

## DSM Science & Technology Awards 2004

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# The Use of Self-Assembly for the Realisation of Immunosensor Interfaces and Detection Systems

## 1. Aim of the research

The aim of this study was to facilitate the realisation of highly sensitive and selective metal-based affinity biosensors. More specifically, this study focused on the use of nanoscaled self-assembly systems of thiol and disulphide molecules, S-layers and PAMAM layers for the construction of functional, selective, sensitive, well-defined and reproducible metal-based immunosensor interfaces. In our work, this general aim has been subdivided into several parts:

- The identification and validation of analytical techniques for a full characterisation of the interfacial immunosensor layer based on self-assembly systems.
- The synthesis of thiols and disulphides with various functional groups.
- The investigation on the (often neglected) role of the gold transducer substrate on the self-assembly processes.
- A multi-technique characterisation of the realised self-assembled monolayers of thiols, disulphides, PAMAM layers and S-layers onto gold substrates.
- Investigation of the immunosensing properties of the self-assembly based biosensor interfaces using the Surface Plasmon Resonance technique. Several kinds of antibodies and immobilisation strategies were elaborated. These surfaces are used to benchmark with commercially available immunosensor interfaces.

This multidisciplinary approach and the optimisation of every step of the biochemical immunosensor interface in combination with the benchmarking study allow us to state that several enhanced biosensor (interface) qualities are identified and presented<sup>1-4</sup>.

Furthermore, a novel, nanotechnology-based biosensing technique has been developed which can possess the desired properties of a portable, cheap and disposable biosensor. This revolutionary way of biosensing is called the Transmission Plasmon Biosensor<sup>5,6</sup>.

## 2. Biosensors

The first biosensors were reported in the early 1960s<sup>7</sup>. The biosensors, having shown the greatest commercial success, have been enzyme electrodes, primarily developed for medical applications such as the detection of glucose in blood<sup>8</sup>. In the past two decades, the biological and medical fields have discovered the great advantages in the development of biosensors and biochips capable of characterizing and quantifying (bio)molecules<sup>9</sup>. The fastest growing area in biosensors research, however, involves affinity-based biosensors and related techniques. These biosensors are expected to revolutionise in areas such as clinical/diagnostics, food processing, military/antiterrorism and environmental monitoring. It is clear that biosensor technologies will play an important role in the development of public health by finding applications in areas where rapid detection combined to high

sensitivity are important<sup>10,11</sup>. The specific applications are currently in the media e.g. bird plague, BSE, dioxins, antibiotics, pig plague, ...

The definition of a biosensor, particularly affinity-based systems, is by no means static, although IUPAC has proposed the following definition<sup>12</sup>: a biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element. Biosensors are therefore analytical devices composed of a *biological recognition element* such as an antibody, a receptor protein, a biomimetic material, or DNA interfaced to a signal *transducer*, which together relate the concentration of an analyte to a measurable electronic signal<sup>9,13-17</sup>.

The work was focussed on affinity sensors and more specifically on immunosensors. This kind of application involves immobilisation of antibodies on the transducer surface. Immunosensors have some advantages directly related to the nature of antibodies: they can be very stable; they can be developed against any kind of analyte and their use is not restricted to the detection of biomolecules; they are very specific in the nature of the binding and can be developed with high affinity. Therefore, antibody based sensors, e.g. immunosensors, are suggested to be more sensitive than enzyme sensors<sup>18</sup>.

These advantages open a wide range of applications for immunosensors<sup>19</sup>. The only restriction on the use of the antibody-antigen system is that the antibodies should be active on the surface, which means that the nanoscaled environment of the immobilised antibodies should be favourable for them. Therefore, they should be immobilised in an oriented and active way, which was performed in this research.

## 3. Results

The optimisation of the biological recognition layer covers the first part of this research with the aim to realise a fully active antibody coated surface onto the transducer substrate. In addition a novel transduction principle has been developed based on nanotechnology, new materials and a novel physical phenomena.

### 3.1 The biological recognition layer

#### 3.1.1 Synthesis of appropriate thiols and disulphide molecules

Several self-assembly approaches have been applied in this study comprising Self-Assembled Monolayers (SAMs) of thiols or disulphides, S-layers and PAMAM dendrimer layers. S-layers and PAMAM layers are commercially available together with several thiol molecules. However, thiol molecules and disulphide molecules with long alkane chains (for enhanced reproducibility and stability) or with preactivated groups (which react with the bioreceptors without any activation step) are not readily available and had to be synthesised.

Thiols and disulphides with the general formula  $\text{HS}-(\text{CH}_2)_n\text{-X}$  or  $\text{X}-(\text{CH}_2)_n\text{-S-S}-(\text{CH}_2)_n\text{-X}$  can form SAMs on gold. These thiols and disulphides have given us the ability to realise mixed SAMs with the essential functional groups. Molecules exhibiting groups which can bind bioreceptors (antibodies) and

groups which prevent non-specific adsorption, have been synthesised. This allows for the realisation of mixed SAMs comprising groups, which can bind the bioreceptors or antibodies and groups, which prevents the adsorption of the matrix of the analyte (antigen). This dual approach enhances the immunosensing capabilities of biosensor as a whole.

### 3.1.2 The role of the gold substrate upon the self-assembly processes

In this study, gold was chosen as the starting material to deposit Self-Assembled Monolayers (SAMs). From an experimental point of view, gold is among the easiest material to use as a substrate. Unlike other metals, it does not readily oxidize upon exposure to the atmosphere and it is compatible with most biosensor transducer systems. It appears that self-assembly of thiols is influenced by three different aspects of the gold substrate, i.e.:

1. the cleanliness of the gold substrate;
2. the crystal structure of the gold substrate;
3. the flatness of the gold substrate.

We have identified the influence of the roughness on the SAM formation and have demonstrated how gold films can be realised with a different crystal structures and a different amount of Au(111). Although there is a clear difference in SAM formation on extremely flat gold and smooth gold, we could not verify any difference in the performance of these SAMs with regard to the degree of antibody immobilisation. Similar conclusions could be drawn from the XRD results for the amount of Au(111) in the gold substrate and its influence on the stability and reproducibility of the SAM formation. We have never observed any differences in antibody coupling or in the stability when SAMs were deposited on gold with a different amount of Au(111).

We believe that a more important issue is the influence of the cleanliness of the gold substrate. The applied SAMs showed only irreproducible antibody immobilisations and unstable behaviour if there was a contamination source during the SAM deposition or when the gold substrate was not subjected to a prior cleaning treatment. Therefore, we state and stress that the pretreatment (cleaning) is a more important factor in SAM formation and subsequent antibody coupling than the amount of Au(111) in the top gold layer and/or the roughness.

The annealing treatments on the gold layers lowered the amount of Au(111) compared to gold without additional treatments, while the blocking and therefore the efficiency of SAM formation was better on the annealed gold. This indicates that the roughness of the substrate is more important than the crystal structure of the gold.

We can therefore state that the important parameters for the SAM formation and reproducible antibody immobilisation can be listed in order of decreasing importance as: cleanliness of the gold > roughness of the gold > the amount of Au(111) in the gold substrate. The UV-O<sub>3</sub> cleaning has been identified to have superior qualities both from environmental as from scientific point of view.

PAMAM dendrimer layers have been realised onto a SAM of thiols and have therefore been subjected to the same conclusions as stated above. We have also identified that S-layers exhibit an improved crystallisation onto UV-O<sub>3</sub> cleaned gold substrates.

### **3.1.3 Surface characterisation on Self-Assembled Monolayers of thiols or disulphides, S-layers and PAMAM layers.**

The understanding of the physical and chemical properties of SAMs of thiols or disulphides, S-layers and PAMAM dendrimer layers and the external influences on the formation and realisation of them have been identified together with the synthetic pathway to realise them. Furthermore, this study has suggested applicable monolayer characterisation techniques to investigate or optimise a particular condition necessary for the self-assembly process. The following surface analytical techniques have been applied: cyclic voltammetry, contact angle geometry, grazing-angle FTIR, x-ray photoelectron spectroscopy, auger electron spectroscopy, atomic force microscopy, scanning tunnelling microscopy and time-of-flight mass spectroscopy.

The UV-O<sub>3</sub> cleaning has been found to be the most adequate cleaning. If this cleaning is applied, 3 h of deposition from a 1 mM solution is in general sufficient for an adequate SAM formation. SAMs of long alkane chain thiol or disulphide molecules are, in general, well-packed and well-structured. No significant change in their properties could be observed if these molecules were used for the formation of mixed SAMs instead of homogeneous SAMs.

The physical and chemical properties of the S-layers and PAMAM layers have been identified with various techniques. Furthermore, we have clearly demonstrated the need and the advantages of using multiple characterisation techniques. The ability to identify different aspects of the self-assembled monolayers has been proven by several examples. In this way, the investigated SAMs could be characterised in all their different aspects, i.e. the influence of the deposition time, the influence of the cleaning procedure on the packing density, the blocking ability, the topography, the structural properties or other physical and chemical properties, e.g. the wettability.

### **3.1.4 Immunosensing properties of the SAMs based biosensor interfaces**

The transducer of a biosensor mainly determines the sensitivity and the stability of the global performance of a biosensor. The immunological interface on the other hand determines, besides the sensitivity and the stability, also the reproducibility and the specificity, which are crucial elements in the final performance of a biosensor or an immunosensor. The immunosensing properties of the antibodies coupled onto the realised surfaces have been determined using the Surface Plasmon Resonance technique. Our biosensor research is focussed towards immunosensing, which implies that an antibody should be covalently bound to the linking layers and the antigen should be specifically recognised. Non-specific adsorption should be limited.

The various linking layers used in this work were based on thiols or disulphides, on PAMAM dendrimers or on S-layers and have been evaluated concerning their immunosensing properties. Five different model systems were used e.g. anti-Human Serum Albumin (anti-HSA) and the corresponding antigen Human Serum Albumin (HSA), anti-Human Transferrin (anti-HT) and Human Transferrin (HT), anti-Prostate Specific Antigen (anti-PSA) and PSA, anti-IgG and IgG and finally preprotein substrates and signal peptidase. These model systems are important for respectively diagnosis diabetic nephrosis, identification of long term alcohol abuse, early prostate cancer diagnosis, autoimmune diseases and drug screening.

We state that several “tools” are available to improve the qualities related to the immunological interface of a biosensor using mixed SAMs of thiols or disulphides. We have shown how the desired recognition signal, the degree of antibody immobilisation or the limitation of the non-specific adsorption are feasible using the different “tools” of the mixed SAMs approach. The different “tools” which influence the recognition signal, the antibody immobilisation and the non-specific adsorption are:

1. Different kinds of thiols can be used, for example thiols with a different structure, length or functional groups.
2. The amount of thiols in the mixed SAMs is directly related to the amount of antibodies which can bind and to the level of non-specific adsorption.
3. Direct antibody coupling procedures (direct binding to the groups of the antibody) and indirect coupling procedures (binding via a crosslinker or an intermediate step) can be used.
4. Antibody modifications can be used to reduce the size of the bioreceptors in order to increase the amount of receptors on the immunosensor interface. In addition it enables to immobilize the bioreceptors in an orientated way.
5. The affinity of the antibodies is directly related to the sensitivity and important for the applied assay e.g. direct detection, sandwich approach, competitive assays, ...
6. The kind or the origin of the applied antibodies can enable higher sensitivity. The camel antibodies, for example, used in this study have a low molecular mass of 15 kDa compared to 150 kDa for conventional mouse antibodies which enables an increased number of bioreceptors on the same sensing surface.
7. The orientation of the antibodies can enhance the sensitivity. Orientated immobilisation means that the antibodies are immobilised onto the surface via specific groups on well-defined places, which are not present in the binding sites involved in the affinity reaction.

Examples and the influence of these different “tools” on the sensitivity and specificity have been identified and shown in this work. The use of S-layers as an immunological interface has been compared with the abilities of the mixed SAMs. A last kind of immunological interface is based on PAMAM dendrimers. The realised surfaces have also been benchmarked with commercial available immunological interface layers (like for example the CM5-chip of Biacore)<sup>1</sup>.

The ability to improve the immunosensing experiments concerning sensitivity and selectivity by using different coupling procedures (direct – indirect and coupling to carboxylic-groups or amino-groups), novel blocking agents or using controlled orientation of the antibodies via different surface chemistries has been shown. Orientated immobilisation strategies have been discussed and the necessary antibody modifications are realised. The utilisation of these antibodies modifications and orientated immobilisation has been shown. The enhanced immunosensing properties using camel antibodies have been clearly demonstrated.

Preliminary experiments have shown that the surface chemistry developed in this work can also be used for other applications than antibody – antigen interactions. Their enhanced qualities compared to several commercial layers have been identified for membrane protein affinity research<sup>3</sup>.

### 3.2 The transducer system – the Transmission Plasmon Biosensor

Surface Plasmon Resonance (SPR) sensors are widely used for biosensing, especially as affinity sensors. These sensors are based on the principle that the surface plasmon resonance is highly sensitive to the refraction index at the interface between the metal film deposited upon a glass prism and a sample upon the metal surface. Similar phenomena also occur on the surfaces of small metal particles, such as gold and silver, and are well known as local surface plasmon resonance. In the present work, the usefulness of UV-Vis absorbance spectroscopy for biosensing on thin gold and silver particle layers has been demonstrated. The change in absorbance properties upon changes in the thickness of the films, upon changes to the substrate and upon the use of different deposition methods for the realisation of gold and silver island and nanoparticle films has been discussed. The realised gold and silver island and nanoparticle films were studied by means of tapping-mode Atomic Force Microscopy (AFM) and UV-Vis absorbance spectroscopy. The particles themselves were characterised both with UV-Vis absorbance spectroscopy and Transmission Electron Microscopy. Different substrates for realising the nanoparticle or island films were used i.e. polymer slides, glass and quartz together with different thin gold and silver nanoparticle layer deposition methods (i.e. sputtering, evaporation, nanoparticle deposition and gold electroless plating). The resulting plasmon resonance and interband absorbance bands in the visible and UV region have been compared. SAMs were used to make the particles films more hydrophobic to induce protein adsorption or to attach covalently antibodies onto a carboxyl-terminated SAM. In a next step, the bound antibodies can recognize the antigens. In addition, a resonant enhancement of the interband absorption was found to occur. The PhD thesis (and the articles written by the author of this work) has first reported on biosensing on quartz substrates in combination with silver and gold particles, based on a resonant enhancement of the interband absorption bands<sup>5,6</sup>. The method presented is versatile, allowing applications in the liquid and gas phases. Furthermore, this novel approach is promising as an easy and cost-effective alternative for conventional biosensing techniques. Future work will focus on the sensitivity and the quantitative interpretation of the absorption differences upon binding to different kind of particles.

The “ELISA like” Transmission Plasmon Biosensor experiments have been proven the large potential and the large application market of this kind of biosensing. Despite these encouraging results some further improvements should be considered to achieve a better sensitivity, which is important for some applications.

In addition this research contributes to the hype existing towards nanotechnology. The linking layers used to covalently immobilize the nanoscaled gold and silver particles are several nanometers thick. The surfaces and the particles have been characterised onto the nanoscale using AFM and TEM and nanoscaled considerations have been used to explain the macroscopic observed phenomena (different colours). The thiol, the antibodies and the antigens are in the nanoscale. However, we believe that this research has an added value to this hype. Instead of focusing on the nanoscaled characteristics of the chemistry and technology, we have used the nanoscaled objects (particles) and the nanoenvironment of these nanoparticles to sense biomolecules. We believe we have brought the nanotechnology to a useful

applicable technology, which is lacking in lots of nanotechnology related research. We therefore have realised to some extent a bridge between the nanoworld and the final user in the macroworld.

## 4. Final conclusions and suggestions for future research

The conclusions of our work allow to state that it is clear that the proposed general strategy for the realisation of metal-based biosensor interfaces has proven to be very successful. In addition, a novel kind of biosensing approach has been presented.

The use and the advantages of the convenient self-assembly techniques for the realisation of immunosensor surfaces have been proven. Furthermore, the enhanced qualities of mixed nanoscaled self-assembled monolayers of thiols and disulphide have clearly been proven and the necessary benchmarking study has been performed. The different “tools” to enable an increased stability, reproducibility, sensitivity and selectivity have been identified. The novel kind of biosensing allows for the realisation of an easy, cheap and disposable biosensor.

Furthermore, this research has shown the potential of multi-disciplinary research. Surface chemistry is combined with inorganic chemistry, organic synthesis and analytical chemistry and is indispensable to allow for the realisation of adequate biosensors and interfaces. In addition, this research is situated in or contributes to the recent “research hypes” called “nanotechnology”, “bottom – up – approach” and “biosensors”. The transmission plasmon biosensor is actually a nice example of a bottom – up – approach to realise via nanotechnology, usable biosensors. To this end, it makes a bridge between the nanoworld and the macroscopic applications.

Similar conclusion can be drawn for the novel realisation of the Transmission Plasmon Biosensor. The potential and the possible application field have been identified and expected to be very promising and successful.

These conclusions and suggestions of our work will lead to the realisation of affinity-based immunosensors which are essential for the development of cheap, miniaturised, integrated bio-analysis systems with significant promise for point of care applications.

Future work must consist of the exploration of a real, portable, multi-analyte sensor combining the biological recognition layers (developed in this work) together with the novel Transmission Plasmon Biosensor. This technology will soon be realised for the detection of PSA (marker for prostate cancer), ampicilline (for the detection of antibiotics in milk) and a fast and cheap blood-typing sensor. This is currently performed in close collaboration with several end users based on the two patents generated in this work. The final goal will be a spin-off activity of this technology.

## 5. References

- <sup>1</sup> Frederix, F.; Bonroy, K.; Laureyn, W.; Reekmans, G.; Campitelli, A.; Dehaen, W.; Maes, G. *Langmuir* **2003**, *19*(10), 4351
- <sup>2</sup> Frederix, F.; Bonroy, K.; Reekmans, G.; Laureyn, W.; Campitelli, A.; Abramov, M.A.; Dehaen, W.; Maes, G. *J. Biochem. Biophys. Meth.* **2003**, *58*(1), 67
- <sup>3</sup> Geukens, N.; Frederix, F.; Reekmans, G.; Lammertyn, E.; van Mellaert, L.; Dehaen, W.; Maes, G.; Anné, J. *Biochem. Biophys. Res. Commun.* **2004**, *314*, 459

- <sup>4</sup> Frederix, F. *Self-assembling monolayers for sensors*, EP1369692A2, US10/454,765 (patent)
- <sup>5</sup> Frederix, F.; Friedt J.-M.; Choi, K.-H.; Laureyn, W.; Campitelli, A.; Mondelaers, D.; Maes, G.; Borghs, G. *Anal. Chem.* **2003**, *75*, 6894
- <sup>6</sup> Frederix, F.; Borghs, G.; Friedt, J.-M. *Spectroscopic detection method for biomolecules*, EP1321761, JP2003240710 (patent)
- <sup>7</sup> Clark, L.C. Jr.; Lions, C. *Ann. Acad. Sci.* **1962**, *102*, 29
- <sup>8</sup> Rogers, K.R. *Mol. Biotechnol.* **2000**, *14*, 109
- <sup>9</sup> Vo-Dinh, T.; Cullum, B. *Fresenius J. Anal. Chem.* **2000**, *366*, 540
- <sup>10</sup> Weetall, H.H. *Biosens. Bioelectron.* **1999**, *14*, 237
- <sup>11</sup> Rand, A.G.; Ye, J.; Brown, C.W.; Letcher, S.V. *Food Technol.* **2002**, *56(3)*, 32
- <sup>12</sup> Thrévenot, D.R.; Toth, K.; Durst, R.A.; Wilson, G.S. *Pure Appl. Chem.* **1999**, *71(12)*, 2333
- <sup>13</sup> Turner, A.P.F. *Sens. Act.* **1989**, *17*, 433
- <sup>14</sup> Marco, M.-P.; Barcelo, D. *Measurement Sci. Technol.* **1996**, *7*, 1547
- <sup>15</sup> Lopez-Avila, V.; Hill, H.H. *Anal. Chem.* **1997**, *69*, 289R
- <sup>16</sup> Göpel, W.; Heiduschka, P. *Biosens. Bioelectron.* **1995**, *10*, 853
- <sup>17</sup> D'Souza, S.F. *Appl. Biochem. Biotechnol.* **2001**, *96*, 225
- <sup>18</sup> Lowe, C.R.; Yon Hin, B.F.Y.; Cullen, D.C.; Evans, S.E.; Sray Stephens, L.D.; Maynard, P. *J. Chromatogr.* **1990**, *510*, 347
- <sup>19</sup> Lippa, P.B.; Sokoll, L.J.; Chan, D.W. *Clin. Chim. Acta* **2001**, *314*, 1