JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 9, No. 6, June 2007, p. 1883 - 1889

SPR imaging antimucin-mucin bioaffinity based biosensor as label-free tool for early cancer diagnosis. Design and detection principle

I. C. STANCU[♣a,b,*], A. FERNÁNDEZ-GONZÁLEZ[♣a,c], R. SALZER[a]
[a]*Technische Universität Dresden, Institut für Analytische Chemie, Dresden, Germany*
[♣]*Marie-Curie post-doctoral fellows*
[b]*Polytechnics University Bucharest, Faculty of Applied Chemistry and Materials Science, Bucharest, Romania*
[c]*Universidad de Oviedo, Departamento de Quimica Fisica y Analitica, Oviedo, Spain*

This paper addresses the actual increasing interest in early cancer diagnosis, by describing a novel approach in designing a modern new and efficient *label-free SPR Imaging biosensor* based on the capacity of antimucin antibodies to prove cancer occurrence by monitoring mucin overexpression. The design, functioning principle of the biosensor and the analysis methods are presented. This new generation of sensors is intended as a revolutionary diagnosis tool exploiting the very sensitive and label-free optical SPR Imaging technique, ideally corresponding to the study of ultrathin biomolecular layers and biological interactions. Saliva is used as biologic fluid to be analysed, mainly due to its non-invasive and stress-free collection. Nevertheless, the described Imaging method allows the acquisition of data for the entire probe simultaneously, this affording the enormous advantage of analysing a big number of samples at once, in a very precise, reproducible and brief procedure, easy to implement in any diagnosis centres.

(Received February 27, 2007; accepted April 26, 2007)

Keywords: Bioaffinity, Biosensor, Cancer diagnosis

1. Introduction

In a century governed by a general explosive evolution of science and technology, when the human genome is almost completely decoded, cancer is one of the biggest health-problems, unfortunately with an increasing occurrence. Cancer cells appear due to genetic change/damage of normal cells, following complex and multicasual phenomena. If these cells are not removed by natural physiological mechanisms, they form tumours in different body organs. Despite advances in diagnosis and therapy, by the time a patient is diagnosed for cancer, the tumour can be already formed or even worse, the cancer can be already metastasised. This is why one of the most efficient fighting-tool against cancer consists in **the early detection** of the disease.

Several new techniques were developed for this purpose, including tests that are not used by conventional physicians. The long list of currently available cancer detection tests includes: 1) non-specific tests (e.g. CA125 detection, darkfield microscopy, Anti-malignin antibody screen test - AMAS) and highly specific ones (e.g. DR-70 blood tests screening for 13 different cancers, use of gold-coated nanoparticles); 2) already formed tumour detection and monitoring (e.g. computer tomography - CT scan, Positron Emission Tomography – PET, Endoscopic ultrasound, Scintigraphy, Thermography, Magnetic Resonance Imaging – MRI); 3) biopsy involving methods (microscopic and histopathological assessment of carcinomas by stained slides); 4) detection before any biopsy (e.g. T/Tn Antigen test, Magnetic Resonance Spectroscopy - MRS); 5) body fluid analyses (e.g. evaluation by Enzyme-Linked Immunosorbent Assay – ELISA, Biological Terraine Assessment – BTA, DR-70, T/Tn antigen); 6) complementary methods (e.g. ElectroDermal Screening – EDS, Maverick Monitoring Test – MMT). Other new developed methods involve Infrared Raman Spectroscopic Mapping of tumours [1-6]. The general research effort of all the scientific community fighting against cancer is aimed at the development of early diagnosis by non-invasive methods, involving easy collecting samples.

One of the most important and developed field consists in the study of **tumor markers** and their possible role in the early detection and diagnosis of cancer. **Tumour markers** are substances overexpressed / secreted by / produced in contact with cancer cells [7-12], they are formed as cancer grows and are detectable even before it reaches a size big enough for detection by other methods. These immunological screening methods are vital for early medical intervention significantly improving the recovery chances. Cancer researchers are turning to proteomics in hopes of developing better screening and treatment options. Proteomics technology is being used to search for proteins that may serve as markers of disease in its early stages, or predict the effectiveness of treatment or the chance of the disease returning after treatment has ended [7-10]. A wide range of tumour markers is found in

different types of cancer (Table1), although their levels can be altered also in patients with noncancerous conditions.

The standard evaluation of tumour markers is represented by ELISA method [9,12-14]. Continuous research efforts have been made for the development of label-free methods for cancer detection and survey. The development of biosensors for Surface Plasmon Resonance (SPR) represents a recent and revolutionary addition to the existing analytical techniques associated with cancer diagnosis and evaluation [15-20]. The application of SPR in biosensing field started in 1983 [21]. SPR is a fascinating label-free optical technique, widely used as a very sensitive tool in the characterisation of biomolecular interactions including DNA-DNA, RNA-DNA, protein-DNA, protein-protein. The newly developed SPR Imaging can detect the presence of a biopolymer on a previously modified metallic surface, by monitoring the change of the local reflectivity upon phenomena of binding/adsorption of biomolecules onto the surface. This is why this method seems ideally designed for the study of ultrathin biomolecular layers. The main advantage of this technique consists in the exploration of bioaffinity interactions **without using molecular labels** and in **the acquisition of data for the entire probe simultaneously** on a CCD camera. It should be mentioned that currently, SPR biosensors compete with the already existing immunological screening tests, widely used due to their low cost highly specific and sensitive response.

Other highly performing and clever technology for detecting proteins by inducing them to stick to and bend a microscopic cantilever, could serve to detect protein markers indicating different cancer [22]. This new technology makes possible the evaluation of multiple markers in a single reaction, becoming potentially much cheaper to perform than a typical ELISA assay [22]. Another advantage of this technique over current assays such as ELISA is that there is a fluorescent-free method.

In this challenging scientific context, our effort addresses the actual increasing interest in early cancer diagnosis, as an efficient tool for in time fighting against this cruel disease. We present a novel approach in designing a new and efficient ***label-free SPR Imaging biosensor*** to be used for early cancer detection. The design, functioning principle of the biosensor and the analysis method are presented in this communication, as first main step in achieving this complex goal.

Table 1. Cancer marker specificity [11].

Cancer	Cancer marker*
Bile Duct	**CA 19-9**
Bladder	HCGb
Brain	HCGb
Breast	CEA, **CA 15-3, CA 125**, IL-6
Bur kitts Lymphoma	Ferr
Cervical	AFP, **CA 125**
Colorectal	CEA, **CA 19-9, CA 125**, PSAF, HCGb
Gastro	CEA, **CA 19-9, CA 125**
Kidney	AFP, CEA, HCGb, IL-2, IL-6, B2M
Larynx	Ferr
Leukemia	Ferr, B2M
Liver	CEA**, CA 15-3, CA 19-9, CA 125**, HCGb
Lung	CEA, **CA 15-3, CA 19-9, CA 125**, HCGb, Ferr, NSE
Lymphoma	B2M
Multiple Myeloma	IL-2, IL-6, B2M
Neuroblastoma	Ferr, NSE
Ovarian	AFP, **CA 15-3, CA 19-9, CA 125**, HCGb, Il-6, B2M
Pancreas	AFP, CEA, **CA 15-3, CA 125**, HCGb
Prostate	PSA, PSAF, PSAC, PAP, B2M, A2M
Testicular	AFP, PAP, HCGb, Ferr,
Thyroid	HTG, NSE
Uterine	CEA, **CA 19-9, CA 125**, HCGb
Wilms Tumor	HTG, NSE

*AFP = alpha fetoprotein, CEA = carcinogenic embryonic antigen, CA 15-3 = carbohydrate antigen 15-3, CA 19-9 = carbohydrate antigen 19-9, CA 125 = carbohydrate antigen 125, PSA = free prostate specific antigen + prostate specific antigen - alpha(1)antichymotrypsin complex, PSAF = free prostate specific antigen, PSAC = prostate specific antigen - alpha(1)antichymotrypsin complex, PAP = prostatic acid phosphatase, hTG = human thyroglobulin, hCG? = human chorionic gonadotropin beta, Ferr = Ferritin, NSE = neuron specific enolase, IL-2 = interleukin 2, IL-6 = interleukin 6, A2M = alpha 2 macroglobulin, B2M = beta 2 microglobulin

Saliva use advantages

The traditional biological samples used as diagnostic media are blood and plasma for circulating concentration and urine for accumulated concentration of analytes. One of the most accessible body fluid to obtain due to non-invasive, stress-free and sufficient volume collection, saliva, was suggested as an alternative for therapeutic and toxicological analyses as early as 1970 (Gorodetzky and Kullberg, 1974, cited in [9]). Saliva is still today a seriously underutilized sample with great potential for biomedical testing. The main advantages of saliva use for cancer diagnosis are well underlined by Lipps [12] as follows: 1) possibility of early cancer diagnosis based upon the concentration of PCM (proteonic cancer marker) versus CT scan of already developed tumours; 2) specific type of cancer prediction; 3) possibility of PCM evaluation before and after chemotherapy, versus the current practice of measuring the size of the tumour; 4) avoiding X-ray exposure and replacement/reduction of classical CT. Furthermore, the storage and shipping costs are lower when compared with those for serum and urine [20]. Despite these undoubtedly important advantages, saliva is still considered as a very challenging diagnostic medium since the concentrations of main marker proteins tend to be lower than in blood or urine. The detection of most of PCM in the salivary environment is very complicated by the presence of contaminants such as mucins, degraded proteins, bacteria and undigested food particles [20]. But if considering mucin as a tumour marker molecule and evaluating its concentration in saliva, these difficulties would disappear. Of course, the remaining question is ***How and why mucin***?

Mucins are a family of large (molecular weights of over 1000 kDa), heavily glycosylated proteins (approximately 80% of the mass of mucins are carbohydrates) involved, for several years, in the pathogenesis of cancer, especially adenocarcinomas [19,23]. Mucin is a good candidate as PCM, since its overexpression or aberrant glycosilation pattern is usually correlated, as shown also in Table1, to the presence of tumours as breast, ovarian, pancreatic, lung, gastric, colorectal, cervical, uterine and liver carcinomas [8,11,23-27]. On the other hand, this protein is easy to identify from saliva, as shown in Table 2, when compared with more complex composition of plasma. The most common mucin-PCM are CA 125, CA 19-9 and CA 15-3. Two of the most well-known serum diagnosis assays for adenocarcinomas recognize epitopes of mucins. CA 15-3 is a circulating antigen from the family of polymorphic epithelial mucins – PEM, produced by the gene MUC 1. CA 19-9 recognizes the sialyl Lewis A oligosaccharide structure [27]. CA 125 is a mucin produced by the gene MUC-16 [7,27]. From the four most studied mucins, the most well characterised is a transmembrane protein presenting 20 amino-acids repeating tandem (rich in the hydroxy-amino acids threonine and/or serine), affording the cancer-specific recognition by appropriate antibodies [19,28].

In the present work, antimucin antibodies are used for the development of SPR imaging sensors for monitoring mucin overexpression, as PCM proving cancer occurrence. As already highlighted, the main aim is to create a functioning SPR Imaging cancer-diagnosis technology, easy to implement in any laboratory, that presents the following properties: 1) sensitivity - high enough to perform useful diagnosis tests on saliva; 2) robust and simple to use; 3) low cost to be compatible with the needs of the clinical diagnostics market. This goal will be achieved by optimising the sensor design with respect to different areas: fabrication, mode of operation, ease of use and data analysis/calibration.

Table 2. Parametric correlation of saliva to plasma (Ritschel and Thompson, 1983) [9].

Parameter	Saliva	Plasma
Volume	500-1500 ml/day	4,3% of body volume
Rate of flow	0,6 (0,1-1,8) ml/min	
pH	6,7 (5,6-7,9)	7,4
Water [%]*	98 (97-99,5)	91,5 (90-93)
Total protein [g/100ml]*	0,3 (0,15-0,64)	7,3 (6-8)
Albumin [g/100 ml]*		4,5 (4-5)
Mucin [g/100 ml]*	0,27 (0,08-0,6)	
Amino acids [mg/100ml]	0,1-40	0,98
Potassium [mM/l]*	8-40	3,5-5,5
Sodium [mM/l]*	5-100	135-155
Calcium [mM/l]*	1,5-2	4,5-5,5
Phosphate [mM/l]*	5,5-14	1,2-2,2
Chloride [mM/l]*	5-70	100-106
Cholesterol [mg/100ml]*	7,5 (3-15)	150-300
Dry substance [g/l]*	6(3,8)	80

*Large variations of some constituents are due to different collection techniques/devices/flow rates.

2. Materials and methods

Materials

Substrate. For the biosensor substrate we use commercially available SPR gold-slides (sSens, Netherlands). These SPR sensors are based on borosilicate glass with a refractive index of 1.51 and a thickness of 0.3 mm. The dimensions of these sensors are 10x10 mm and they are compatible with common, commercial SPR instruments and also with modular set-ups. The gold evaporation procedure that is applied to manufacture the substrates was optimised with respect to gold layer thickness and crystallinity. For good adherence of the gold to the glass a thin titanium layer was applied [29].

Gold substrate should be used immediately after rinsing with piranha solution (75% of concentrated H_2SO_4 and 25% of 30% H_2O_2) for approximately 1 minute, followed by distilled water cleaning and drying under a nitrogen stream. (Recommended time for rinsing with piranha is up to 5 minutes, in order just to remove the contaminants and to avoid the increase of the roughness of the gold surface [30].)

Thiols. Different thiols, with different chain length and amine- or carboxyl ending groups can be used: Cysteamine, 3-Mercaptopropionic acid, 16-Mercaptohexadecanoic acid and 11-Mercaptoundecanoic amine. The thiols can be used without further purification, as 1mM freshly prepared ethanolic solution.

Activation mixture: For the activation of the carboxylic goups, N,N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), as 20mM and 5 mM in phosphate buffer solutin, pH 7,4.

Sensing phase. Monoclonal Anti-Human Gastric Mucin, Clone 45M1 antibody can be used as solution in phosphate buffer pH 7,4.

Proteins. Mucin (M) from porcine stomach (Type III partially purified powder) can be used. Bovine Serum Albumine (BSA) will serve as surface blocking agent against non-specific interactions.

Phosphate buffer. Phosphate buffer pH 7,4 is prepared by using 0,0312% monosodium phosphate monohydrate and 0,2075% disodium phosphate heptahydrate in distilled water. Phosphate buffer pH 6.8 can be prepared by using 0,1214% monosodium phosphate monohydrate and 0,0322% disodium phosphate heptahydrate in distilled water.

Analysis sample. In order to validate the proposed biosensor principle, synthetic media mimicking saliva with respect to pH and containing variable mucin content will be used. After principle validation, real saliva samples can be used.

Methods

Biosensor design

The key step in the design of the biosensor consists in the optimisation of the immobilisation of the antibody on the gold slide.

A. Antibody immobilisation

Self assembled monolayers (SAMs) are created on neat gold via direct exposure (immersion or spotting) of the metallic surface to 1mM freshly prepared thiol solution in ethanol (Fig. 1, way 2). Different thiols can be used, containing amine- or carboxyl-ending groups: Cysteamine, 3-Mercaptopropionic acid, 16-Mercaptohexadecanoic acid and 11-Mercaptoundecanoic amine. Thiols can be used as purchased, without further purification. Thiol spontaneous adsorption and self-organization on gold is considered achieved after 24h, at room temperature. Teflon dishes are recommended in order to avoid any parasite retention of mercaptans on glass during SAMs formation. When removed from the adsorption solution, the modified gold should be extensively cleaned with a low speed flow of ethanol and then dried with a stream of dry nitrogen or kept in liquid media. After preparation, the samples can be characterized by Contact Angle goniometry, Ellipsometry, X-ray Photoelectron Spectroscopy, Atomic Force Microscopy and SPR angular interrogation and Imaging. These techniques can provide the necessary information in terms of molecules homogenous repartition on the sensor surface, molecules self-assembling, SAMs homogeneity and thickness etc.

Following SAMs formation, the antibody molecules are stoichiometrically bound on the thiol SAMs (way 3, Fig. 1), after a corresponding activation with EDC/NHS mixture (30 minutes, room temperature, dark). The immobilisation can be achieved by direct exposure of the SAMs-covered slides to the protein solution (inside a flow cell or by spotting), for 3 hours, at room temperature. Several concentrations (e.g. 5, 10, 25, 50, 100 ppm in phosphate buffer pH 7.4) should be used and evaluated for optimising the antibody immobilisation procedure, in terms of homogenous binding and sufficient sensitivity for SPR Imaging. Rinsing with phosphate buffer solution pH 7.4 must be performed at the end of the incubation time, to remove any non-reacted molecules.

B. Design features

The biosensor active surface will contain a big number of antigen recognition spots in order to allow an increased number of samples/patients evaluation at once. This will increase the practical/economical efficiency of the diagnosis tool by making possible the illness detection for a big number of samples in a single-step fast method.

SPR Imaging

The SPR Imaging principle was briefly described in the Introduction, with emphasis on the label-free detection and simultaneous detection of the whole surface of the array.

Among commercially available devices, the two GenOptics systems (InteractorTM and SPRiLAB TM) are the most commonly known. The research teams in the field of SPR imaging are usually building up their own modular devices.

Considering the idea of implementing this technology in any laboratory we have designed a SPR Imaging modular set-up. The scheme is presented in Fig. 2. Monochromatic light (wavelength 632,8 nm) is provided by a He-Ne laser LGK 7653-8 (LASOS GmbH, Ebersberg, Germany) (1 in Fig. 2). The laser beam is p-polyrised (2 in Fig. 2) and then expanded 10 times using a collimator (3 in Fig. 2). A system of tilting mirrors (5) allows the

modulation of the beam direction in order to respect the necessary incidence angle on the surface of the prism, corresponding to the SPR occurence. The prism used in the set-up is made of BK7 glass (n=1.51), 90°. The sensing phase/sensing system is fixed on the prism surface, by using a drop of oil with the same refractive index as the glass. The reflected light is magnified by a system of lenses (6) and brought by a mirror (4) on a CCD camera (Retiga 1300 Qimaging, Burnaby, British Columbia, Canada) (4 in Fig. 2).

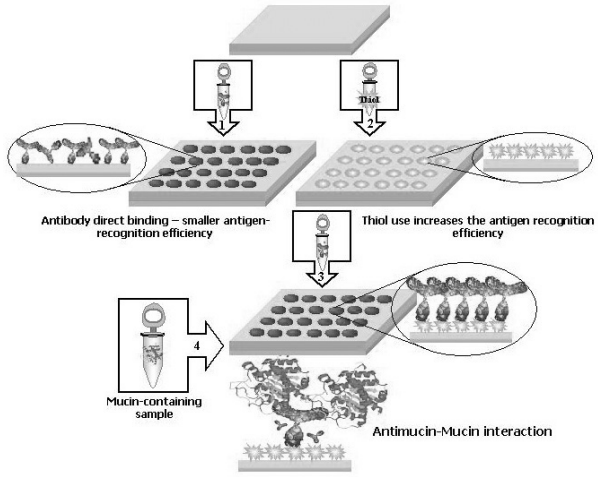

Fig. 1. Biosensor design. 1 – Antibody direct immobilisation on neat gold; 2 – Thiol SAMs patterned-coating of gold; 3 – Antibody immobilisation on thiol SAMs; 4 – Mucin-containing sample analysis, with antigen specific recognition and binding by the antibody.

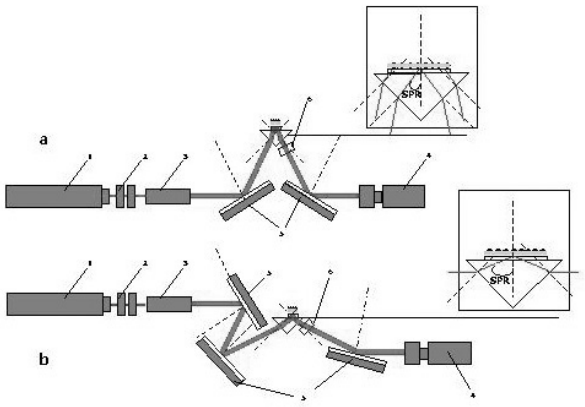

Fig. 2. Modular SPR Imaging set-up schemes. a – in air/gas analysis; b – in liquid media analysis.1 – He-Ne laser, 2 – polarysors, 3 – collimator, 4 – CCD camera, 5 - tilting mirrors, 6 – optical objective.

Analysis and SPR Imaging detection principle

The biosensor exploits the ability of antimucin antibody to specifically interact with the mucin molecules present in the saliva. Once the sensing phase immobilised, synthetic/saliva samples carrying different concentrations of mucin are used for SPR Imaging exploring.

In order to prevent mucin non-specific adsorption, before the analysis step, a blocking step is performed by exposure of the sensor surface to a BSA solution in phosphate buffer (pH 7,4), for 15 minutes, at ambient temperature.

Multiple operation modes are possible: analysis at gold SPR angle (where the minimum reflectivity takes place), exploring at the SPR angle corresponding to the minimum reflectivity of the antibody layer or analyses at SPR angles corresponding to the minimum one for different mucin levels in the analyte samples.

The easiest evaluation we propose is the antimucin-mucin interaction monitoring at non-reacted antibody SPR angle. This will allow a wider range of reflectivity variations following different interaction intensities, the minimum reflectivity corresponding to the antibody (the darkest signal in terms of grey level) and the reflectivity signal increasing (lighter grey level) with the intensity of the interaction antibody-antigen. The interaction intensity will be different depending on the concentration of PCM in the analysis medium. The negative control for cancer diagnosis would be represented by the signal obtained following the interaction antibody–mucin in healthy people saliva. This interaction will be weak in intensity due to the low concentration of mucin in healthy persons. The corresponding refraction index change (inducing a shifting in the SPR angle) will be modest and a non-significant reflectivity change will be noticed on the registered SPR image, when compared with the image for the non-reacted antibody (darkest spots). When the mucin level in the sample solutions overreaches the physiological level, a stronger signal (lighter grey level) will be obtained depending on how important the interaction is. This will give direct information on the mucin overexpression related to cancer occurrence. The analysis principle is schematically presented in Fig. 3.

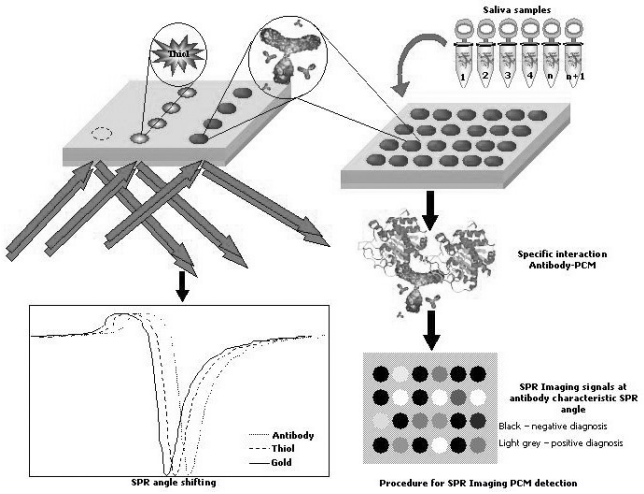

Fig. 3. Left – SPR angle shifting due to Antibody immobilisation on gold; Right – How the SPR Imaging works for PCM detection - schematic view.

3. Results and discussion

In cancer diagnosis, several arrays were already developed against epitopes on the MUC1 core protein, but the sensitivities are in the range 50-90% and specificities in the range 50-80% for diagnosis of patients with advanced carcinomas [Rye P.D. et al., 2001 and Norum L.F. et al., 2001 cited in [27]). These modest performances of these assays are due to the non-cancer-specific high-level occurrence of mucins in serum. None of these assays was selected for early cancer diagnosis. They are usually used to confirm a malignancy and therapy progress.

Our biosensor addresses this problem, by offering an alternative by proposing as analysis medium saliva. The multiple advantages of using saliva were detailed in the Introduction. The biosensor is intended as possible efficient tool associated with an easy to perform method for PCM label-free analysis of multiple saliva samples from cancer supposed-patients. The approach is very challenging and several aspects are to be considered of major importance, for the successful achievement of this idea. This paper emphases the biosensor design and the detection method.

Biosensor design

When designing a sensor surface the main concerns are represented by the **immobilisation** of the biological recognition element and the design features. Two simple procedures of antibody immobilisation could be envisaged, as schematically presented in Fig. 1. The easiest method would consist in the simple adsorption of the antibody molecules on the neat gold surfaces freshly cleaned with piranha solution (way 1 in Fig. 1). This leads to randomly oriented bioaffinity molecules, reducing the efficiency of the biosensor with respect to the recognition of the analyte species [30-32]. This is why, despite the easiness of this technique, a different one is recommended, involving a preliminary modification of the sensor surface by self assembled monolayers (SAMs) of thiol molecules carrying amine- or carboxyl- ending groups (way 2 in Fig. 1).

Neither this latter procedure is not perfect with respect to the orientation of the antibody molecules, but it is recommended to the more specific techniques involving perfect-orienting molecules like avidin / biotin, protein A, protein G and specific anti-IgG capture antibodies. The reason is extremely simple. The quality of the SPR Imaging biochip strongly depends on the mass bound to the sensor surface, so, in order to keep a high sensitivity of the device just SAMs and the antibody should be immobilised on the surface [33]. The SAMs dimensions and morphology are also important. The smaller the molecules, the higher the sensitivity of the biochip against the antigen, due to a modest refractive index change by the thiol. Despite this consideration, it was shown that longer-chain thiols could afford a better assembling on the gold. Atomic Force Microscopy, Contact Angle, Ellipsometry and Photoelectron Spectroscopy are the techniques usually used relative to characterise aspects like the homogeneity, hydrophilicity and thickness of the SAMs.

Nevertheless the immobilization procedure should be optimized with respect to the homogeneity and thickness of the sensing phase. These parameters are essential for the high-end research and diagnosis purposes involving SPR Imaging technology. This highly sensitive optical technique claims perfectly controlled self-assembled and functional biochemical sites inducing an easy to detect change in the refractive index once they interact with the target molecules. The **optimum antibody concentration** to be used for the biosensor surface is considered the concentration allowing a sufficient and homogeneous covering of the sensor surface and the evaluation of the widest range of antigen concentrations for cancer detection. It should be **low enough** to detect a high antigen concentration in the analysis samples by respecting the sensitivity of the SPR imaging and **high enough** to provide uniform layer and bioaffinity sites for antigen specific recognition. This means that the optimization of the immobilization procedure can be performed only by permanently combining state-of-art of modern ultrathin layer chemistry with SPR Imaging interrogation of the designed platforms.

The antibody immobilization time is also a very important parameter and it should be optimized by performing SPR Imaging/angular interrogation kinetic monitoring of the binding phenomenon, in the flow cell of the device.

The design features are also very important when foreseeing the efficiency of the biosensor. The multi-spot format offers the possibility of multiple sample analyses. It should be mentioned that the spot dimension and shape should be appropriate to the well known SPR Imaging exigencies: spot-to-spot reproducibility and homogeneity, spot edge identifying, spot signal-to-noise ratio. Sample easy identifying should be provided, the simplest method consisting in a letters/ciphers encoding system.

SPR imaging and detection method

With respect to the SPR Imaging technology, it is well known that the main advantage of this method against classical angular or wavelength interrogations consists in the analysis of the whole biosensor surface with multi-spot format in a single step.

Actually, the refractive index shifting is recorded by a CCD camera as reflectivity variation and an image as response of the biosensor is obtained (Fig. 3).

Once the antimucin immobilized on the biosensor surface, the sensitivity can be tested by direct checking of the **interaction with mucin** from different-concentrated samples. Kinetic studies of the interaction mucin-antimucin are necessary, in order to indicate the duration of the analysis. As already underlined, it is of main importance to block the surface with BSA in order to avoid any non-specific mucin binding on gold or SAMs.

When performing measurements at the antibody SPR angle, it is easy to distinguish between different

reflectivity units corresponding to different antibody-mucin interaction intensities.

Calibration tables/curves are to be drawn, relating different reflectivity values with the corresponding mucin concentrations. The negative control corresponds to the lowest reflectivity unit, characterising the physiological level of mucin in saliva of healthy people. The recorded images can be automatically converted by appropriate computer-assisted algorithms in cancer detection answer.

4. Conclusions

The method is expected to be very sensitive and to provide a valuable tool for early detection of cancer, by exploiting the overexpression of a tumour marker in an easy-to-collect sample, saliva. The possibility of simultaneous monitoring of an important number of samples, in a very simple label-free protocol is very promising. Nevertheless we are addressing the problem of fighting against one of the most killing disease in our century by the early detection of this one. The development of the new SPR Imaging bioaffinity biosensor and of the non-invasive detection-associated method could represent an important progress in the field of cancer diagnosis and survey techniques.

Acknowledgements

The 5th EU Frame Program is gratefully acknowledged for the project "*Molecular Imaging for Label-Free Detection in Functional Proteomics*" (HPMD-CT-2001-00077).

References

[1] C. Krafft, S.B. Sobottka, G. Schackert, R. Salzer, The Analyst, **130**(7), 1070-7 (2005).
[2] W. Steller, J. Einenkel, L. C. Horn, U.-D. Braumann, H. Binder, R. Salzer, C. Krafft, Anal. and Bioanal. Chem., **384**(1), 145-54.3 (2006).
[3] C. Krafft, S. Miljanic, S. B. Sobottka, G. Schackert, R. Salzer, Proc. SPIE **514**1, Diagnostic Optical Spectroscopy in Biomedicine II; p. 230-236, (2003).
[4] C. Krafft, S. B. Sobottka, G. Schackert, R. Salzer, J. Raman Spectrosc. **37**, -3:367–375 (2006).
[5] C. Krafft, Anal. Bioanal. Chem. **378**, 1:60-62 (2004).
[6] G. Shetty, C. Kendall, N. Shepherd, N. Stone, H. Barr, British J. Cancer **94**, 1460-1464 (2006).
[7] M. F. Pichon, Immuno-analyse & Biologie spécialisé **19**, 241-249 (2004).
[8] A. Moore, Z. Medarova, A. Potthast, G. Dai, Cancer Res. **64**, 1821-1827 (2004).
[9] K. M. Höld, D. de Boer, J. Zuidema, R. A. A. Maes, Intern. J. Drug Test., 34p, http ://www.criminology. fso.edu/journal/hold.html.
[10] http://www.cancer.gov/newscenter/pressreleases/proteomicsQandA, 22/05/2006.
[11] http://www.spendloveresearch.org/ResearchPages/tumor&cancertypes.htm, 22/05/2006.
[12] B. V. Lipps, Novel Saliva test for early diagnosis of breast, colon, liver and ovary cancer, http://www.ophidia.com/Saliva%20Test%20for%20Diagnosis%20of%20Cancer.pdf - 16/05/2006.
[13] M. Coelho, P. Gauthier, M. Pugnière, F. Roquet, A. Pèlegrin, I. Navarro-Teulon, British J. Cancer **90**, 2032-2041 (2004).
[14] S. von Mendsdorff-Pouilly, M. M. Gourevitch, P. Kenemas, A. A. Verstraeten, S. V. Litvinov, G. J. van Kamp, S. Meijer, J. Vermorken, J. Hilgers, Eur. J. Cancer **32A**, 8 :1325-1331 (1996).
[15] J. Homola, S. S. Yee, G. Gauglitz, Sens. Actuators B: Chemical **54** (1-2): 3-15 (1999).
[16] J. Deka., J. Kuhlmann., O. Muller, Eur. J. Biochem. **253**: 591–597 (1998).
[17] V. Regnault, J. Arvieux., L. Vallar, T. Lecompte, Journal of Immunological Methods **211**, 191–197 (1998).
[18] E. Gorodkiewicz, A. Fernández-González, A. Akkoyun, G. Steiner, R. Salzer, Chem. Anal. (Warsaw) **50**, 103-116 (2005).
[19] L. M. Pericleous, J. Richards, A. A. Epenetos, N. Coutenay-Luck, M. P. Deonarain, British J. Cancer **93**, 1257-1266 (2005).
[20] C.-A. Yang, E. Brooks, Y. Li, P. Denny, C.-M. Ho, F. Qi, W. Shi, L. Wolinsky, B. Wu, D. T. W. Wong, C. D. Montemagno, Lab on a Chip, **5**: 1017-1023 (2005).
[21] B. Liedberg, C. Nylander, I. Lundström, Sens. Actuators **4**, 299-304 (1983).
[22] http://www.berkeley.edu/news/berkeleyan/2001/09/06_.html, 22/05/2006.
[23] V. Apostolopoulos, G. A. Pietersz, I. F. McKenzie, Curr. Opin. Mol. Ther. **1**, 98-103 (1999).
[24] G. Sölétormos, D. Nielsen, V. Schiøler, H. Mouridsen, P. Dombernowsky, Eur. J. Cancer, **40**: 481-486 (2004).
[25] G. G. Bon, G. J. van Kamp, R. A. Verstraeten, S. von Mensdorf-Pouilly, J. Hilgers, P. Kenemans, Eur. J. Obst. & Ginec. Reprod. Biol., **83**: 67-75 (1999).
[26] M. Andrianifahanana, A. Agrawal, A. P. Singh, N. Moniaux, I. van Seuningen, J.-P. Aubert, J. Meza, S. K. Batra, Oncogene **24**: 6143−6154, (2005).
[27] M. A. Hollingsworth, B. J. Swanson, Nature Reviews, Cancer **4**: 45-60 (2004).
[28] N. W. Toribara, J. R. Jr. Gum, P. J. Culhane, R. E. Lagace, J. W. Hicks, G. M. Petersen, Y. S. Kim, J. Clin. Invest., **88**:1005-1013 (1991).
[29] http://www.ssens.nl/.
[30] A. Ulman, Monolayers, An Introduction to Ultrathin Organic Films from Langmuir-Blodgett to Self-Assembly, Academic Press, 442 p. (1991).
[31] K. Rogers, Principle of Affinity-Based Biosensors, Mol. Biotechn., **14**,109-128 (2000).
[32] K. L. Brogan, K. N. Wolfe, P. A. Jones, M. H. Schoenfisch, Anal. Chim. Acta, **496**, 73-80 (2003).
[33] F. Darain, S.-U. Park, Y.-B. Shim, Biosens. Bioel., **18** (5-6): 773-780 (2003).

*Corresponding author: izabela.cristina.stancu@gmail.com