad) was used for injecting of the QDs sample on the nitrocellulose chromatography paper. The membrane was placed into the unit and the vacuum was on. Different volumes (5, 2, 1 a 0.5 μl) of QDs were pipetted on 12 x 8 spots platform with simultaneous drying. The prepared QDs membranes were analyzed by Carestream In-vivo Xtreme Imaging System (Rochester, USA). Excitation wavelength : 460 nm, emission wavelength: 700 nm, exposure time: 2 s, binning: 2x2 pixels, field of view: 7.2 x 7.2 cm.

Results and Discussion

The fluorescence of the quantum dots conjugated to the hemagglutinin was tested in solution The concentration of quantum dots in solution of quantum dots and hemagglutinin was 1/2, so it had better fluorescence properties (Fig. 2). But after the application of the sample on the nitrocellulose membrane, the quantum dots with hemagglutinin had higher intensity of the fluorescence (Fig. 3). Maximum intensity of the quantum dots with hemagglutinin was 1,5 times higher. The hemagglutinin caused different distribution of the quantum dots on the membrane, which could be the reason for the better fluorescence.

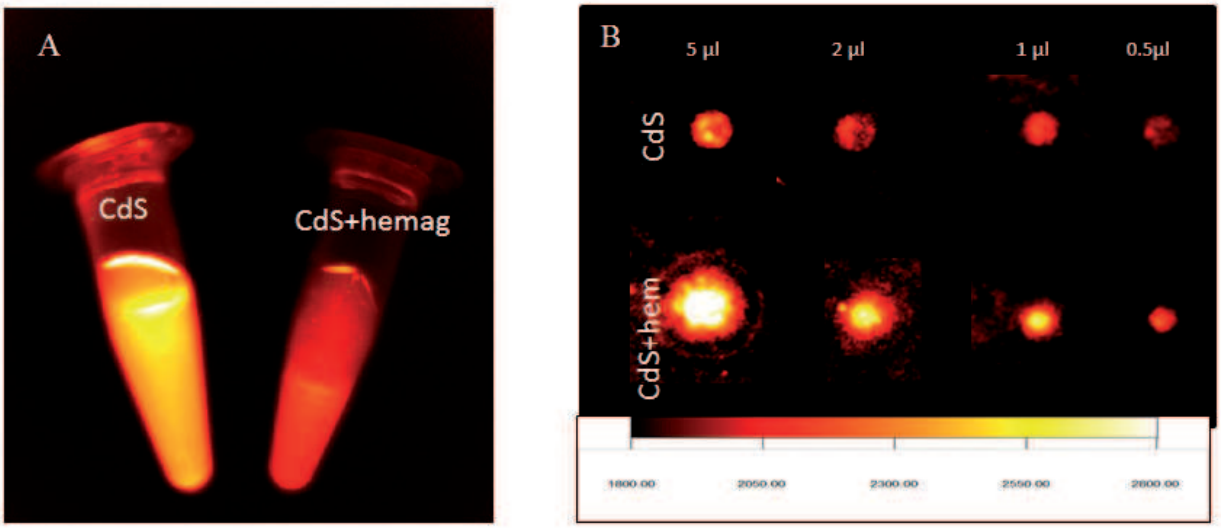

FIGURE 2. Fluorescence of the CdS quantum dots and CdS quantum dots with hemagglutinin: A) quantum dots in solution in microtubes, B)quantum dots on chromatography nitrocelulose membrane

Conclusion

The viral protein hemagglutinin can be visualized by staining with quantum dots. The CdS quantum dots stabilized with mercaptopropionic acid interact with hemagglutinin and allow their detection. The useage of nitrocelulose membrane is good for visualisation of the marked hemagglutinin and can be used in the detection of the influenza virus.

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Kvantové tečky jako nástroj pro současnou detekci tří chřipkových virů v jednom vzorku

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Abstrakt

Cílem této studie bylo navržení biosensoru pro detekci tří chřipkových subtpyů v jednom vzorku současně s využitím paramagnetických částic (MPs) a nanočástic (NPs). Navržený proces detekce obsahoval tří základní kroky: (i) hybridizace cílové DNA na modifikované MPs, (ii) hybridizace anti-sense řetězce značeného NPs na cílovou DNA a (iii) elektrochemickou detekci izolované DNA a NPs značky. Dále byl sledován efekt různých inkubačních teplot a koncentrací cílové sekvence na účinnost hybridizace.

WHD_16_2013

Úvod

Chřipka je infekční onemocnění dýchacích cest, které způsobují viry z čeledi Orthomyxoviridae, která obsahuje tři rody: chřipka typu A, B a C. Nejvíce virulentní je chřipka typu A [1]. Virulence je dána dvěma povrchovými antigeny neuraminidázou (NA) a hemaglutininem (HA), oba antigeny se vyskytují ve více subtypech (H1-16 a N1-9)[2]. Mutační změny obou antigenů představují riziko vzniku nového subtypu, který by způsobil takovou pandemii, jakou byla

Španělská chřipka (asi 50 mil. obětí)[3]. V boji proti vzniku a šíření epidemií je nejúčinnější včasná a správná diagnostika.

Cílem naší studie bylo navrhnout biosensor pro stanovení více chřipkových subtypů v jednom vzorku. Proces izolace se skládal z dvojité hybridizace a byl zakončen elektrochemickou detekcí. Byl zkoumán efekt hybridizačních teplot a koncentrace cílové sekvence.

Materiál a metody

1 Příprava NPs (CdS, PbS a ZnS) a značení komplementárního řetězce pomocí NPs

Chemikálie použité v protokolu byly dodány firmou Sigma-Aldrich. Příprava a značení anti-sense řetězce pomocí NPs byly provedeny podle protokolu v předchozí studii [4], následně byly skladovány ve tmě při 4 °C.

2 Automatická izolace cílové molekuly a próby pomocí NPs

Automatická pipetovací statice EP Motion 5075 (Eppendorf, Germany) byla použita pro automatizaci izolace nukleové kyseliny (Obr.1). MPs: Dynabeads Oligo (dT)25 (Invitrogen, Oslo) byly napipetovány do jamek v mikrodestičce (PCR 96, Eppendorf, Germany). Detička byla přenesena na magnet kde byl odpipetován uchovávací roztok a MPs byly promity fosfátovým pufrem I (PBI), (pH = 6.5, 0.1 M NaCl + 0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4). Následovala první hybridizace. Do jamek byla přidána cílová DNA a hybridizační pufr (HB)(0.1 M fosfátového pufru, 0.6 M guanidin thiokyanátu, 0.15M Tris, pH = 7.5), směs byla inkubována (15 min, 25°C) a poté promyta PBI. Následovala druhá hybridizace. Do jamek byly přidány próby značené NPs a HBI, tato směs byla inkubována (15 min, 25°C) a pak promyta PBI. Posledním krokem byla eluce: eluční pufr (fosfátový pufr II - 0.2 M NaCl + 0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4),(5 min, 85°C, míchání). Po eluci byla destička přenesena na magnet a produkt byl odpipetován do nových jamek. Automatizovaná izolace modifikovanými MPs byla popsána i v předchozích publikacích [4, 5].

2.3 Elektrochemická detekce CA a kovového peaku ODN-SH-NPs komplexu

Měření byla provedena v elektrochemické cele ve standartním tří-elektrodovém zapojení, v prostředí elektrolytu: acetátový pufr 0.2 M CH_3COOH + 0.2 M CH_3COONa (pH 5.0). Měřícím přístrojem byl 663 VA Stand (Metrohm, Switzerland). Výsledky byly vyhodnoceny programem GPES 4.9. Pro detekci byly vybrány dvě voltametrické metody.

Square wave voltametrie (SWV) pro detekci DNA (CA pík) a diferenční pulzní voltametrie (DPV) pro NPs (Cd, Pb, Zn pík). Parametry pro SWV a DPV byly nastaveny podle předchozí studie[4].

Výsledky a diskuze

Byla popsána metoda pro izolaci a detekci tří subtypů chřipky v jednom vzorku, výsledky byly vyhodnoceny na základě rozdílné elekroaktivity NPs - různé kovy (Zn, Pb a Cd) dávají signál při různých potenciálech. Schéma izolace popisuje obrázek Obr.1.

3.1 Elektrochemická detekce ODN-SH-NPs – CA a kovový pík

K detekci komplexu ODN-SH-NPs byly použity dvě voltametrické metody. Pomocí SWV byl detekován CA pík, potenciál: -1.4V. Pomocí DPV byl detekován pík kovu z NPs: -1.03 V (Zn); -0.63 V (Cd);-0.45 V (Pb). Byly změřeny kalibrace pro CA pík oligonukleotidu a kalibrace pro jednotlivé kovy z NPS.

3.2 Optimalizace hybridizační teploty

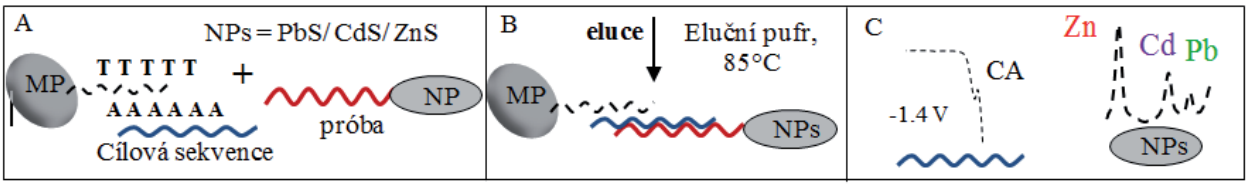
Cílem této části bylo sledovat vliv teploty hybridizační reakce a koncentrace cílové molekuly ODN. Byl sledo-

ván vliv čtyř koncentrací cílové molekuly (2.5; 5; 10 and 20 µg/ml) a vliv čtyř různých teplot hybridizace (15°C, 20°C, 25°C and 30°C). Následně byly vyhodnocené CA signály Obr.: 2. A, B, C a Cd, Zn, Pb signály Obr. 2. D, E, F. Nejvyšší efekt byl pozorován při teplotě 25°C. Oproti předpokladu, že s rostoucí teplotou poroste efekt hybridizace, byl efekt hybridizace při teplotě 30°C mnohem menší a to z toho důvodu, že teplota Tm oligonukleotidů byla 28°C.

matického pipetovacího systému a elektrochemické detekce představuje unikátní nástroj pro detekci jednotlivých chřipkových subtypů.

Poděkování

Za finační podporu děkujeme těmto projektum CEITEC CZ.1.05/1.1.00/02.0068 a NANOLABSYS CZ.1.07/2.3.00/20.0148.



Obrázek 1: Schéma izolace a detekce tří specifických chřipkových sekvencí. (A) MPs modifikované cílovou sekvencí (vazba T-A) a NPs modifikace of anti-sense. (B) Hybridizace NPs značené próby na cílovou sekvenci a následná eluce. (C) Elektrochemická detekce NPs a cílové sekvence

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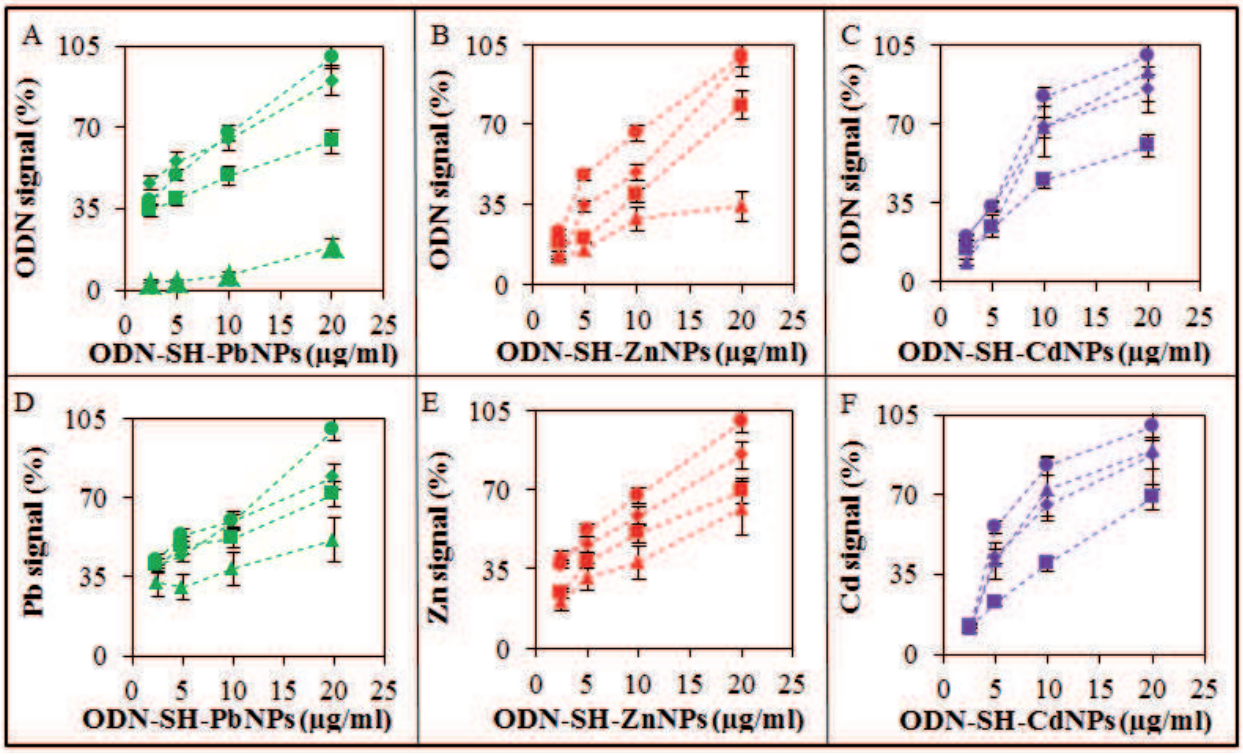

Obrázek 2: Závislost relativní výšky píku (%) na koncentraci ODN-SH-NPs (µg/ml), (A,B,C- výška CA píku (ODN); D,E,F –výška kovového píku; A+D ODN-SH-PbNPs; B+E ODN-SH-ZnNPs; C+F ODN-SH-CdNPs. Hybridizační teploty: ●30°C ; ● 25°C; ●20°C;●15°C

Závěr

Byla navržena a optimalizována metoda pro současnou detekci tří chřipkových oligonukleotidů značených pomocí NPs v jednom vzorku. Dále byl sledován efekt hybridizační teploty a koncentrace cílových sekvencí na výšku CA a NPs píku. Jako optimální teplota hybridizace byla zvolena teplota 25°C. S rostoucí koncentrací cílové sekvence rostla výška CA i NPs píku. Kombinace MPs, NPs, auto-

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PARAMAGNETIC PARTICLES BASED AUTOMATED ISOLATION COUPLED WITH ELECTROCHEMICAL ANALYSIS OF QUANTUM DOTS LABELED INFLUENZA HEMAGLUTININE

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Abstract

This study presents biosensor, which can rapidly analyze influenza virus by glycan modified paramagnetic particles (MPs), which can selectively bind to viral antigen hemaglutinin (HA), which was labelled by quantum dots (QDs). Our assay consists of two parts. The first one is HA-QDs isolation by glycan modified MPs and the second is electrochemical detection of isolated HA-QDs complex. Optimized detection of cadmium sulfide quantum dots (CdS QDs), respectively HA-CdS complexes was performed using differential pulse voltammetry.

WHD_17_2013

Introduction

Influenza represents one of the greatest pandemic threats today. According to the World Health Organization (WHO), influenza is responsible for several million illnesses and 250 000 - 500 000 deaths each year [1].

Influenza viruses (influenza A,B,C) are RNA viruses that make up three genera of the Orthomyxoviridae family. Type A viruses are most virulent and can be subdivided into different serotypes based on combination of viral antigens: hemagglutinin (HA) and neuraminidase (NA). HA mediates glycan receptor binding for viral entry in host cell. NA conducts receptor-destroying activity for virus release[2]. More than 500 human cases of H5N1 have been reported, with mortality nearly 60 %. Meta-analysis published in Science shows 12,677 participants infected with H5N1 have subclinical or mild infections [3]. It is possible that deaths documented by WHO, are also underestimated. A standardized approach for large scale studies is examined.

Electrochemical biosensors have attracted considerable interest due to its high performance and low cost [4]. Conventional enzyme and electroactive labels have incorporated due to their improved sensitivity in protein detection. QDs are interest because of their size and optical properties, but our interest was placed on their electroactivity.

This study aimed to develop a low cost isolation and rapid CdS quantum dots based biosensor to virus detection. The results are confirmed by differential pulse voltammetry (DPV). We applied protocol of streptavidin coated MPs modified with biotinylated glycan and by them selective isolated HA-CdS complex.

Materials and Methods

Preparation of CdS and HA-CdS complex

Preparation of CdS and labeling of HA (A/H5N1/Vietnam) by QDs (CdS) were performed by protocol presented in previous study [5] and were subsequently stored in the dark at 4 °C

Automatic MPs based separation

Automatic pipetting station (epMotion 5075) was used for automatic isolation. MPs modified by streptavidin was pipetted to microplates (PCR 96, Eppendorf). Stored solution was drained and MPs were washed by 100 µl of phosphate buffer (PB) (0.3 M, pH 7.4, made from NaH_2PO_4 and Na_2HPO_4). 20 µl of biotinylated glycan were added to each wells and incubated (30 min, 25 ° C, 400 rpm) Fig.1/A. After incubation, the sample was washed three times by 100 µl of PB. Subsequently 20 µl of HA complex was added. It was further incubated (30 min, temperature was optimized parameter, 400 rpm) and washed by 100 µl of PB. 35 µl of PB was added followed by the treatment of ultrasound needle (2 min) Fig.1/B. The plate was transferred to the magnet and the supernatant was pippeted to the new wells and measured using DPV. The detected substance was identified as hemaglutinin and/or cadmium (quantum dots) Fig.1/C.

3. Determination of HA and Cd

DPV measurements were performed with a 663 VA Stand (Metrohm, Switzerland) in standard cell consisting of three electrodes, a cooled sample holder (4°C) (Julabo F25, Julabo DE, Germany). GPES 4.9 was used for data analysis. The analysed samples were deoxygenated by argon (99.999 %).

For HA detection was selected Adsorptive transfer technique (AdT) of DPV method. As supporting electrolyte was used Brdicka solution 1 mM $Co(NH_3)_6Cl_3$ and 1M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The parameters of method were as follows: initial potential of -0.7 V, end potential of -1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, Eads = 0 V. The sample volume was 5 µl with a total volume of 2 ml in the measurement cell (1995 µl of Brdicka).

For determination of cadmium ions Anodic stripping technique of DPV was selected. As electrolyte acetate buffer (0.2 M CH_3COONa + CH_3COOH, pH 5) was used. The

parameters of the measurement were as follows: purging time 120 s, deposition potential -0.9 V, accumulation time 240 s, equilibration time 5 s, modulation time 0.057 s, interval time 0.2 s, initial potential -0.9 V, end potential -0.3 V, step potential 0.00195 V, modulation amplitude 0.02505 V, sample volume: 5 µl, volume of measurement cell 1 ml (5 µl of sample; 995 µl of acetate buffer).

Results and Discussion

We designed and implemented a biosensor, which consisted of paramagnetic particles based isolation of viral proteins modified by QDs with subsequent electrochemical detection of HA and QDs as a new type of label.

Determination of HA and Cd

Calibration curves of HA and Cd were provided using AdT DPV (HA detection) and ASV DPV (Cd detection). The results were used to study of the incubation temperature effect of binding between HA-CdS complex to glycan modified MPs.

MPs for HA-CdS isolation

The first step of influenza infection, involves HA, which binds to a host cell's surface glycans via a terminal sialic acid (Sia) with α2-3 and α2-6 linkages [6, 7]. Due to this fact we use streptavidin coated MPs which were modified by biotinylated glycan. This tool was used for isolation of HA-CdS complex by fully automated pipetting robot (epMotion 5075). Streptavidine conjugated beads were incubated with biotinylated glycan (30 min, 25°C, 400 rpm). After glycan modification of MPs. HA-CdS was added and incubated by condition of (30 min, 400 rpm). Temperature of incubation was optimized (5, 25, 35, 45, 55, 65°C) Fig.2. After that samples were treated with ultrasound needle (2 min.). The plate was transferred to a magnet and supernatant was transferred to separated well. Product from each well was analysed using DPV.

3.5 Optimization of incubation temperature

Incubation temperature and its effect were tested to design a biosensor. The influence of varying temperatures on binding between glycan and HA-CdS was investigated. Changes in cadmium(II) ions peak height showed increasing dependency on the temperature, but only to temperature of 45°C, At condition by 55 and 65°C Cd peak decreased Fig.2/A. Dependence of HA peak height was slightly different. HA peak increased to incubation temperature of 45°C, by temperature of 55°C HA peak decreased, but at temperature of 65°C was HA peak again increased Fig.2/B. Electrochemical determination of metals peak are generally more sensitive than protein and DNA detection, so we accepted the temperature of 45°C as the optimal.

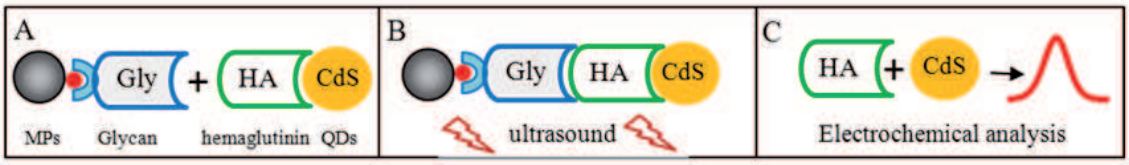

Figure1: Scheme of isolation A+B and electrochemical detection C of hemaglutinine HA labeled by quantum dots (QDs). A reaction between biotinyled glycan bounded on streptavidin modified paramagnetic particles (MPs) and HA binding to QDs, B isolation of HA-QDs folowed by ultrasound breaking, C electrochemical ection of HA and QDs by voltammetry

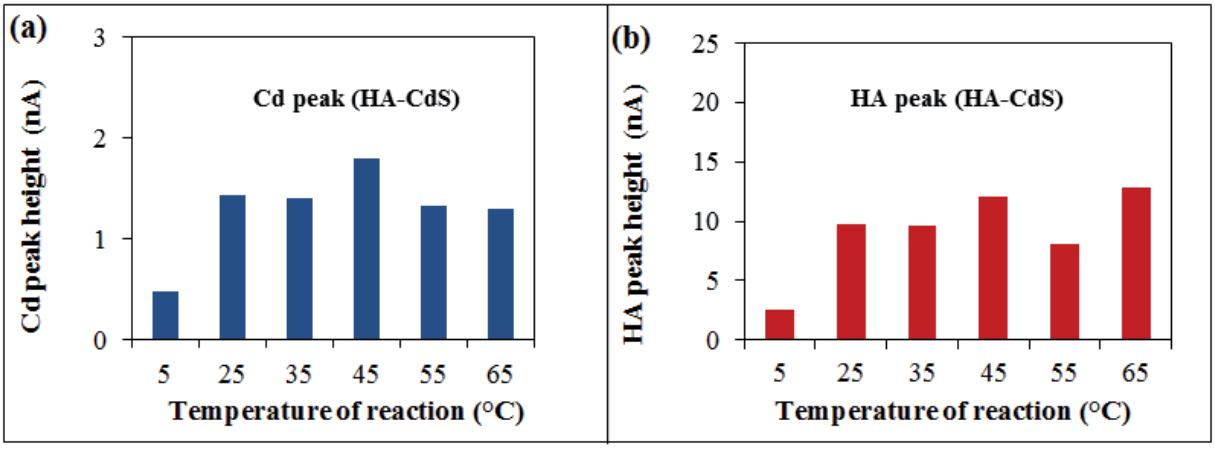

Figure 2: Optimization of applied temperature to the binding proces of HA-QDs to MPs modified by glycan. Effect of temperature condition (°C) was detected by electrochemical analysis of Cd peak (a) and HA peak (b). (a)For Cd detection ASV DPV was used . (b) For HA used AdT DPV.

Conclusion

Most cases of human infection due to H5N1 virus have involved close contact with infected poultry. Effective routine surveillance may be impossible in countries lacking basic public health resources. For a global anti-epidemic strategy a protocol that allows sensitive detection is still required. A rapid detection method using an automated MPs-based technology may test accuracy for the detection of a broad range of disease markers.

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PRŮTOKOVÁ ANALÝZA VIROVÝCH SEKVENCÍ NA ZÁKLADĚ DETEKCE ADENINŮ

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Abstract

V současnosti je jeden z důležitých směrů molekulární biologie a instrumentální analýzy detekce specifických sekvencí nebezpečných virových onemocnění, které mohou vyvolat světovou pandemii. V našem experimentu jsme se zaměřili na detekci viru eboly, chřipky a HIV pomocí injekční analýzy v zastaveném toku s elektrochemickou detekcí. Pro tento účel byla modifikována pracovní elektroda ze skelného uhlíku CuS nanočásticemi pro detekci adeninu ve virových sekvencích. Pro jejich stanovení byla optimalizována jejich kyselá hydrolýza pomocí mikrovlnného zařízení. Optimální podmínky byly koncentrace HCl 100 mM, výkon mikrovlnného zařízení 800 W a doba hydrolýzy 30 minut.

WHD_18_2013

Úvod

Pro studium nukleových kyselin jsou zejména vhodné elektrochemické metody a to především díky jejich senzitivitě, selektivitě [1-3], možnosti miniaturizace a také možnosti modifikace pevných elektrod [4]. K tomuto účelu jsou vhodné pracovní elektrody ze skelného uhlíku, který byl prokázán jako vhodný nástroj pro konstrukci DNA biosenzorů [5]. V literatuře je také popsáno mnoho způsobů modifikací těchto elektrod, z nichž nejlepších výsledků je dosaženo modifikací pomocí uhlíkových nanočástic [6]. Jiné experimenty využívají měďnatých iontů pro senzitivnější detekci adeninu, se kterým tvoří stabilní komplex [7]. Taktéž bylo publikováno sestrojení modifikované elektrody elektrodepozitovaného Cu nanooxidu [8]. V naší práci jsme se zaměřili na elektrochemickou detekci adeninu ze sekvencí DNA eboly, chřipky a HIV s modifikovanou elektrodou CuS nanočásticemi pomocí mikrofluidního systému injekční analýzy (SFIA).

Materiály a metody

Sekvence virových nukleových kyselin (HIV, chřipka, ebola) byl zakoupeny od firmy Sigma Aldrich (St. Louis, USA). Adenin byl zakoupen od firmy MP Biomedicals (Německo). $CuCl_2$, NaH_2PO_4, Na_2HPO_4, KCl, NaOH, metanol, H_2O v ACS čistotě, byly zakoupeny od firmy Sigma Aldrich (St. Louis, USA). CuS nanočástice byly připraveny reakcí $Cu(OAc)_2 \bullet H_2O$ s $C_4H_6O_4S$. Následně byl přidán NH_4OH a $Na_2S \bullet 9H_2O$ (vše Sigma Aldrich).

Pro elektrochemickou detekci adeninu byl použit mikrofluidní systém SFIA. Tento systém byl složen z programovatelné nástřikové pumpy (Model eVol, SGE Analytical Science Pty Ltd, Austrálie), tříkanálového dvoupozičního ventilu (Valco, Instruments Co. Inc, USA), dávkovací kapiláry, která přímo vstupovala do elektrochemické průtokové cely (CH Instruments, Inc., USA) a prototyp miniaturizovaného potenciostatu (910 PSTAT mini (Metrohm, Švýcarsko). Elektrochemický detektor byl složen z uhlíkové pracovní elektrody, platinové pomocné elektrody a Ag/AgCl 3M KCl referentní elektrody. Pomocí automatické pipety bylo do průtokové elektrochemické cely o objemu 500 nl nadávkováno 50 µl vzorku. Elektrochemická cela byla vždy po každé analýze promyta 200 µl 50% metanolem a stabilizována 200 µl elektrolytu (0,5 M PBS, pH 7). Elektrochemická detekce byla provedena pomocí diferenční pulzní voltametrie (DPV). Data byla zpracována pomocí PSAT softwaru 1.0 (Metrohm, Švýcarsko). Pro modifikaci uhlíkové pracovní elektrody byl do elektrochemické cely nadávkován 0,1 mM KCl s přídavkem 20 µM $CuCl_2$ nebo 200 µM CuS nanočásticemi. Následně byla provedena elektrochemická deponace při -0,4 V 200 s. Pro vytvoření oxidu byl do elektrochemické cely nadávkován 0,1 M NaOH a bylo provedeno 20 skenů cyklické voltametrie od -2 do -0,4 V s rychlostí skenu 0,1 $V.s^{-1}$.

Hydrolýza virových sekvencí byla provedena pomocí 50 µl 10 mM HCl, která byla smíchána s 50 µl vzorku. Směs byla poté zahřívána 80 min při 800 W v mikrovlnném reakčním systému (Multiwave 3000, Anton Paar, Rakousko). Následně byly vzorky neutralizovány 10 mM NaOH a odpařeny pomocí vakuové odparky (Ultravap, Porvair, Velká Británie) při 60 °C. Nakonec byly vzorky zředěny 0,05 M PBS pufrem (pH 7) a analyzovány.

Výsledky a diskuze

V tomto experimentu jsme se zaměřili na detekci adeninu, pomocí injekční analýzy v zastaveném průtoku využívající jako pracovní elektrodu skelný uhlík modifikovaný CuS nanočásticemi. Parametry detekce metodou diferenční pulsní voltametrie a modifikace elektrody byly optimalizovány v našich předchozích experimentech. Pro stanovení adeninu z řetězce sekvencí nukleových kyselin eboly (ACCTCACTAGAAAA), chřipky (TAATAACCATTGGA) a HIV (GAGCAGTGGGAATA) bylo důležité tento řetězec rozštěpit pomocí kyselé hydrolýzy asistované mikrovlnami. Na základě literatury [9] jsme se rozhodli použít k hydrolýze HCl, jejíž koncentrace byla optimalizována spolu s dobou hydrolýzy a výkonem mikrovlnného zařízení. Další parametry jsou uvedeny v materiálech a metodách.

Pro zjištění optimální koncentrace kyseliny jsme testovali vliv 1, 10, 100 a 500 mM HCl na výšku píku adeninu při koncentraci virové sekvence eboly 100 $\mu g.ml^{-1}$. Výsledky byly následně přepočítány na procenta. Z grafu Obr. 1A je patrné, že glykosidické vazby v řetězci sekvence nukleových kyselin jsou nejlépe štěpeny za použití 100 mM HCl a

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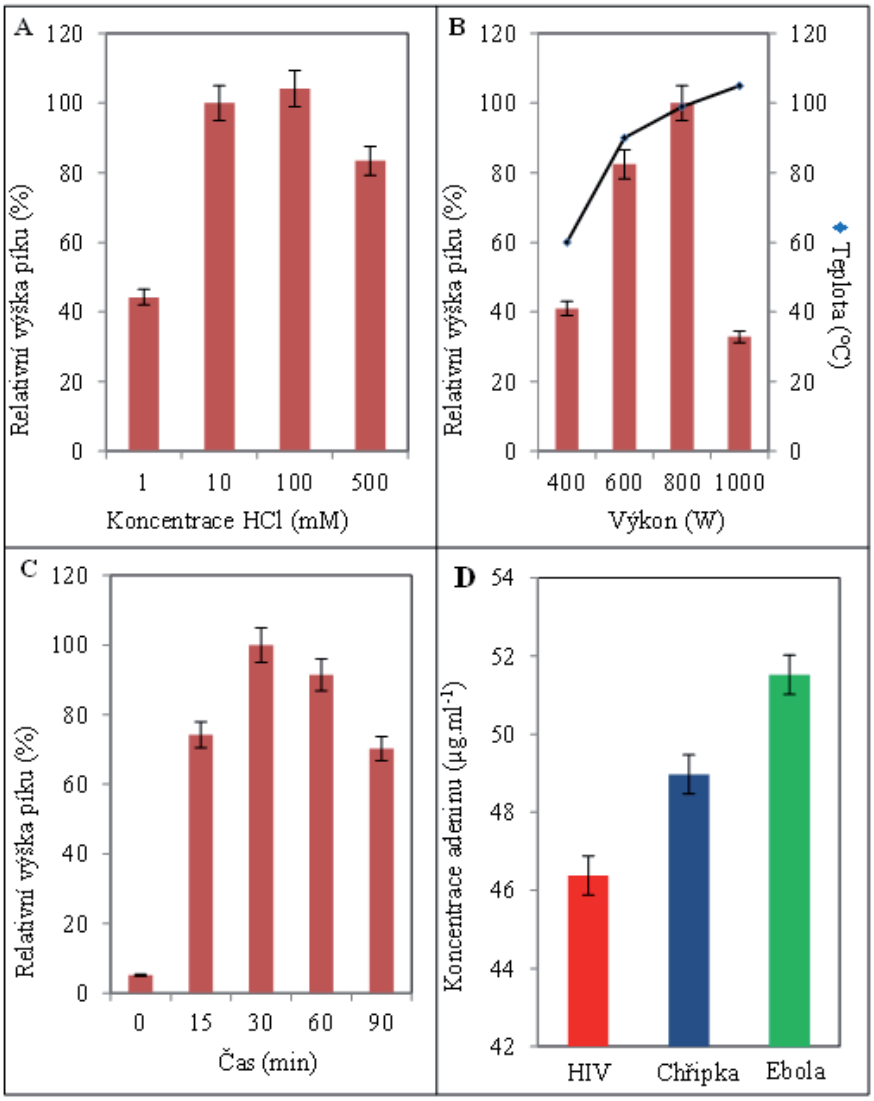
nižší (1 mM) a vyšší (500 mM) koncentrace způsobují nedostatečné štěpení vazeb v řetězci, což se projevuje poklesem signálu adeninu. Na grafu (Obr. 1B) je znázorněna závislost průběhu hydrolýzy při výkonu 400, 600, 800 a 900 W. Je zřejmé, že zvýšením výkonu a tím i teploty na 800 W a 99 °C dochází k výrazně vyššímu štěpení řetězce nukleových kyselin na jednotlivé báze, jak dokládají další studie [10], [11]. Avšak při dalším zvýšení výkonu na 900 W a teplotě 105 °C dochází jen k nepatrnému zvýšení výtěžnosti adeninu. Je známo, že při vyšších teplotách hydrolýzy může docházet ke vzniku degradačních produktů, které mohou zkreslovat elektrochemickou analýzu. Nakonec byla sledována doba mikrovnami asistované hydrolýzy 0, 15, 30 a 90 min (Obr. 1C). Ze získaných výsledků je zřejmé, že s narůstající dobou hydrolýzy se zvyšuje elektrochemický signál adeninu a to do 30 min. Následně je zaznamenán pokles signálu při 60 a 90 min a to pravděpodobně díky vzniku degradačních produktů.

Optimalizovanými podmínkami hydrolýzy byly připraveny vzorky sekvencí nukleových kyselin o koncentraci 100 $\mu g.ml^{-1}$ eboly, chřipky a HIV a analyzovány pomocí metody DPV injekční analýzou v zastaveném toku. Na obrázku 1D je vidět, že elektrochemický signál je přímo úměrný koncentraci adeninu v sekvenci nukleových kyselin jednotlivých virů. Prokázali jsme, že tato metoda je vhodná pro detekci sekvencí nukleových kyselin virů eboly chřipky a HIV. Díky miniaturizaci a vysoké senzitivitě se jedná o velmi perspektivní metodu vhodnou pro využití v biosenzorech.



Obr. 1: Optimalizace hydrolýzy virových sekvencí nukleových kyselin eboly 100 µg.ml-1. Vliv A) koncentrace HCl, B) výkonu mikrovlnného zařízení a C) času hydrolýzy. D) Koncentrace adeninu virových sekvencí eboly, chřipky a HIV získaná pomocí injekční analýzy v zastaveném toku s elektrochemickou detekcí s využitím modifikované elektrody CuS nanočásticemi ze skelného uhlíku. Další parametry jsou uvedeny v kapitole "Materiál a metody"

Závěr

V tomto experimentu byla vyvinuta metoda pro stanovení nebezpečných virů eboly, chřipky a HIV založená na zjištění koncentrace adeninu těchto virových sekvencí pomocí injekční analýzy v zastaveném toku s elektrochemickou detekcí využívající modifikovanou elektrodu ze skelného uhlíku CuS nanočásticemi. Tato metoda je vysoce senzitivní a umožňuje tak stanovit i velmi nízké koncentrace adeninu v řetězci nukleových kyselin.

Poděkování

Práce byla podpořena projekty
NANOLABSYS CZ.1.07/2.3.00/20.0148
a CEITEC CZ.1.05/1.1.00/02.0068.

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METODY PURIFIKACE A CHARAKTERIZACE VIROVÝCH PROTEINŮ

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Abstract

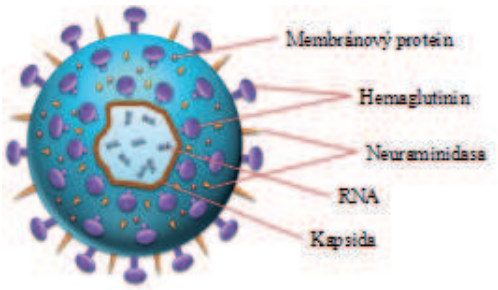
Viry jsou všudypřítomné struktury doprovázející buněčné formy života. I přes to, že mají některé vlastnosti podobné buněčným organismům, nemohou vykonávat celou řadu biologických procesů, které jsou pro živé organismy typické. Viry jsou schopné se množit pouze v buňkách živých organismů a mohou vyvolat virová onemocnění, mezi která se řadí například chřipka, klíšťová encefalitida ale také AIDS. Každé z vyvolaných onemocnění, ať už je více či méně závažné, je třeba stále detailněji zkoumat a hledat možnosti další léčby, ale také i způsob jejich včasné diagnózy. K těmto účelům lze použít i studium virových proteinů, které tvoří velkou část viru.

Virový protein

Viry jsou nebuněčné parazitické částice, které jsou schopné se množit pouze v buňkách živých organismů. Viry se dělí dle obsažené nukleové kyseliny na RNA-viry, DNA-viry a retroviry, které obsahují reverzní transkriptázu katalyzující transkripci virové RNA do DNA. Nukleové kyseliny virů jsou uloženy v kapsidě, která určuje tvar viru, umožňuje transport viru na specifická místa a v neposlední řadě chrání genetickou informaci. Mimo kapsidy mají některé viry i obal lipidové povahy. Schématický nákres viru chřipky je uveden na Obr. 1.

Mnoho virů způsobuje závažná onemocnění, jako jsou AIDS, klíšťová encefalitida, mononukleóza, ebola, ale také ichřipka. Chřipka patří mezi často se vyskytující onemocnění a pro většinu populace běžně nepředstavuje závažný problém, oproti tomu u oslabených a náchylných jedinců může působit vážné zdravotní problémy, nebo dokonce úmrtí. Virus chřipky je mimo jiné nebezpečný i schopností vyvolat epidemie až pandemie. Viry a virovými onemocněními se zabývá medicinský obor virologie.

Virové proteiny jsou obsažené zejména v kapsidě [1]. Tyto proteiny mohou přispět k detailnímu poznání jednotlivých virů a přispět k objevu dalších metod léčby jednotlivých virových onemocnění.



Obr. 1: Schématický nákres viru chřipky

Purifikace virových proteinů

Pro purifikaci proteinů je nejdůležitější metodou kapalinová chromatografie. Její použití k tomuto účelu se stále více rozšiřuje, a to i díky vylepšením v této oblasti a na to navazujících novinkách jak v chromatografických médiích, tak v kolonách. Mezi základní purifikační chromatografické metody se řadí gelová chromatografie (založená na rozlišení dle velikosti částic) [2], iontově výměnná chromatografie (dochází k dělení peptidů dle náboje)[3], hydrofobní interakční chromatografie (interakce nepolární stacionární fáze s hydrofobní částí analytu) [4]a afinitní chromatografie, která je založena na tvorbě specifické vazby analytu se stacionární fází [5]. Pro purifikaci proteinů pomocí afinitní chromatografie je velmi často využívána fúzní sekvence jako je např. histidinová kotva (soubor šesti po sobě jdoucích histidinů).Ta se může vázat na kovové ionty, které jsou imobilizované v purifikační koloně a tím může usnadnit purifikaci proteinu. Po několikanásobném promytí nosiče dojde k odstranění nespecificky vázaných proteinů a při použití elučního pufru dochází k uvolnění navázaného proteinu [6]. Podobně se využívají i další fúzní sekvence jako jsou např. glutathion S-transferasa (GST), hemaglutinin (HA), 8 aminokyselinový epitopFLAG. Při použití iontově výměnné chromatografie (IEC)je stacionární fází iontoměnič, který může obsahovat buď kladně (anex) či záporně (katex) nabité skupiny. K dělení peptidů dochází na základě rozdílných isoelektrických bodů. Eluce probíhá na v důsledku změny pH nebo iontové síly. K desorpci dochází na základě změny náboje nebo vytěsněním solemi. Analyty jsou detekovány pomocí UV či fluorescenčního detektoru, na který pro účely purifikace navazuje sběrač frakcí [7].

Charakterizace virových proteinů

Charakterizaci proteinů je možné provést řadou metod, jako jsou gelová elektroforéza, kapilární elektroforéza a zejména hmotnostní spektrometrie. Ta je založena na rozdělení nabitých částic podle jejich molekulové hmotnosti. Vhodnou metodou pro charakterizaci proteinů je z důvodu jejich velikosti ionizace laserem za přítomnosti matrice (MALDI) v kombinaci s detektorem doby letu (TOF). Pomocí specifického enzymatického a chemického štěpení lze při použití MALDI TOF MS vyhodnotit i sekvenci konkrétního peptidu, a to porovnáním hmotností štěpů získaných experimentálně s hmotnostmi štěpů, které byly odvozeny na základě znalostí primární struktury proteinů a štěpících míst [8][9].

Poděkování

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SEPARATION OF INFLUENZA VIRUSES USING MICRO-ULTRACENTRIFUGES

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Abstract

Influenza is a viral infectious disease, having mainly a predisposition to the respiratory system, but also in particular circumstances giving multi-organ complications. Due to the on going volatility and continuous evolution of the biology of the virus, the fight with it, and effective prevention is still a challenge for researchers. There are three types of influenza viruses: A, B and C. Human influenza A and B viruses cause seasonal epidemics of disease almost every winter in the United States. The emergence of a new and very different influenza virus to infect people can cause an influenza pandemic. Influenza type C infections cause a mild respiratory illness and are not thought to cause epidemics.In this short communication, we show the procedure for isolation of influenza viruses by micro-ultracentrifugation

WHD_20_2013

Introduction

Influenza viruses are spherical-shaped enveloped RNA viruses between 80 and 120 nm in diameter classified in Orthomyxoviridae. There are three kinds of influenza viruses depending on serotype: type A, type B, and type C. However antigenic changes within these subtypes results in the extreme diversity of viral strains and makes an annual reformulation of the influenza vaccine necessary [1]. In recent years highly virulent avian and swine flu viruses have been raising serious concerns about flu pandemics[2, 3]. Following is an example protocol for the separation of influenza viruses by means of the S140AT-2035 fixed-angle rotor that is developed for the new Cs 150NX micro-ultracentrifuges (HITACHI).

Material and Methods

Photo: CS150NX Micro-ultracentrifuge

Centrifuge Conditions:

All centrifuge steps were performed using the CS-150NX micro-ultracentrifuges with the S140AT-2035 fixed-angle rotor (up to 10 tubes can be contained) with 1.5 mL open-top polycarbonate (PC) thick-walled tubes.

Separation procedures:

1. Centrifuge the infected allantoic fluid or infected cell culture medium at 6,000 rpm for 20 minutes to remove host-derived coarse foreign substances. Depending on volume, this step can be completed in a floor model superspeed centrifuge such as the S140AT-2035 Hitachi.

Photo: 1) S140AT rotor and 2) open-top polycarbonate thick--walled tubes

2. Pour the supernatant into the 1.5 mL PC thick--walled tubes.

3. Perform centrifugation using the S140AT fixed-angle rotor with the following parameters: 32,000 rpm, 45 minutes, 4°C, Acc.9, Dec. 7

4. Remove the supernatant and add 0.3 mL of Veronal buffer solution including 0.9 mL $CaCl_2$ to the sediment, then suspend again. To minimize the formation of virus clumps, add a small amount of buffer solution to the sediment. Leave it on overnight at 4°C and perform pipetting to resuspend.

5. Layer the concentrated virus fluid on 1.5 mL of 10 to 40% (w/v) sucrose continuous density gradient solution in each PC thick-walled tube.

6. Perform centrifugation using the S140AT fixed-angle rotor with the following parameters: 32,000 rpm, 45 minutes, 4°C, Acc.9, Dec. 7.

7. A white layer is formed slightly above the center of the tube. The virus layer can be observed, in a dark room, by exposing light to the tube. Collect the minimum amount of virus layer.

8. Dilute the fractionated virus fluid with buffer solution about 1.5 times. Layer the diluted virus fluid on 1.5 mL of 30 to 60% (w/v) sucrose continuous density gradient solution in each PC thick-walled tube.

9. Perform centrifugation using the S140AT fixed-angle rotor with the following parameters: 32,000 rpm, 45 minutes, 4°C, Acc.9, Dec. 7. Collect the formed virus layer and dilute it with 2.5 times or more buffer solution.

10. Perform centrifugation using the S50-A fixed-angle rotor with the following parameters: 32,000 rpm, 1 hour, 4°C, Acc. 9, Dec. 7. Add buffer solution to the sediment and resuspend.

Conclusion

This protocol in this brief allows the researcher to efficiently isolate viral particles through a sucrose gradient using the new fixed-angles140AT and CS 150NX HITACHI micro-ultracentrifuge. This protocol allows for the isolation of virus particles in a small volume previously reserved for standard ultracentrifuges and rotors.

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3D TISKÁRNA JAKO NÁSTROJ PRO VÝROBU MOKROFLUIDNÍCH SYSTÉMU (LAB ON A CHIP)

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Abstract

Tato studie se zaměřuje na návrhy mikrofluidních systémů, které umožňují separaci, eluci a elektrochemickou detekci. Byly navrženy 4 mikrofluidní čipy pro elektrochemické analýzy. Pro výrobu mikrofluidního systému byla využita 3D tiskárna a metoda Popyjet. Čipy byly uzpůsobeny pro separaci analyzovaného vzorku pomocí paramagnetických částic. Separace a eluce probíhala v kosúheníkových komůrkách. Pro docílení účinnější separace a následné eluce mají vývodní kanálky esovitý tvar. Vytištěný čip byl osazen elektrochemickým detektorem s klasickým tříelektrodovým zapojením. Jako pracovní elektroda byla použita uhlíková komerčně vyráběná špička, referenční elektroda byla vyrobena z grafitové náplně (tuhy) o průměru 0,5 mm. Pomocná elektroda byla vytvořena z platinového drátku. Použité zapojení a materiály splňují podmínky pro standardní elektrochemické analýzy.

WHD_21_2013

Úvod

Mikrofluidní techniky zaznamenaly v posledních letech prudký rozvoj v důsledku nároků na menší spotřebu reagencií, rychlejší a citlivější analýzy [1]. Výroba těchto zařízení není finančně nákladná a umožňuje integrovat několik procesů analýz (dávkování, míchání, separaci a detekci) do jednoho čipu [2]. Elektrochemické detektory bývají v mikrofluidních systémech upřednostňovány před optickými z důvodu jednodušší a levnější instrumentace [3]. Velkých pokroků v této oblasti bylo dosaženo využitím metod molekulární biologie, pro tvorbu biosenzorů [1, 4]. Mikrofluidní systémy mohou být snadno vytvářeny pomocí 3D tisku. Tato technologie vznikla v druhé polovině dvacátého století. Poprvé byla prezentována Charlesem Hullem, který si v roce 1986 nechal patentovat technologii zvanou stereolitografice. Tato technika je založena na principu trojrozměrného laserového tisku s využitím UV laseru a tekutého polymeru.

V tomto experimentu jsme se zaměřili na výrobu elektrochemických mikrofluidních čipů (lap on a chip) pomocí 3D tiskárny, které umožňují separaci, eluci a elektrochemickou detekci. Čipy byly vytisknuty metodou Popyjet, která vyniká vysokou kvalitu tisku a nízkými provozními náklady.

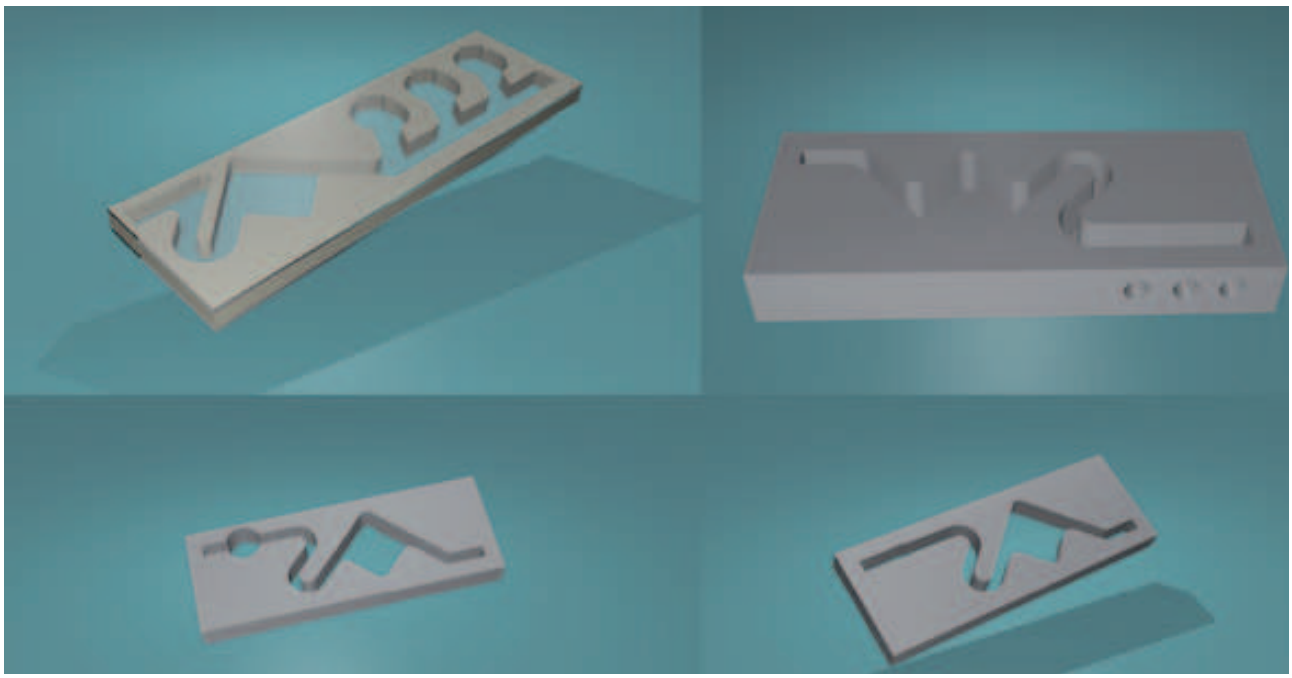
Materiál a metody

Čip byl vytvořen 3D tiskárnou EASY 3D MAKER (DO-IT Strážnice, Česká republika). Pro tisk byl použit polylaktid bílé barvy. Tento materiál byl nanášen extruderem (tavná hlavice tiskárny) při teplotě 210 °C na vyhřívanou podložku (40 °C).

Potřebný tiskový materiál (PLA) i 3D tiskárna (EASY-3DMAKER) včetně softwaru potřebného pro její obsluhu (G3DMAKER) byla zakoupena od společnosti DO-IT s.r.o. Ostatní software, pro tvorbu modelu čipu, byl jako volně šiřitelný stažen z internetu: Blender 2.65 (program je vyvíjen komunitou z celého světa) a netFabb (Parsberg, Německo).

Výsledky a diskuze

Námi navržené čipy, které byly vytištěny metodou Popyjet, slouží k izolaci zkoumané látky pomocí paramagnetických částic a následné detekci. Při jeho návrhu byl kladen důraz zejména na to, aby se paramagnetické částice držely jen v té části čipu, ve které je to žádoucí. O tuto vlastnost se stará komora kosoúhelníkového tvaru a magnet, který je umístěn v dokovací stanici čipu. Pod kosoúhelníkovou komorou se dále nachází ochrana proti úniku a následnému vyplavení paramagnetických částic. Tato ochrana je řešena esovitým zkroucením vývodního kanálku v místě, kde stále působí magnetické pole. Pokud by tedy došlo k úniku paramagnetických částic z kosoúhelníkové komory, tato ochrana by je neměla již dále pustit. V poslední části čipu, těsně před jeho vývodem, se nacházejí elektrody sloužící k zaznamenání elektrochemického signálu v reálnem čase.



Obrázek 1:A – Návrh mikrofluidního čipu s dávkovacími rezervoáry pro separaci a eluci analytu; B – Návrh čipu s elektrochemickou detekcí; C – Návrh čipu pro stacionární měření; D – Návrh čipu pro měření průtokového uspořádání

Závěr

V této práci jsme definovali algoritmus, který umožňuje za pomocí drobných modifikací, výrobu libovolného mikrofluidního čipu. Samotná výroba je omezena přesností tisku 3D tiskárny, což ovlivňuje především velikost a architekturu čipu. Navržené mikrofluidní čipy splňují podmínky pro standardní elektrochemické aplikace.

Poděkování

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CAPILLARY ELECTROPHORESIS OF CYTOSTATIC DRUGS

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Abstract

Since its appearance, capillary electrophoresis (CE) has been widely applied in various areas of analysis – inorganic and organic ions, small organic molecules and/or biomolecules (aminoacids, peptides, proteins, nucleic acids). In this research, applications of capillary electrophoresis on cytostatics drugs were introduced.

WHD_22_2013

Introduction

Anticancer drug monitoring is necessary for the successful development of anticancer drugs and their optimal use in cancer treatment.The first developed methods for the analysis of cytotoxic compounds are based on the use of liquid chromatography with UV detection (LC-UV)[1]. These methods showed satisfactory quantitative performance for the analysis of samples containing high concentrations of target drugs (i.e. development of pharmaceutical formulations, stability studies…). Nevertheless, a sample preparation step allowing a pre-concentration of target compounds has to be applied before the LC-UV analysis when the samples have small amount of cytotoxics. In the 1990s, the high selectivity and sensitivity of mass spectrometry (MS) revolutionized the whole analytical procedure by simplifying and reducing the sample preparation step. Today, LC–MS is one of the powerful techniques for the analysis of anticancer drugs with very effective analytical performance[1]. Moreover, other analytical techniques were also introduced to monitor anticancer drugs such as capillary electrophoresis coupled to UV-detection (CE--UV),amperometric detection, laser-induced fluorescence (CE-LIF) or mass spectrometry (CE-MS)[2-6].Platinum adducts are the critical cytotoxic lesions in DNA after platinum-containing anticancer therapy. Different adducts are formed by the interaction of platinum complexes with nucleotides, but the contribution of individual adducts to the antitumor activity and toxicity of platinum complexes has yet to be fully investigated [4]. The separation and identification of DNA nucleotide platinum adducts by capillary zone electrophoresis, and capillary zone electrophoresis coupled to MS, has been reported recently [7]. The method was used to analyse the formation of adducts between cis--diaminedichloroplatinum (cisplatin) and DNA nucleotides. Besides, CE method was used to determine cisplatin and cis-diammineaquachloroplatinum(II) (recognized as the major aquated metabolite form) in human serum[8]. A combination of two CE modes differing in separation mechanism, micellar electrokinetic chromatography (MEKC) and capillary free-zone electrophoresis (CZE) was applied to acquire the concentration information for characterization of the cisplatin speciation.

Material and Methods

Capillary electrophoresis with UV detection

Capillary electrophoresis system (3DCE Hwelett Packard)with DAD detection (λ1 = 200 nm, λ2 = 272 nm, λ1= 293 nm, λ1 = 360 nm) was used for electrophoretic measurement. Uncoated fused silica capillary (ltot = 64.5 cm, leff = 56 cm and ID = 50 μm) was used. Separation was carried out at 20 kV with hydrodynamic injection for 20 s by 3.4 kPa. 0.2M borate pH 9 wasused as background electrolyte.

Solutions of cisPt in concentrationrange from 0.125 to 1mM and thymine oligonucleotides (5´-TTTTTTTTTTTTTTTTTTTT) with concentration of 125μg/ml and 62.5μg/ml were prepared. Furthermore, thymine oligonucleotide (5´- TTTTTTTTTTTTTTTTTTTT) with the concentration of 125μg/ml was used to mix with cisPt in concentration range from 0.125 to 1 mM (1:1 ratio).

Results and Discussion

The result from electrophoretic measurement of mixture of cisPt in concentrationrange from 0.0625 to 0.5mM and thymine oligonucleotides (5´-TTTTTTTTTTTTTTTTTTTT) with the

concentration of 125μg/ml was shown in figure 1. Figure 1 shows that the height of peak of thymine oligonucleotides changed due to the changes of cisPt concentration.

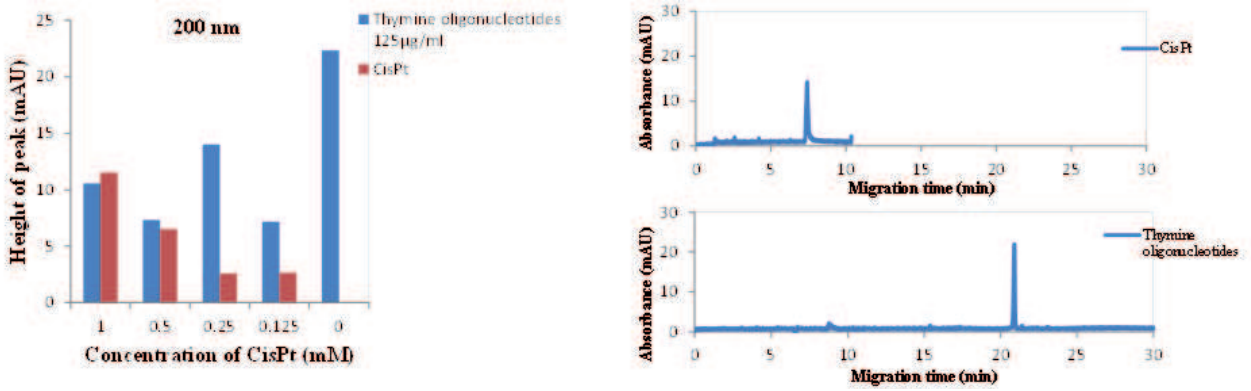

Fig. 1: a) The dependence of the peak heights of cisPt and thymine oligonucleotide on the concentration of cisPt in the mixture, b) Typical electropherogram of cisPt 0.5mM at 200 nm, c) Typical electropherogram of thymine oligonucleotide 62.5µg/ml at 200 nm (for separation conditions see experimental part)

Conclusion

The behavior of cisPt with thymine oligonucleotides (5´-TTTTTTTTTTTTTTTTTTTT) should be investigated in the future. Furthermore, interactions of cisPt with other oligonucleotides containing adenine, guanine or cytosine should also be discovered.

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FIA ANALYSIS OF QUANTUM DOTS

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Abstract

Microfluidic detection of quantum dots is the aim of this experiment. In our laboratory there were prepared Cd, Pb and Cu QDs. For the determination of Cd, Pb and Cu quantum dots the fully automate microfluidic system SFIA-ED has been designed. Flow injection analysis of QDs was preceded by optimization methods for $Cd(NO_3)_2 \bullet 4H_2O$, $Pb(NO_3)_2$ and $Cu(NO_3)_2 \bullet 3H_2O$. These quantum dots were used for labeling and detection of the specific influenza virus H5N1 protein. The concentration of Cd, Pb and Cu ions were determined by electrochemical methods of differential pulse voltammetry. The measured data were edited in programs PSTAT (Metrohm AG, Switzerland) and GPES (Eco Chemie, Netherlands).

WHD_23_2013

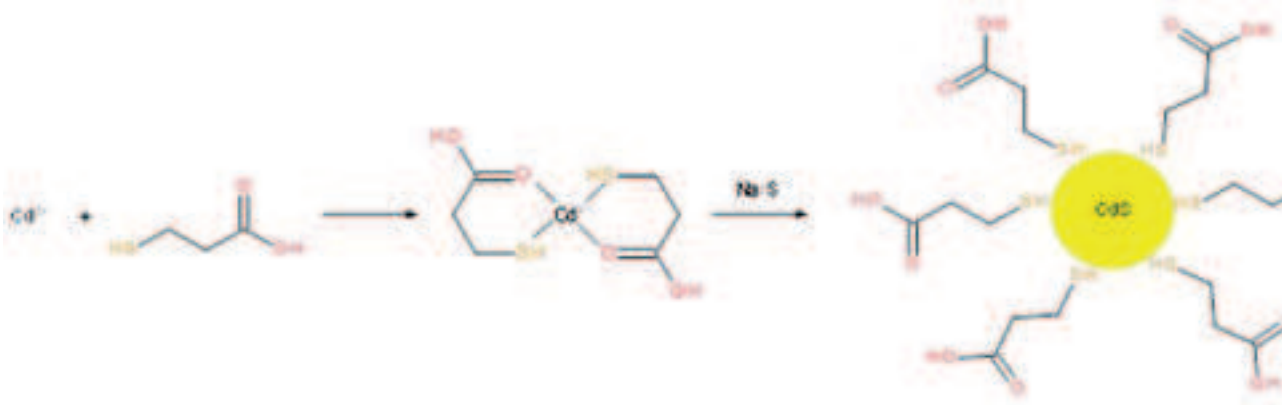
Introduction

QDS could be effectively used as a biosensor for the detection of viruses too. [1] One such virus is the influenza virus.Influenza represents one of the greatest threats in society today [2, 3]. The World Health Organization (WHO) initiated the Global Influenza Program, which provides member states with technical support, strategic guidance and coordination of activities essential to better prepare healthcare systems against seasonal, zoonotic, and pandemic influenza threats to populations and individuals (http://www.who.int/influenza/en/) [1, 3-5]

This study aimed to develop a low-cost isolation and rapid CdS, PbS and CuSquantum dots-based biosensor to detection of specific influenza protein – hemagglutinin (HA). Influenza virus contains two major surface proteins, hemagglutinin (HA) and neuraminidase (NA). HA mediates glycan receptor binding and membrane fusion for cell entry. NA functions as the receptor destroying enzyme in virus release [6]. Viral quantity was determined through cadmium(II), lead(II)and copper(II) ion concentrations abstracted from quantum dots using the system of stopped flow injection analysis (SFIA).[2, 7, 8] The main attention was focused on quantum dots detection and optimization of the whole system.

Material and Methods

The first step of the experiment was preparation of quantum dots (QDs). CdS, PbS and CuS quantum dots were prepared with a slightly modified method published in [9]. The next step was the preparation of samples. A/H5N1/Vietnam/1203/2004 protein (accession no. ISDN 38687, Prospec-Tany TechoGene, Tel Aviv, Israel), (200 µL, 100 µg/mL) was mixed with a solution of CdS, PbS, CuS QDs (100 µL).Followed was separation of samples using magnetic nanoparticles and detection of metal parts.The next step was a flow injection analysis. Microfluidic system for fully automated electrochemical detection was suggested (SFIA-ED).The electrochemical flow cell includes one low volume (1.5 µl) flow-through analytical cell (CH Instruments), which consisted of doubled glassy carbon (GC) working electrode, Ag/AgCl electrode as the reference electrode and output platinum tubing as an auxiliary electrode. Electrochemical flow cell was connected to miniaturized potentiostat 910 PSTAT mini (Metrohm, Switzerland) as a control module. The differential pulse voltammetry (DPV) as measuring method was used and parameters were as follows: initial potential -1.2 V, end potential -0.2 V, modulation amplitude 0.05 V, step potential 0.001 V. All experiments were carried out at laboratory



Scheme 1. Formation of CdS QD covered by 3-mercaptopropionic acid. Possible structure of intermediate chelate formed in reaction of Cd2+ and 3-mercaptopropionic acid is depicted

temperature. Acetate buffer (0.2M, pH 5) was used as the supporting electrolyte. Every measuring sample consists from 15 µl of sample solution which was diluted in aceta-

te buffer. The data obtained were processed by the PSTAT software 1.0 (Metrohm, Switzerland). Flow injection analysis of QDs was preceded by optimization methods for $Cd(NO_3)_2 \cdot 4H_2O$, $Pb(NO_3)_2$ and $Cu(NO_3)_2 \cdot 3H_2O$. The calibration curves of cadmium, lead and copper were measured in the concentration range from 0.02 µM - 1.56 µM. Optimized parameters were: time of accumulation (30, 60, 120, 180, 240, 360, 420 sec.) and deposition potential (-1.2, -1.1, 1.0; -0.9, -0.8 and -0.7 V). Parameters and measurement system was the same as the system for the detection of QDs.

Results and Discussion

Isolation procedure of influenza protein using specific glycan binding had the influence on the amount of quantum dots bounded with protein after this procedure. It was expected decrease of detected electrochemical signal because the effectiveness of the individual steps through the procedure is not completed. This phenomenon was the subject of our interest. Fig. 1A, 1B and 1C show the influence of applied concentration of QDs, respectively theirs metal parts, to the detected amount of individual metal related to the protein amount. The blue columns represented the individual metal concentration detected in the product of isolation process. These dependences had linear character for all three types of QDs, for cadmium as follows: $y = -0.0002x + 0.0009$, $R^2 = 0.998$; for lead $y = -0.0008x + 0.0041$, $R^2 = 0.998$ and for copper $y = -0.0002x + 0.0009$, $R^2 = 0.966$. It is obvious that these dependences had very similar equations (with minimum differences) because the detected metal concentration was related to the protein amount.

The red columns represented the purified part of the mixture, the metal concentration which was lost through the isolation process. The dependence for each quantum dots had polynomial character with reliability R^2=0.99. Specifically the cadmium dependence was as follows: $y = 0.0145x^2 - 0.1322x + 0.3155$, $R^2 = 0.994$; lead dependence: $y = 0.0145x^2 - 0.1315x + 0.3122$, $R^2 = 0.994$; copper dependence: y = 0.0145x2 - 0.1322x + 0.3156, $R^2 = 0.994$. The cadmium and copper dependences showed very similar equations. For the other site the lead equation differed from the others (mainly in the second member of the equation). This fact is in accordance with the difference in linear equations at the detected metal concentrations (blue columns) where the direction at lead QDs is differed from the others.

Fig. 1D, 1E and 1F show the dependences of detected metal concentrations on applied protein concentration which create complex with individual QDs. The protein amount was related to the 10 µl volume which corresponds to the one dosage of mixture in the electrochemical cell in SFIA system. The obtained dependences had polynomial character, for cadmium as follows: $y = -21.943x^2 + 16.605x - 0.2389$, R^2=0.98; for lead $y = -46.469x^2 + 37.634x - 0.4304$, R^2=0.98 and for copper $y = -43.455x^2 + 30.626x - 0.4429$, R^2=0.96.

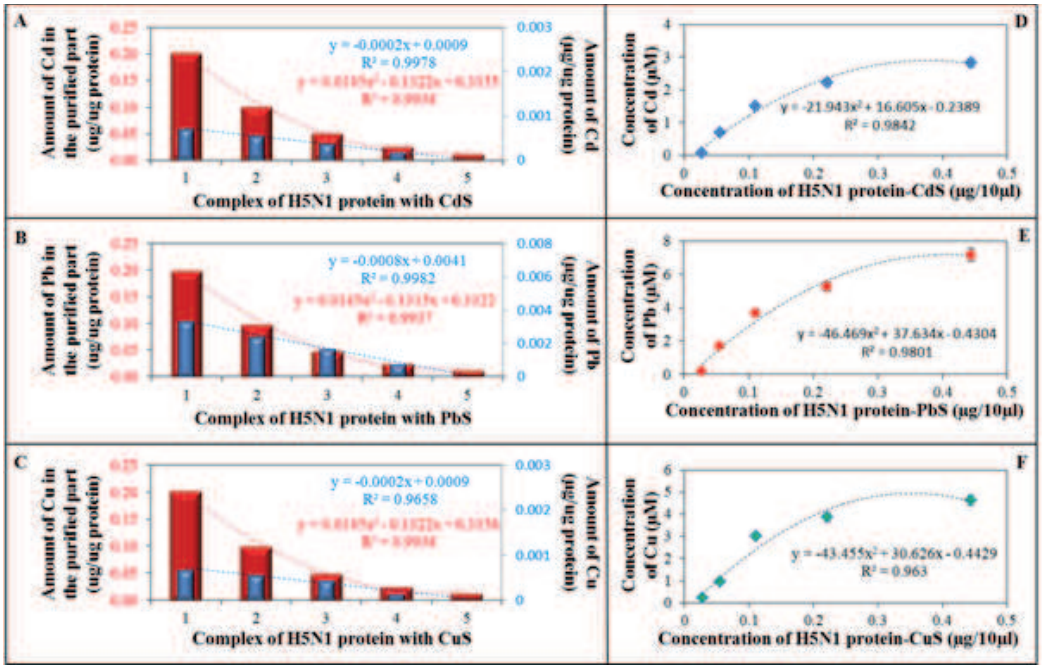


FIGURE 1 (A), (B), (C) Dependence of cadmium, lead and copper concentration (relate to protein amount) on various concentrations of complex H5N1 protein (protein concentration is 0.0444; 0.0222; 0.0111; 0.0055; 0.0028 µg/µl) with CdS, PbS, CuS quantum dots (five concentrations: 0.2025; 0.1013; 0.0506; 0.0253; 0.0127 µg/µg of protein). Blue part correspond to real measured signal of Cd(II), Pb(II) and Cu(II). Red part represend concentration of Cd(II), Pb(II) and Cu(II) in the purified part of solution. (D), (E), (F) Dependence of Cd(II), Pb(II) and Cu(II) detected concentration on H5N1 protein complex with individual quantum dots concentration. Differential pulse voltammetry on glassy carbon (as a working electrode) as a measuring method was apllied Reassembly route of preparation of drugs in apoferritin cavity

Conclusion

Differential pulse voltammetry in connection with microfluidic injection system was proofed as a a suitable method for the electrochemical detection of Cd, Pb

and Cu quantum dots.This system was used for influenza protein determination as a possibility of future biosensor construction.

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Rejstřík

Moderní nástroje pro zobrazování biologicky významných molekul pro zajištění zdraví

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