Biocybernetics and Biomedical Engineering
2011, Volume 31, Number 4, pp. 3–16

Analytical Microsystems for Biomedical and Environmental Applications

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Two types of analytical microsystems for the detection of species of interest in biomedical diagnosis and in environmental monitoring are specifically described in this paper.
We describe a novel device that will measure whole blood concentration of D-dimer, a recognized biomarker of increased blood clotting activity and that will then offer opportunity to use the test in the point of care setting. The device combines innovation in antibody bio-engineering for high specificity immunoassay-based diagnostics and nano/micro engineered impedimetric analysis electrodes incorporating a biocompatible polymer substrate with development of a disposable microfluidic manifold, enabling diagnostics at the point-of-first-contact.
The feasibility of a generic microsytem integrating a microfluidic system of concentration and a module of electrochemical detection is demonstrated for the four metals of the European directive (DCE 2000/60/EC) for the quality of water resource: cadmium, mercury, lead and nickel.

K e y w o r d s: analytical microsystems, fluidic microsystems, Electrochemical Impedance Spectroscopy, Deep Venous Thrombosis, water resource, heavy metals, diamond like carbon

1. Introduction

Analytical microsystems allow to integrate the different functions necessary for the detection of species of interest in a biomedical or an environmental sample: uptake of the sample, temperature control, filtration, mixture, preconcentration, separation, labeling, quantification… on a single chip.Two types of analytical microsystems for the detection of species of interest in biomedical diagnosis and in environmental monitoring are specifically described in this paper.

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Received 29 November 2010; accepted 31 March 2011

Diagnosis and hence treatment of disease is often reliant on an interpretation of chemical parameters within biological samples. Currently many of these measurements are performed in centralized laboratories, requiring specialized equipment and expert personnel. This approach, whilst delivering high quality results, can delay diagnosis and hence delay commencement of treatment. There is an emerging requirement for diagnosis to be made at point of first contact with the patient, requiring clinical measurements being made outside the laboratory using compact, portable devices that can be operated by non-specialised people. This approach, known as point-of-care testing, is particularly applicable when there is a need for rapid diagnosis and fast initiation of treatment.

Monitoring of water resources and natural environments is a major environmental challenge for the coming years involving numerous actors (local governments, water agencies, regulators, managers of water treatment, citizens). In particular the Framework Directive on Water WFD 2000/60/EC, applicable in France and Europe, requires knowledge and monitoring of a large number of substances called priority with the objective, the return to good status of water bodies before 2015. In this context it is essential to develop tools to monitor in real time the condition of water bodies.

A chemistry of the environment to learn about the quality and operation of water systems must adapt to their dynamic and geomorphological features. To do this we must develop methods of investigation with the following qualities: economic resources to be able to increase the number of measurements; self to limit maintenance costs, fast to follow transient events, reliable and accurate in order to keep the quality of current measurements and finally non-disruptive not to modify the concentrations around the point of measurement.

If the analysis in laboratory provides deployment of a wide range of analytical techniques allowing accurate detection of the specific analytes, it does not meet these requirements. Development of the micro-analytical systems suitable for continuous measurements, opens up an innovative mean for quantification of pollutant flows in the environment and their monitoring in real time.

2. Medical Diagnosis Device for Deep Vein Thrombosis

Venous thromboembolism represents a single disease entity with two patterns of clinical presentation: deep vein thrombosis and pulmonary embolism. The deep vein thrombosis (DVT) is an internal clot formed in one of the body's deep veins, usually one of those in a leg. If a part of the clot breaks free and moves into the lung, it can lead to the pulmonary embolism (PE), an often a fatal condition. The venous thromboembolism afflicts an estimated 71 per 100,000 persons yearly, and DVT and PE are major causes of unexpected mortality in hospitals throughout the Europe [1–4]. It is essential to make the diagnosis quickly and accurately and to start the treatment promptly.

During 2005 number of the reported incident cases of DVT and PE totalled 1.3 million in France, Spain, Germany, Italy, United Kingdom, Japan and United States alone. This figure is expected to rise to 1.4 million per annum by 2011, due to increase in population. Within these countries it is estimated that the healthcare systems invest up to €6.6 billion annually in assessment of patients suspected to be suffering from the thromboembolitic event, whose diagnosis proves to be negative.

Clinical practice guidelines [5–7] indicate that accuracy of the diagnosis of DVT/PE by measurement of D-dimer concentration in combination with radiological or laboratory diagnostic tests is superior to traditional methods of diagnosis [8, 9]. Hence, D-dimer, a recognised biomarker for the diagnosis of a thrombus, is routinely measured in hospital laboratories using a traditional ELISA technique. D-dimer tests are generally highly sensitive but not highly specific, suggesting that the negative tests are much more useful for ruling out DVT than are the positive tests for ruling in DVT [10–13]. The negative predictive value (proportion of patients with a negative test who do not have DVT) for patients with a negative D-dimer blood test and a low clinical probability of DVT is higher than 99% [13–15].

However since the most quantitative D-dimer tests are performed in the laboratory, these can generally only be offered in a hospital clinic hence patients cannot be fully assessed within primary care: they are referred for assessment to a secondary care setting, such as a clinic or hospital, where a combination of laboratory based blood test and expert clinical assessment is performed. This causes potentially life-threatening delays to the diagnosis and commencement of the treatment for patients who do indeed have a blood clot; it also causes unnecessary delays for patients who do not.

An analysis of the current medical diagnostic practice and the available tests indicates that practitioners in primary care, such as GP surgeries and community clinics would welcome access to a reliable point of care D-dimer test as it would improve quality of the care that they can provide to their patients, improve DVT healthcare diagnosis and treatment services, and hence improve patient outcomes.

Development of an innovative medical diagnostic device to measure whole blood D-dimer concentration at point of care is described. It comprises a novel immunochemical biosensor and detection system within a disposable cartridge and with a reader for data management and transfer.

The development of a thin film biosensor, comprising an antibody captured onto the surface of a printed electrode and capable of measuring of concentration of a biomarker in whole blood, is described and development of the impedimetric detection, with the appropriate blood handling, for measurement of the biomarkers in whole blood is discussed. Concentration of D-dimer antigen in a sample of whole blood is measured by a novel impedimetric measurement system. The biosensor and detection system lie within a disposable cartridge, containing the innovative whole blood handling capabilities and the test and data management and transfer system are held within the base unit.

Finally, the development of a disposable liquid handling cartridge, with a microfluidic system designed to manipulate a whole blood sample and capable of delivering the sample, in appropriate form, into the impedimetric measurement system, is described.

2.1. Detection of the Biomarker in Whole Blood Using a Thin Film Biosensor with Impedimetric Detection

Whole blood D-dimer concentration is measured through detection of change in impedance at the measurement electrode as a result of binding of D-dimer molecules to antibodies that have been bioengineered and immobilized onto the electrode surface, thus creating a highly specific immunoassay.

2.1.1. Development of the Biosensor

Biosensors increasingly use thin films incorporating active biological molecules. Immobilisation of the active molecules on the transduction part can take a number of forms; for example, as monolayers directly attached to the sensor, as components in thin polymer films, or immobilised on supports, such as nanoparticles. In electrochemical biosensors the active layer is interfaced with electrodes which react to changes in the electronic structure of the active layer, and which are detected by the electrochemical measurement. These changes, which are produced by chemical interactions between the molecules in the active layer and the analyte of interest, can be detected using a number of measurement techniques [16].

Commonly, microelectrodes produced from suitable conducting materials, such as gold, platinum, and silver are used as the interface electrodes. However, the development of techniques such as nanoimprint lithography (NIL) [17], focused ion beam (FIB) milling [18] and laser ablation, complemented by high-resolution etching techniques such as reactive ion etching (RIE) [19], have lead to the production of nanoelectrodes [20]. These nanoelectrode geometries potentially have advantages with respect to their micro-fabricated analogues because they have improved spatial resolution and reduced signal noise [21].

Interfacing of the active layer molecules to nanoelectrodes is not a trivial subject, and we describe our evaluation and selection of an appropriate technique. For example, if the molecules can be incorporated into a monomer precursor, they can be immobilised onto the electrodes using electropolymerisation [22]. In this case, the polymer itself may need to be conducting to facilitate electron transfer to the electrode surface [23]. Common electrically conducting polymers used in sensing applications include poly(pyrrole) [24] and poly(aniline) [25] and their derivatives. The active (bio)molecules can be covalently bonded to the polymers, using suitable coupling reactions, which may involve the use of reactive moieties, such as amine, acid or thiol groups, or simply immobilised in the polymer matrix. Positional control

of the active layer using this technique is facile, as the polymer grows predominantly on the electrode surface. However, the addition of the supporting polymer in the sensing system introduces a level of complexity when modelling the sensors and when attempting to produce a number of sensors with high reproducibility.

Direct immobilisation of the active (bio)molecules to the surface of an electrode can be completed using covalent bonding. One of the most commonly used techniques for this is the interaction between a thiol group and a gold electrode. For example, this technique has been extensively used for immobilisation of the (bio)molecules through self assembly [26]. Such self-assembly can be used to immobilise biological material [27] and can be positionally controlled using a soft lithography method, called micro-contact printing [28], down to nanometric scales [29]. Formation of a thiol linkage between the gold and the thiolated (bio)molecule means that the active molecule is directly linked to the electrode. Non-specific immobilisation either side of the electrodes should be avoided as long as the thiol group has no affinity for the underlying substrate. The same molecules can be deposited using dip-pen lithography [30].

We have produced the nanoelectrodes on the polymeric substrates and describe immobilization of the active (bio)molecules using electropolymerisation, micro-contact printing or dip-pen lithography techniques, ready for incorporation of the functionalised electrodes into our microfluidic cartridge system for the detection of D-dimer in whole blood at levels of < 300 ng/ml. A variety of data extraction approaches are available including the measurement of impedance across a number of frequencies [31].

2.1.2. Electrochemical Impedance Spectroscopy (EIS) Label-free Detection

To achieve a sensitivity of 1ng/ml in a range of 0–3000 ng/ml of D-dimer in whole blood there is a need to measure impedance change on the electrode with high precision.

Electrochemical Impedance Spectroscopy (EIS) is a rapidly developing electrochemical technique for the characterization of biomaterial-functionalized electrodes and especially for the transduction of biosensing events at the electrodes. Experiments have indicated that antibody loaded electroconductive matrices (particularly polypyrrole) give concentration dependant responses when interrogated with EIS [32, 33]. The electroconductive matrix presents a high surface concentration of grafted biomolecules and the change of conformation brought by biological recognition induces a change of the polymer conjugation, which results in amplification of the electrical phenomenon. A particular enhancement of the sensitivity of detection was observed in presence of a redox probe [34]. EIS, under appropriate experimental conditions, represents a label-free technique for immunodetection.

We have developed an approach to optimize the orientation control of the reduced antibody-scFV fragments in the electroconductive matrix. Further, we control

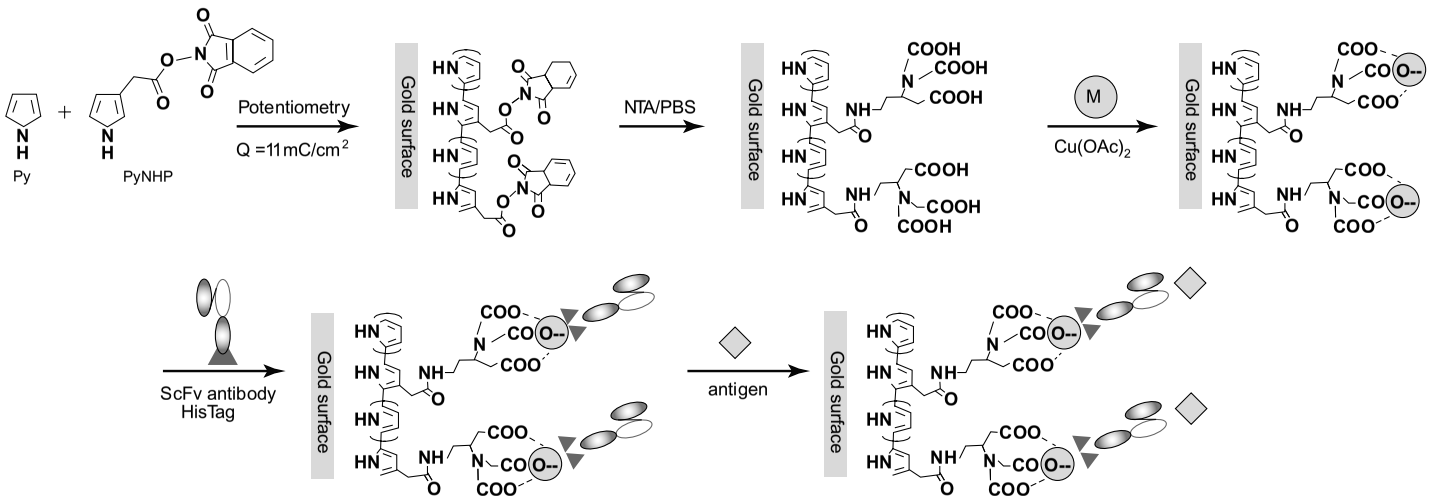

Fig. 1. Immobilisation procedure of the reduced antibody-scFV fragments on the NTA functionalized polypyrrole polymer layer

the electronic exchange by inclusion of a specific redox species (copper ions) in the polymer structure, in order to enhance the sensitivity of detection and limit a non specific adsorption onto the functionalised electrode surface, by grafting a specific group onto the pyrrole monomer. The immobilization procedure of the reduced antibody-scFV fragments is presented in Fig. 1.

The impedance of the biofunctionalised electrode is modified when different concentrations of D-dimer comes in contact with the reduced antibody-scFV fragments as presented in Fig. 2. The Nyquist diagrams represent the imaginary part of the impedance versus the real part of it.

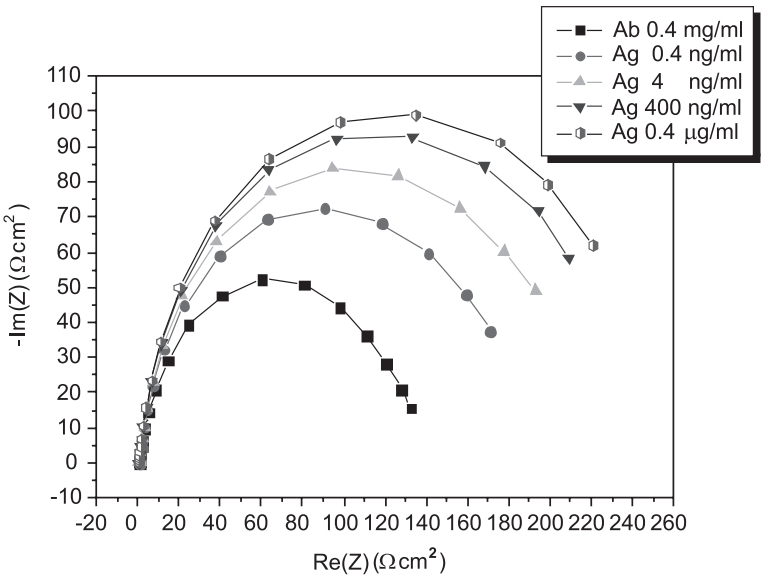

Fig. 2. Nyquist diagrams of the biofunctionalised electrode before and after contact with increasing concentrations of D-dimer

The equivalent electrical circuit of the biofunctionalised electrode/electrolyte interface is presented in Fig. 3.

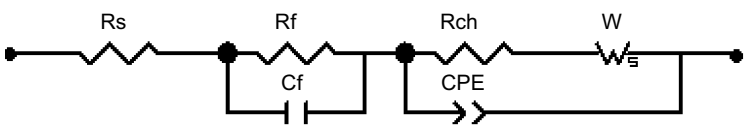

Fig. 3. Equivalent electrical circuit of the biofunctionalised electrode/electrolyte interface.
Rs – solution resistance, Cf – capacitance of the copolymer film, Rf – polymer film resistance, CPE – constant phase element, Rch – charge transfer resistance, W – Warburg Impedance

The charge transfer resistance (Rch) is the parameter highly influenced when the antibody-antigen complex is formed. Its variation versus the concentration of D-dimer allows to determine the calibration curve of the immunosensor. Its analytical features are the following ones: Detection limit: 100 pg/ml, Dynamic range: 0.1 – 400 ng/ml, Sensitivity: 11081 Ω cm^2/(μg/ml).

Comparison between the result obtained with our DVT-IMP device and with a D-dimer chemiluminescent enzyme immunoassay (CLEIA) method, from Pathfast (Mitsubishi Kagaku Iatron, Inc.) in a human plasma sample from a patient gives the following results:

D-dimer level CLEIA- Pathfast : 167 ng/ml, D-dimer level DVT-IMP : 144.56 ±14.25 ng/ml.

2.2. Development of a Novel Microfluidic System, Device and Reader

2.2.1. Disposable Cartridge and Liquid Handling System

We have designed a cartridge to ensure robustness of sample and reagent transfer and, therefore, to focus on fluidic transfer properties rather than additional functionality.

The cartridge is designed and fabricated using established technologies in polymer microfabrication. These include hot embossing to define the microfluidic channels and chambers, photolithography and nanoimprint lithography to structure the impedimetric electrodes and laser ablation for the passivation layer. A photo of the cartridge is presented in Fig. 4.

Our design and choice of mass manufacturing approach takes into account consideration of future scalability.

The nano- and microelectrodes for the impedimetric measurement are housed in a sample chamber that can be accessed from both sample and reagent reservoirs.

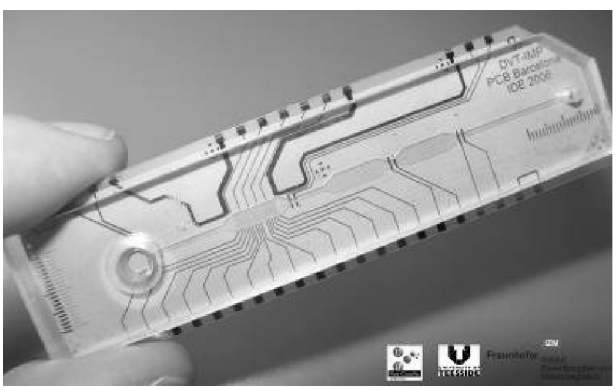

Fig. 4. Photo of the cartridge including: 5 sets for redundancy/signal averaging and a negative control for calibration of the sensor electronics, temperature control via a feedback loop with a heater incorporated in the reader, fluid manipulation via fill sensors

Immobilisation of the D-dimer antibody onto the electrode is performed in an open cartridge configuration. It facilitates the immobilisation process and allows easy quality control of the biological receptor layers. However, it puts very high demand on the joining of the cartridge parts. We describe our choice of the existing and novel joining processes that minimize temperature, UV and solvent load to the biological receptor molecules while guaranteeing robust functionality of the cartridge.

2.2.2. Reader – Design and Functionality

During the measurement phase the 'cartridge' and the 'reader' will operate as a one compound system to provide all required functionalities. We describe, therefore, our design for the device as a whole, which has been conceptualized and developed to be user friendly, and able to be operated by non-expert users, such as practitioners, paramedics and ultimately by patients.

The reader itself is a microcontroller driven device, part of a complex system consisting of four component parts that, when combined, give rise to a device capable of measuring biomarker concentration under Point-of-Care conditions.

We have developed an approach to drive the blood sample from point of entry to the electrode and then to remove the unwanted blood sample components (plasma, red blood cells, other proteins, etc.) from the measurement site. It relies on fluidic interconnection between the cartridge and the reader and on a micro-pump situated in the reader that will move buffer solution and blood sample through the cartridge.

3. A Generic Microsystem for Monitoring of Quality of Water Resource

We demonstrate feasibility, here the principle of an integrated microsystem, integrating a microfluidic filtration and concentration system with a microdetection module (an electrochemical microsensor). We illustrate the feasibility of our concept by taking the four metals cited in the DCE/2000: Cadmium, Mercury, Lead and Nickel; Cadmium and Mercury are classified as "priority hazardous substances", that is to say, toxic, persistent and bio-accumulative and whose waste should be eliminated before 2015.

3.1. Fluidic Microsystem of Concentrating Extraction

The micro-fluidic system is based on the selective transport of metals in an aqueous phase, the water resource previously filtered containing the metals, into an organic phase receiver. By concentrating first metals initially present in the aqueous phase, it is possible to achieve lower limits of quantification required by the specifications.

Transport through the liquid membrane is performed using a lipophilic ligand specific for ion target. The schematic diagram of the concept of extraction/concentration applied to the detection of a metal (Mt) is given in Fig. 5. The concept has been presented in [35, 36].

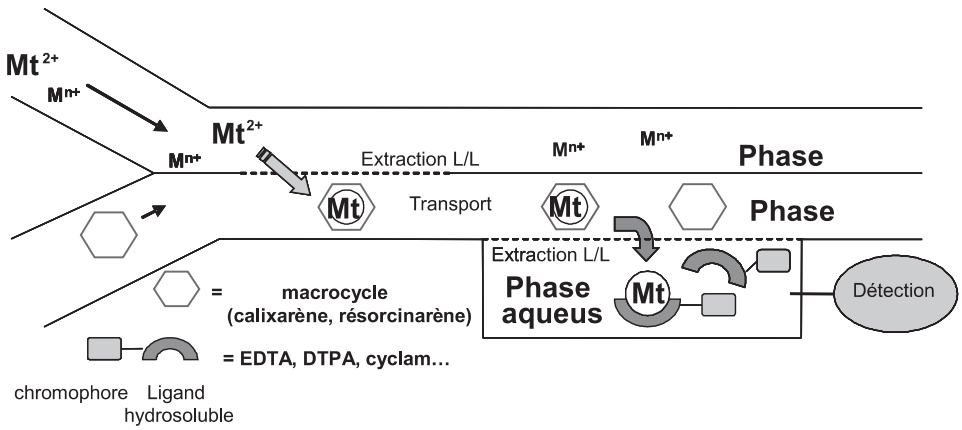

Fig. 5. Diagram of the operating principle of the concentrating extraction process

The metal extraction can be achieved by changing the physicochemical conditions (pH ...) or using a water-soluble extractant incorporating a chromophore and present in an organic receiving phase with a volume well below that of water phase supply. In the process, the receiving phase is enriched with metal when a large amount of aqueous phase to be analysed passes through the component.

3.2. Electrochemical Microsensor

The previous findings have shown that thin films of Diamond Like Carbon (DLC) are excellent candidates for using the method of stripping voltammetry; detection limits of 1–2 ppb for lead and 0.4–1 ppb for cadmium with an excellent reversibility were obtained, these deposits being performed using CVD technique [37]. This method allows the selective detection of the selectedmetals . The detectable chemical forms are free forms and forms that are weakly complexed which are the forms generally the most bioavailable. The microsystem combining a preconcentration stage with such electrochemical microsensor will lower the detection limits of the overall microsystem by a factor of 30 or better.

However, fabrication of diamond films demands some critical conditions, for example, a high substrate temperature of about 800°C or above [38], which is not compatible with microfabrication of the microelectrode arrays. DLC films are also chemically stable and can achieve designed physical and mechanical properties,

with a Csp^2/Csp^3 ratio depending on the deposition conditions [39] and can be easily deposited at room temperature using the PLD technique. In the present study, femtosecond pulsed laser ablation is used to deposit pure a-C and a-C:B (8%) films under vacuum onto SiO_2/Si and Si_3Ni_4/Si substrates at room temperature. These films were used, for the first time [40], for simultaneous electrochemical detection of four heavy metals namely, Cd^{2+}, Pb^{2+}, Ni^{2+} and Hg^{2+}, using square wave anodic stripping voltammetry (SWASV) technique, in aqueous solution.

Standard solution of 10 ppb concentration of each metal of Cd^{2+}, Pb^{2+}, Ni^{2+} and Hg^{2+} in 0.1 M acetate buffer pH 4.2 were used to evaluate the ASV responses of the (a-C/SiO_2), (a-C/Si_3N_4), (a-C:B 8%/SiO_2) and (a-C:B 8%/Si_3N_4) electrodes. Figure 4 shows overlaid SW anodic stripping voltammogram *I-E* curves for the metals, obtained in 0.1M acetate buffer pH 4.2 after deposition at -1.3 V for 90s. The presented results demonstrate capability of the DLC electrodes to detect simultaneously the four metals with a detection limit of 1 µg/L and a dynamic range from 2 to 25 µg/L for each. The different sensitivities obtained are 63, 218, 11 and 70 $\mu A.\mu g^{-1}.L$ for Cd^{2+}, Pb^{2+}, Ni^{2+} and Hg^{2+} respectively.

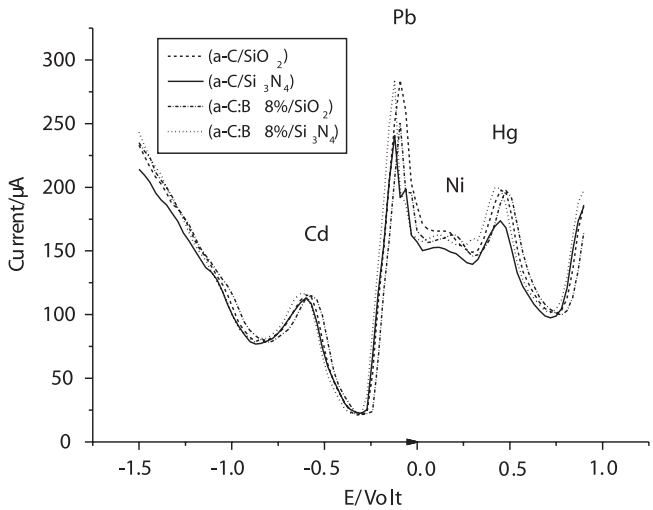

Fig. 6. Overlaid anodic stripping voltammogram (DPASV) curves for 10 ppb concentration of each metal of Cd^{2+}, Pb^{2+}, Ni^{2+} and Hg^{2+} in 0.1 M acetate buffer pH 4.2 with (a-C/SiO_2), (a-C/Si_3N_4), (a-C:B 8%/SiO_2) and (a-C:B 8%/Si_3N_4)DLC electrodes. t_{dep} = 90s ; E_{dep} = –1.3V

4. Conclusion

Two examples of analytical microsystems were presented in this paper: the medical diagnosis device for Deep Vein Thrombosis and the generic microsystem for monitoring of quality of water resource. The focus was done on the electrochemical sensor (materials, fabrication process) and on the microfluidic system (materials,

design), showing the difficulty for the harmonization of the fabrication processes of the different part of the analytical microsystem.

Acknowledgments

This work was supported by European Commission through DVT-IMP project (FP6-IST-34256) and by ANR PRECODD through ANR-07-ECOT-006.

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