FINAL PUBLISHABLE JRP REPORT

JRP-Contract number	HLT04
JRP short name	BioSurf
JRP full title	Metrology for the characterisation of biomolecular interfaces for diagnostic devices
Version numbers of latest contracted Annex Ia and Annex Ib against which the assessment will be made	Annex Ia: V#1.0 Annex Ib: V#1.0
Period covered (dates)	From 01 May 2012 To 30 April 2015
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Report Status: PU Public

EMRP
European Metrology Research Programme
Programme of EURAMET

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union

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1 Executive Summary

Overview
This project developed methods to reliably and consistently measure the performance of biochemical interfaces used in in-vitro diagnostic devices (IVDs). We created the first reference biomolecular interfaces for IVDs, and developed techniques to more accurately characterise the properties of probe and target molecules at these interfaces. These developments will allow IVD manufacturers to develop increasingly accurate and reliable devices for a broader range of health conditions. Resulting in faster diagnoses at the point of patient care, ultimately helping to more effectively treat disease and drive down healthcare costs in Europe.

Need for the project
Healthcare costs are rising rapidly throughout Europe as populations' age and rates of chronic disease increase. The World Bank estimates that public expenditure on healthcare in the EU could rise as high as 14% of GDP by 2030 (up from 8% in 2000), continuing to rise thereafter. IVDs are handheld devices that have the potential to reduce healthcare costs through rapid diagnosis at the point of care (at home or in the field), lowering the costs of diagnosis by replacing expensive laboratory techniques and equipment, and by easing the demand for hospital services.

Although IVDs have great potential, only a small number are currently produced for a limited number of medical conditions, as it is challenging to manufacture devices that satisfy the performance requirements of the European IVD directive (98/79/EC). IVDs work by detecting target molecules in patient samples that are indicative of disease or other adverse health conditions. Target molecules are identified by using a probe molecule, which binds specifically with the target molecule. These probe molecules are attached to an interface, a surface that makes contact with the patient sample. The probe molecules on the interface ensure that the target molecules are held, and all other molecules in the sample rejected. The IVD detects changes at the interface, and needs to be sensitive enough to detect the small number of bound targets. The number of probe molecules present at the interface, and their ability to function, is the key determinant of IVD performance. However, there are no standard, industry-wide techniques to measure the properties and performance of interfaces, and lack of consistency in interface chemistry is the chief cause of accuracy and reproducibility issues in IVDs. Standard, accurate techniques are needed to understand the properties of biomolecular interfaces, in order to manufacture high-quality, reliable devices.

Specifically, techniques are required to quantify the amount of probe present at the interface, and the efficiency by which targets bind with probes, in order to accurately identify levels of target molecule in a sample. The development of innovative techniques to characterise the distribution, orientation and structure of probes would also further enhance diagnostic accuracy. As would the ability to detect multiple different targets with one interface, as health conditions can produce a range of target molecules, and their combined measurement would provide more detailed diagnoses. IVDs predominantly use flat interfaces, but interfaces based on nanoparticles are attractive, as they can indicate target binding events simply and clearly. But despite a number of proof-of-principle demonstrations, few nanoparticle-based IVDs are used in clinical practice. Measurement techniques need to be validated for both a reference flat interface, and a nanoparticle interface, to support the current use of flat interfaces and encourage the development of nanoparticle-based devices.

Scientific and technical objectives
The following objectives were set to achieve the overall goal of providing techniques to better characterise the properties and performance of biomolecular interfaces:

1. Produce reference flat and nanoparticle interfaces: Establish accurate, traceable and comparable methods to determine the amount of biomolecular probe immobilised on each interface. An inter-laboratory comparison will validate these methods using the reference interfaces produced.
2. Use innovative approaches to determine the orientation and structure of biomolecules at an interface, and develop useful measurement approaches for the research, development and quality control of biomolecular interfaces in diagnostic devices.

3. Develop novel approaches for the measurement of interface structure that can be correlated with activity and binding efficiency. Develop methods to measure and predict the activity of immobilised probes, by measuring the activity of diagnostic surfaces, quantifying and modelling the interaction between probes and targets.

4. Assess and evaluate the capabilities of new, emerging techniques and approaches to biomolecular sensing which enable multiplexed, label-free identification and quantification of bound targets.

Results

1. Produce reference flat and nanoparticle interfaces: Establish accurate, traceable and comparable methods to determine the amount of biomolecular probe immobilised on each interface:

Versions of the same IVD with different amounts of probe will capture varying proportions of target molecules from the same sample, and may give different diagnoses. Therefore it is vital to understand how much probe is present at an interface. As interface chemistry varies between different IVDs, in order to develop accurate and traceable benchmark measurement methods, standardised reference surfaces must first be developed.

We achieved this by:

- Developing the first ever flat reference interface, based on biotin molecules (a commonly used probe) attached to a gold surface, and developing the first reference nanoparticle interfaces, also based on gold.
- Probe density on these interfaces could be accurately measured, and could be adjusted from 100% to 0.1% coverage, a ten-fold increase in precision over previous methods.

A major project highlight was the achievement of the first successful inter-laboratory study of nanoparticle interface chemistry, in which consistent results were achieved across each laboratory. The collaboration demonstrated that reproducible interface measurements could be achieved in different locations, using the reference interfaces, if standardised preparation and data analysis procedures were followed.

2. Use innovative approaches to determine the orientation and structure of biomolecules at an interface:

The amount of probe is not always the only guide to the functional performance of interfaces, as interfaces with poorly distributed (clumped) probes capture less target than surfaces with more evenly distributed probes. Probe molecules have active sites where they bind with target molecules, if probes are in the wrong orientation, or have lost the structural shape of their active site, they will not be able to bind with their targets. Techniques that provide information on probe distribution, orientation and structure will further enhance diagnostic performance.

We developed proof-of-concept techniques for these measurements by testing two methods previously identified in the literature as possible approaches:

- We demonstrated the first use of soft X-ray spectroscopy to measure properties of the interface, and developed a novel measurement device to analyse probe molecule distribution, orientation and structure.
- Also for the first time, we used Secondary Ion Mass Spectrometry (SIMS) to measure both the distribution and structure of probes on flat interfaces.

The techniques developed represent a vital first step in the measurement of these additional probe properties, and present a direction for further research to incorporate the techniques into commercial devices.

3. Develop novel approaches for the measurement of interface structure that can be correlated with activity and binding efficiency:

In addition to understanding the properties of probe molecules at the interface, it is also vital to understand the efficiency by which the probes are binding with their target, to accurately assess the level of target molecules in a sample.

We studied the binding of avidin target molecules with the biotin reference surface, as avidin selectively and strongly binds with biotin, and is used routinely in IVDs. Two techniques were used. Ellipsometry measures changes in the speed of light at the interface caused by target binding. The quartz crystal microbalance (QCM) method measures changes in mass and stiffness at the interface caused by target binding.

- Simultaneous measurements with ellipsometry and QCM demonstrated that the sensitivity of the QCM method dropped as target density increased. Therefore accurate QCM measurements can only be performed if the probe molecules are separated by a specific minimum distance. This result demonstrates the importance of the reference surfaces in establishing the response of detection systems.
- The two methods revealed a novel approach to determine the type of binding between biotin and avidin targets – avidin bound by two biotin probes was significantly stiffer than avidin bound by one biotin probe. This binding was easily detected using QCM.

To study target binding of avidin with the reference nanoparticle surface, optical spectroscopy was used to detect changes in colour of nanoparticles as they bound with targets. This approach is potentially very accurate, but can be made uncertain if targets bind with multiple nanoparticles (agglomeration). To ensure measurement accuracy, agglomeration must be accounted for.

- We developed a method for measuring the number of target molecules attached to each nanoparticle by combining the optical spectroscopy with particle size measurement.
- We used high resolution sedimentation techniques to demonstrate that genetically modified avidin molecules with a single binding site did not produce agglomerates, but normal avidin molecules did form agglomerates.

4. Assess and evaluate the capabilities of new, emerging techniques and approaches to biomolecular sensing which enable multiplexed, label-free identification and quantification of bound targets:

Health conditions may produce multiple target molecules, and measuring more than one can provide a more accurate diagnosis. For example, in the treatment of heart attacks, three target molecules are measured to determine the time since the heart attack, and to treat the patient appropriately. In most situations, it is necessary to 'tag' target molecules with 'label' molecules that can be more easily detected. However, sometimes this is not possible, either because the target has not previously been identified, or the presence of the label changes the behavior of the target. In these cases a multiplexed method is preferable, which detects multiple targets without using label molecules.

- A novel optical waveguide device was developed and demonstrated, capable of ultra-sensitive detection of molecules on surfaces without using labels. The waveguide device uses light to cause target molecules bound by probes to fluoresce, so that they can be detected directly. This device offers major advantages over conventional methods in terms of ease of use, the adaptability to different illumination wavelengths, a larger field of view, and the ability to detect unlabelled targets directly.
- Additionally, the ability of secondary ion mass spectrometry to detect multiple target molecules using unique labels was demonstrated for the first time. And label-free detection of targets on a diagnostic surface using ambient surface mass spectrometry was demonstrated.

Multiple techniques were developed for multiplexed, label-free target identification, providing avenues for further research to refine the techniques for commercial exploitation.

Actual and potential impact

Dissemination of results

To promote the uptake of the outputs of this project, results have been shared through the publication of 15 papers in international journals (listed in the next section), and over 60 presentations delivered at international conferences and workshops, including five invited talks. The number of invited talks is testament to the fact that our project consortium is seen to be leading the European effort to provide measurement methods for biological interfaces. Stakeholder engagement was greatly enhanced by the consortium bringing together world-leading experts at two international workshops: the 2012 65th IUVSTA workshop on "Measuring molecular Adsorption at the Solid-liquid Interface", and the 2015 "Nanoparticle concentration, chemistry and interfaces" workshop. Two press releases highlighted the work of the consortium, and resulted in an interview with the journal "The Analytical Scientist". In addition, five training courses were organised during the project to share best practices amongst the consortium members and external stakeholders. BioSurf consortium members have also been consulted by several research groups,

including the University of Washington, the Technical University of Vienna and the University of Manchester, on the best approaches to understand and measure nanoparticle surface chemistry.

Impact on standardisation

This project will have a fundamental impact on standards through enabling the initiation of the first ever European standardisation activities for measuring the properties of interfaces. During the project, consortium partners participated in the Versailles Project on Advanced Materials and Standards (VAMAS) and ISO meetings: VAMAS TWA 2 “Surface Chemical Analysis”, ISO TC 201 “Surface Chemical Analysis” and ISO TC 229 “Nanotechnology”. Resulting in a request for an ISO TC 201 SC4 New Work Item on “Surface chemical analysis - Characterization of Glass substrates for biosensing applications”, arising from the work by BAM. As a result of an ISO TC 201 request, an inter-laboratory comparison was launched to use the reference gold nanoparticle interfaces to develop an ISO standard on the preparation of nanomaterials for surface chemical analysis.

Early impact on industry

Through participating in the VAMAS study, we shared our improved methods for measuring nanoparticle interface properties with 14 research organisations, 6 national laboratories (including the NMIs of Brazil, China and Korea) and 4 instrument manufacturers. These institutes perform measurements for the developers of IVD systems, and more accurate measurements will support the production of new and improved IVDs.

Two patent applications have been prepared by two academic partners. A researcher based at Technische Universität Berlin has filed an application for their experimental cell for soft X-ray fluid interface analysis. A researcher based at Chalmers Tekniska Högskola AB has filed an application for their novel optical waveguide device, which has gained interest from both the public and private sector, including pharmaceutical company AstraZeneca.

Potential future impact on industry

The reference interfaces and traceable methods developed in this project are expected to have a direct impact on the manufacture of IVDs in Europe. Almost all European manufacturers of IVDs, and private test laboratories offering clinical diagnosis services, are SMEs that lack the financial and technical infrastructure required to develop standardised measurement techniques for biomolecular interfaces. In contrast to Europe, National Measurement Institutes (NMIs) in North America and Asia already support manufacturers by providing such standardisation activities. With this project we have initiated a new area of activity for European NMIs, creating the foundational infrastructure from which high-performance IVDs can be developed and tested. The results of this project will allow European IVD manufacturers to produce devices that consistently meet the requirements of the EC IVD directive, and which will be competitive in European and international markets.

Specific, high-potential innovations we developed include:

- The novel waveguide technique opens up new opportunities for carrying out measurements with both improved sensitivity and increased ease. The potential commercialization of the technique will likely have significant social impact as it will facilitate a more sensitive, simpler, faster and more precise detection of disease biomarkers, considerably improving diagnoses and the selection of successful treatments. To demonstrate this, we successfully detected Amyloid-beta molecules, a biomarker for Alzheimer´s disease, using a gold nanoparticle interface.
- The possibility of monitoring scattered light from surface immobilized nanoparticles has raised an interest in using the waveguide device for nanoparticle size determination, which could have a considerable impact within the nanotechnology community because of the proposed EU definition of nanomaterials and their potential regulation. The waveguide device has also gained interest from the private sector, particularly from the pharmaceutical company AstraZeneca, which sees the waveguide as a possible tool for carrying out screening of drug candidates with extraordinarily high sensitivity.

- There has also been a considerable interest within the research community to use the waveguide device for studying the interaction of virus and bacteria with interfaces. Collaborations have been initiated with BOKU University in Vienna and the University of Göttingen, who want to use the device to study bacteria and the kinetics of platelet activation.

We anticipate the results of this project will be used by manufacturers to develop more accurate and reliable IVDs for a broader range of medical conditions. This will lead to a more competitive European IVD industry, will aid in the treatment of disease, and will ultimately provide a much needed means to help tackle the rise in public healthcare expenditure throughout Europe.

2 Project context, rationale and objectives

In-vitro diagnostic devices are vital for the drive for cost effective healthcare, point of care monitoring and personalised medicine. Without the rapid and reliable biomolecular analysis offered by advanced IVD devices such as immunoassays, these goals cannot be achieved. The global market in *in-vitro* diagnostics is consequently growing and as a result, EU member states have placed a high priority on the development of, for example, portable immunoassays. Portable IVD immunoassay systems must be simple to use, have a shelf-life of many months and provide results that are both consistent and accurate. These criteria require the integration of many components, the most important being 1) those that enable recognition of biomarkers (i.e. molecules that signify a disease) and 2) those that detect the recognition event. These two key components of an *in-vitro* diagnostic device represent the two major limiting components in a device and consequently there is a need to understand 1) the efficiency of the recognition component and 2) the performance of the detection component.

Many IVD diagnostic devices rely upon surface (i.e. interface) functionality as their recognition component for detecting the presence, activity or concentration of target biomolecules. Furthermore, there is widespread acknowledgement that poor surface (i.e. interfacial) quality is one of the most important limiting factors in diagnostic performance. Therefore, there is substantial interest in the development of methods to improve the performance, reproducibility and shelf-life of diagnostic interfaces. However, these developments are hindered by the lack of adequate and validated measurements of interfacial quality. In addition, the detection method cannot be adequately described without a recognition component that has a known and reproducible performance, and currently there are no accepted standard surfaces against which the performance of detection methods can be assessed.

The European IVD Directive 98/79/EC places strict requirements on the manufacturers of IVD devices to specify and guarantee performance characteristics such as sensitivity, specificity, accuracy, repeatability, reproducibility and limits of detection. They must also ensure that performance of the IVD devices is not affected by storage and transport and provide the date after which the IVD device should not be used. These requirements, coupled with the need to provide a quality control system for production of IVD devices need substantial evidence gathered in accordance with appropriate standards. However, there are currently no documentary standards that relate to the assessment of the reproducibility and lifetime of biomolecular interfaces (used in IVD devices).

Therefore, there is a need for: accurate measurements of biomolecular concentration, distribution and structure at relevant interfaces used in IVD devices. Such relevant interfaces increasingly include those surfaces with nanoscale topography and nanoparticles, as well as a reference surface for biomolecular recognition which may be used to characterise the performance of the detection method in used in IVD devices in terms of repeatability, reproducibility, linearity and accuracy.

The use of nanoparticles in IVD devices is rapidly increasing, due to the attractiveness of these systems which revolves around the simplicity with which binding events can be detected. Binding of a target (such as a biomarker to an interface) results in a local refractive index change close to the (nano)particle and/or aggregation of the (nano)particles. Both of these binding events cause a change in particular electron oscillation frequencies in the (nano)particle and this change may be detected using optical spectroscopy or by simply observing a colour change in the (nano)particle suspension. However, there are many possible interferences and detailed analysis of the response requires a metrological understanding of the nanoparticle interface, which is currently unavailable. This lack of metrology may be why, despite the many reports of the

proof-of-principle of IVD devices, there are as yet few examples of nanoparticle-based diagnostic systems used in clinical practice. Indeed, stakeholder engagement by the consortium has established that one of the key weaknesses of IVD devices is poor reproducibility related to poor understanding and control of surface (interface) chemistry.

The concept of molecular IVD devices such as immunoassays relies upon a known 'probe' biomolecule attached to a surface (or interface) that selectively captures suspended 'target' biomolecules that are markers for disease. The development of these IVD immunoassays into microarray formats has led to the potential for multi-target/biomolecule detection which allows either the detection of more than one disease, or the more certain diagnosis of a particular disease.

Early diagnosis of disease is an important aim in health care research, as the time to diagnosis is a key factor for the chances of rapid and successful recovery. As a consequence, early diagnosis reduces the need for advanced and expensive treatment and, most importantly, increases the quality of life of the patient. However, in order to achieve early diagnosis, powerful methods and devices are required that not only are capable of reliable diagnosis at a very early stage of disease, but that can also diagnose more than one disease (in parallel), in order to provide the necessary screening at an acceptable cost and effort level for the patient and the health care system. For example, ideally, a single blood sample taken once a year could be sufficient to detect more than one disease biomarker and determine risk factors for many of the most frequent and serious diseases well before clinical symptoms appear, thus making early diagnosis and treatment possible.

Therefore, the following objectives were set to achieve the overall goal of providing techniques to better characterise the properties and performance of biomolecular interfaces:

1. Produce reference flat and nanoparticle interfaces: Establish accurate, traceable and comparable methods to determine the amount of biomolecular probe immobilised on each interface. An inter-laboratory comparison will validate these methods using the reference interfaces produced.
2. Use innovative approaches to determine the orientation and structure of biomolecules at an interface, and develop useful measurement approaches for the research, development and quality control of biomolecular interfaces in diagnostic devices.
3. Develop novel approaches for the measurement of interface structure that can be correlated with activity and binding efficiency. Develop methods to measure and predict the activity of immobilised probes, by measuring the activity of diagnostic surfaces, quantifying and modelling the interaction between probes and targets.
4. Assess and evaluate the capabilities of new, emerging techniques and approaches to biomolecular sensing which enable multiplexed, label-free identification and quantification of bound targets.

3 Research results

3.1 Produce reference flat and nanoparticle interfaces: Establish accurate, traceable and comparable methods to determine the amount of biomolecular probe immobilised on each interface. An inter-laboratory comparison will validate these methods using the reference interfaces produced.

3.1.1 Introduction

The importance of this objective relates to the need for a valid and useful reference surface through which a known and reproducible response to the presence of a target biomolecule may be obtained. The necessary steps are to establish robust reference systems that will be transferrable and acceptable to the majority of IVD system developers. The chemistry and the shelf life of the reference surface must be established and requires the development of metrological tools capable of determining the interfacial chemistry with accuracy and precision. Finally, the inter-laboratory comparison serves two purposes: ensuring that reproducible measurements can be as a precursor to standardisation and to disseminate the methods developed in this project to expert laboratories.

The aim of this objective was to produce standard platforms and reference surfaces for use in the other objectives of the project and to develop the methods by which linker, passivator and probe density can be measured. Here, the appropriate measurand is the amount of substance expressed as an area mass density, thickness and/or fractional coverage. Extraction of this measurand from data always relies upon a number of assumptions for which rigorous uncertainty budgets are not currently available. Therefore, one of the key outputs of this objective was to provide guidance on the calculation of these uncertainty budgets and the conversion between units.

This work was supported by the project's stakeholders/collaborators in laboratory medicine and industrial bio-assay developers and there was extensive stakeholder and collaborator engagement through participation in the inter-laboratory comparison using the reference materials. There was effective cooperation between all project partners and work between BAM, PTB and NPL has opened new routes for a traceable quantification of functional group densities on silicon oxide surfaces using X-ray photoelectron spectroscopy (XPS) (NPL, BAM) and TXRF/NEXAFS (PTB). This could not have been accomplished by either one of the project partners alone, since PTB does not possess the sample manufacturing expertise and neither BAM nor NPL possess the needed traceable XRR set-up at a synchrotron radiation source. This is an example of synergistic cooperation and provides assurance that the developed reference surfaces produced in this project had accurately defined linker and probe densities. In addition, the collaboration between BAM, NPL, PTB, REG(Charité) and Scienion AG has allowed the project to generate peptide surfaces, both flat and nanoparticle, as well as planar carbohydrate reference surfaces and arrays both with exceptional quality and activity.

The reference surfaces produced and used within the project were:

1. Carbohydrate reference surfaces; innovative saccharide microarrays on silicon oxide surfaces for high-throughput analysis of carbohydrate–protein interactions,
2. Protein-binding reference surfaces; protein probes attached to planar gold surfaces and
3. Peptide and protein reference nanoparticles; peptide probes attached to gold nanoparticles. These three systems acted as models for conventional and emerging diagnostic assays.

The protocols for producing these reference materials (surfaces) were developed and applied successfully to produce reference surfaces with different probe concentrations. Each different sample was characterised using XPS, in order to measure thickness, composition and reproducibility. Protocols to measure the density of linkers and probe molecules were completed and used to investigate the shelf-life and storage conditions for the reference surfaces. In all cases, storage conditions were found to ensure that these reference surfaces had a shelf-life of many months, which made them suitable for the inter-laboratory comparisons.

3.1.2 Carbohydrate reference surfaces

The protocol for producing planar carbohydrate reference surfaces and arrays was developed using an efficient one-step amination reaction strategy employing different carbohydrate molecules and silicon oxide surfaces modified with amine reactive epoxy moieties. For example simple monosaccharide molecules e.g. glucosamine, mannosamine, galactose-ß-aminopropyl and some oligosaccharide molecules, e.g., amino-cyclodextrin were used. The yield of carbohydrate (probe) attachment on these surfaces could be determined using chemical-derivatisaion XPS. These protocols and results were disseminated in a publication by the project [C Nietzold *et al*., Functional Group Quantification on Epoxy Surfaces by Chemical Derivatization (CD)-XPS].

That protocol for producing planar carbohydrate reference surfaces and arrays was applied successfully to produce carbohydrate reference surfaces with different probe concentrations as demonstrated using XPS. It became evident from the nitrogen content that the probe (glucose) density on these surfaces increased when using higher concentrated carbohydrate solutions for the immobilisation reaction. Based on the protocol REG(Charite) also produced carbohydrate reference arrays using a set of selected complex carbohydrates acting as probes for lectins (proteins that bind carbohydrates), the specific probe in this case being Concanavilin A. These oligoglucose molecules (complex carbohydrates) were prepared by REG3(Charité) and had between 4-10 glucose units. TXRF analyses are described in section 3.2 and provided traceable quantification of elements at the carbohydrate surface enabling a traceability chain to be established for XPS.

A protocol on the quality control of the production of reference carbohydrate surfaces was also produced by the project. As part of this, the effect of storage time (i.e shelf life) and conditions for the reference surfaces was tested and validated using XPS, SIMS and fluorescence spectroscopy. Two different carbohydrate reference array layouts for the SIMS/XPS testing and fluorescence analysis are shown in Figure 1. The imaging capabilities of ToF-SIMS and XPS were combined with optical fluorescence data to study the spot morphology and heterogeneity (elemental and chemical) as illustrated in Figure 1c-f. Photoelectron spectroscopy in single spots were used to identify carbohydrate specific acetal and alkoxy moieties, cf. Fig. 1f. From the results no alterations of the reference surfaces and reference arrays to different storage times (up to one year) and conditions could be found in SIMS or XPS data. Moreover, fluorescence data showed an almost constant activity of the probe molecules over time.

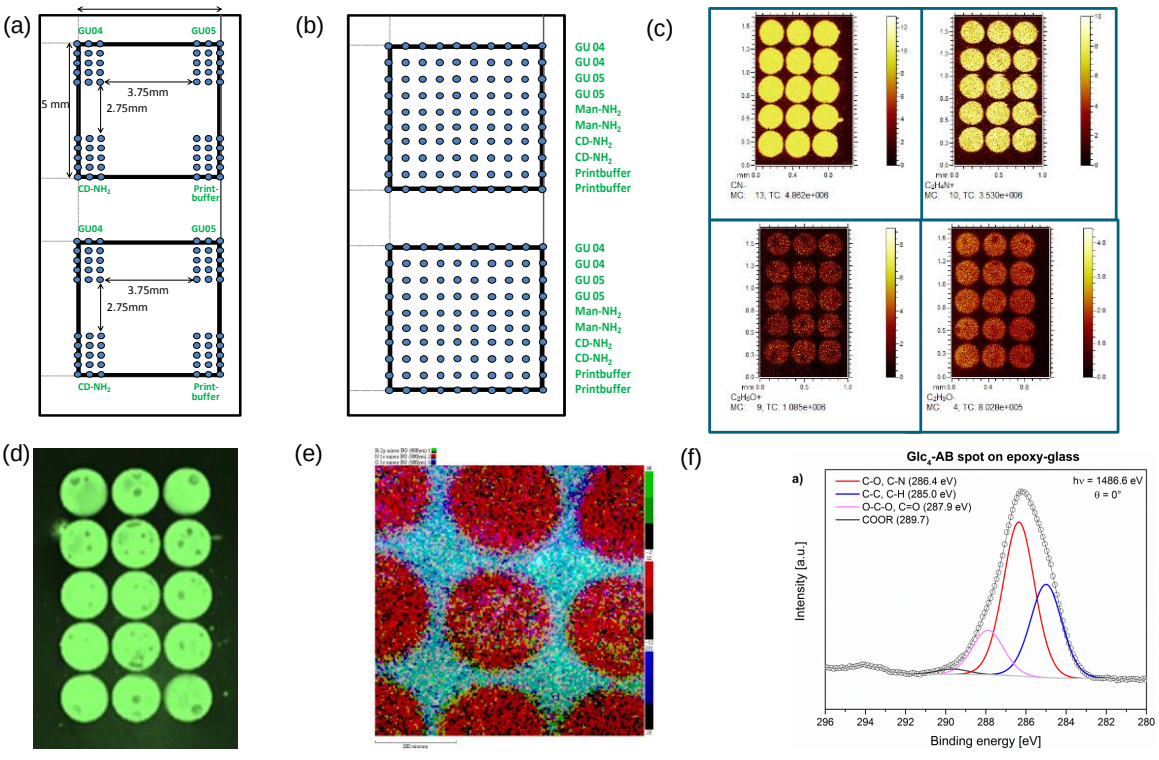

Figure 1. Slide layouts for (a) SIMS/XPS analysis and (b) fluorescence measurements during the storage and shelf-life study. Images of the slides using (c) ToF-SIMS, (d) fluorescence (e) XPS of a GU04 subarray representing the spot morphologies and elemental distributions. A high-resolution C 1s corel-level spectrum measured in the central spot of image (e) is shown in (f) clearly identifying the ether environment (red peak) of the saccharide probe.

3.1.3 Protein-binding reference surfaces

Following the suggestions of the project's stakeholder advisory board, it was decided that the most acceptable reference surface for stakeholders was the well known biotin-avidin system. This was established upon a gold surface, which is the most commonly used material with the most commonly employed detection systems: (i.e. quartz crystal microbalances and surface plasmon resonance), but is also compatible with most other forms of detection. Efforts were therefore directed at producing reference surfaces formed from molecules with three key components: 1) a thiol group to attach to the gold, 2) a biotin unit as the protein probe and 3) the hydrogel polyethyleneglycol (PEG) to eliminate non-specific attachment. Finding reliable suppliers of the appropriate compounds presented an initial challenge and the stakeholder advisory board was extremely helpful in identifying the best sources.

Methods were developed to reliably produce the reference samples with controllable surface density of the probe.Two compounds were used to create the surfaces, the first consisted of thiol and biotin end-groups on a PEG chain 10 units long. The second was a diluent to modify the biotin surface coverage and was a PEG chain 6 units long with a thiol group on one terminus

The project developed new XPS and SIMS data analysis methods to measure the thickness of an organic substance on a gold surface (using XPS) and to measure the composition of a mixed thiol overlayer (using SIMS). The latter method was a direct result of the project's investigation of structure and orientation in objective 2 and is described in more detail in section 3.2. The data may be directly converted into the density of probe molecules and details of this reference surface, including the SIMS analysis and protein binding (from objective 2) were disseminated in a paper [Ray *et al*., Neutralized Chimeric Avidin Binding at a Reference Biosensor Surface]. Figure 2 demonstrates the excellent reproducibility of the protein-binding reference surfaces and comparability of the new measurement methods.

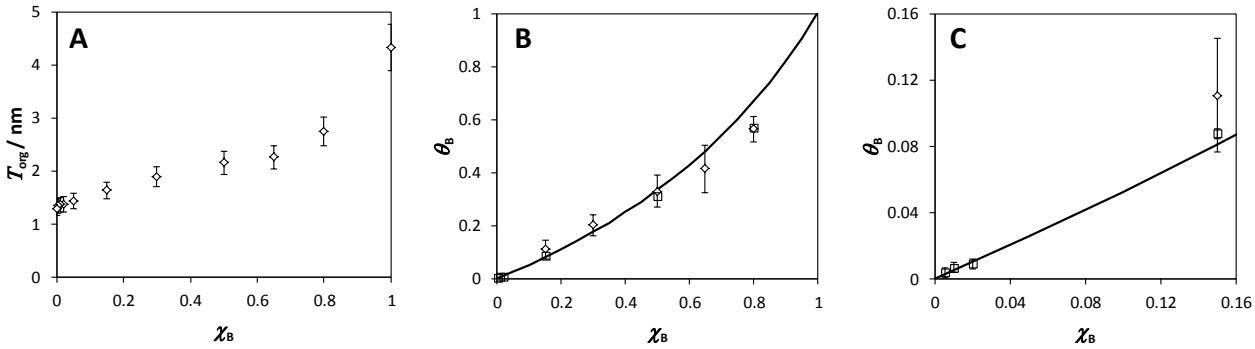

Figure 2. (A) The thickness of the organic probe layer on gold surfaces (T_{org}) as a function of the relative composition of thiols in the preparative solution (χ_B, χ_B = 1 is pure biotin-PEG-thiol). Here, the error bars represent the uncertainty (k = 1) of the measurement. The precision is similar to the size of the symbols. (B) Surface fraction of the biotin, θ_B, compound as a function of χ_B. Diamonds are XPS results, squares are SIMS results. (C) Expanded view of Fig. 1B showing the order of magnitude sensitivity improvement using SIMS.

Collaboration between project partners NPL and PTB validated the probe densities measured by XPS through the use of reference-free X-ray Fluorescence (XRF). Reference-free XRF analysis allows for the quantification of functional groups containing light elements such as C, N and O or even slightly heavier elements like sulphur in a first-principles based approach. Near Edge X-Ray Absorption Fine Structure (NEXAFS) spectroscopy is suitable to identify distinct bonds and therefore can be used to identify different molecules. Grazing Incidence X-Ray Fluorescence analysis (GIXRF) can be used to determine the amount of deposited atoms which can be converted into a surface packing density of the attached molecules. The results for the molecular surface coverage for the protein-binding reference surfaces determined by reference-free XRF quantification from the GIXRF at S Kα count rate and from the NEXAFS at the C Kα

count rate are shown in Table 1. These provide important verification of the laboratory-based XPS results and the concordance of the novel SIMS method with both of the quantitative techniques provides a level of confidence that this method is valid in the dilute regimes (<10% probe fraction) where only SIMS has the requisite sensitivity for detection of probes.

Table 1. Results for the measured surface coverage of the biotin reference surfaces in comparison to the XPS and SIMS determined packing densities. Note that no uncertainty statement can be provided for the SIMS result because the novelty of the method means that an uncertainty budget has not yet been developed.

	Density (molecules per nm^2)			Fraction of biotin probe (%)		
biotin fraction in solution (%)	GIXRF at S Kα	NEXAFS at C Kα	XPS	XRF	XPS	SIMS
100	3.8 ± 0.4	2.9 ± 0.9	3.6 ± 0.4			
50				24 ± 10 %	33 ± 6%	31%
15				8 ± 4 %	10 ± 3%	9%
0		1.8 ± 0.6	2.4 ± 0.3			

3.1.4 **Peptide and protein reference nanoparticles**

The project carried out a detailed investigation of the physical principles of particle size analysis and local surface plasmon resonances (LSPR) responses, along with a careful experimental study of the attachment of IgG antibodies to gold nanoparticles. The results enabled, for the first time, a consistent description of the major methods employed by laboratories to assess diagnostic nanoparticles. Through a combination of LSPR data and hydrodynamic size the project developeed a novel method and proved that it was possible to measure the average number of protein probes attached to a nanoparticle. Typical results are summarised in Figure 3, where it is clear that, for small nanoparticles, it is difficult to attach more than 100 IgG molecules per particle. This has direct implications for nanoparticle functionality and reproducibility as it is often the case where only a few percent of such probes are active. This method was disseminated in a paper [N. C. Bell *et al*. Quantitation of IgG protein adsorption to gold nanoparticles using particle size measurement].

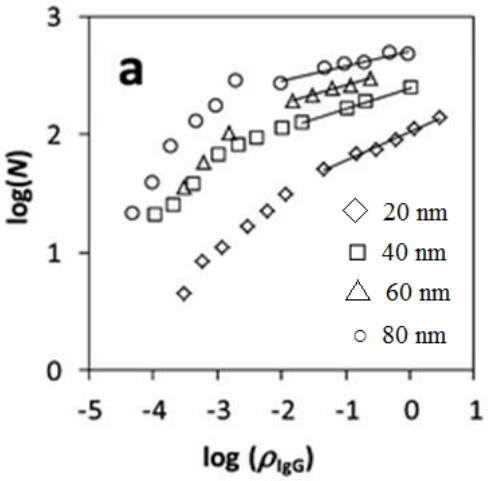

Figure 3. The average number of probe molecules per nanoparticle, *N*, plotted against the concentration of probe molecules in the preparative solution ρ_{IgG} (g/L). Four different gold nanoparticle sizes are shown and the data are plotted on $\log_{10}$ scales.

Strong collaboration between the project partners PTB and NPL on the traceable measurement of the amount of protein probe on nanoparticles enabled a comparison of small angle X-ray scattering (SAXS) with methods commonly available to stakeholders, such as dynamic light scattering (DLS). As the electron

density contrast between gold and lighter elements meant that the protein shell on gold particles was invisible to SAXS, the project used polystyrene particles and demonstrated that laboratory methods, such as DLS, were reliable provided that no agglomeration of particles had occurred. The typical relative uncertainty for the protein layer thickness of 10 nm or less using SAXS was 20 %, and although other techniques demonstrated a bias (for example DLS consistently overestimated the protein layer thickness) the agreement was wihin the combined uncertainty of the methods. These results was disseminated in a paper [C. Minelli *et al*. Characterization of IgG-protein-coated polymeric nanoparticles using complementary particle sizing techniques].

Within the project, the analysis of peptide and protein probes on gold nanoparticles was performed by XPS, and a new, simple method of analysing the XPS data, which provided both the composition and thickness of the organic material coating the particle, was developed. Using this method it was possible to directly calculate the average number of probes attached to each nanoparticle. The uncertainty of this method has not yet been established, however it is possible to compare the results with analysis of particle size and LSPR response, as described above. The agreement between these methods is shown in Figure 4 and the method was disseminated as a paper [N. A. Belsey *et al*. Analysis of protein coatings on gold nanoparticles by XPS and liquid-based particle sizing techniques].

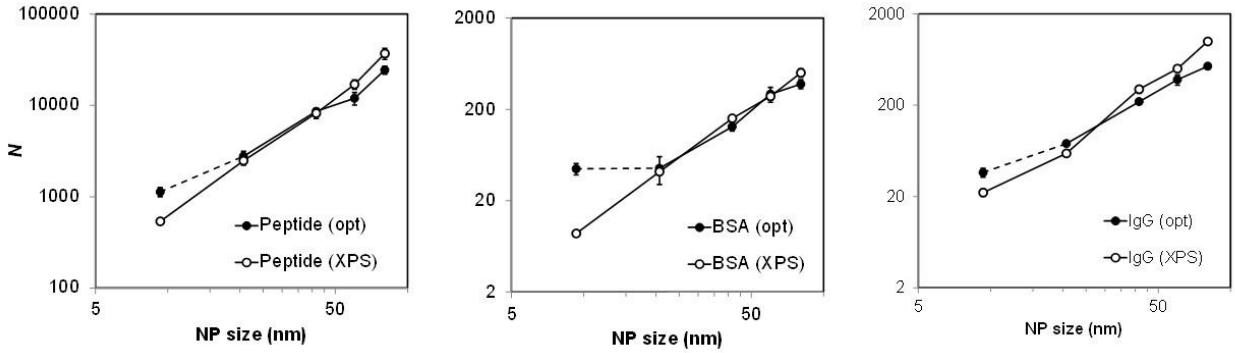

Figure 4. The average number of probe molecules per nanoparticle, *N*, plotted against the particle diameter and measured using LSPR (opt) and XPS. Note that the peptide probe is smaller than the protein molecules BSA and IgG and therefore a larger number of probes may attach to a particle.

3.1.5 **Interlaboratory comparison.**

A major highlight of the work in this objective was the use of the project's reference materials (surfaces) in a VAMAS interlaboratory comparison on the accurate measurement of chemistry and average thickness of nanoparticle coatings by X-ray Photoelectron Spectroscopy (XPS) and Low and Medium Energy Ion Scattering (LEIS and MEIS respectively). The project's protocols for producing reference surfaces on silicon oxides and gold with peptide-functionalized gold nanoparticles as probe molecules were sent to 24 participating institutes and companies, and 3 types of sample were provided: 1) peptide-functionalized gold nanoparticles (59 nm diameter gold core) ready-deposited (dried) onto silicon wafer; 2) a concentrated aqueous solution of the same peptide-functionalized gold nanoparticles for 'in-house' sample preparation; 3) a peptide-functionalized flat gold surface and; a cleaned flat gold substrate.

The VAMAS interlaboratory comparison finished at the end of the project and the key findings from the results of the interlaboratory comparison were that all participants obtained useful data from the peptide-functionalized gold nanoparticle ready-deposited samples. Although most interlaboratory comparison participants were unable to interpret their data, the resulting shell thickness agreed with a relative scatter of less than 20 %. Results from the interlaboratory comparison for the 'in-house' sample preparations (from a concentrated aqueous solution of the same peptide-functionalized gold nanoparticles) were highly scattered and highlighted the need for standardised procedures for nanoparticle sample preparation.

3.1.6 Conclusions

Versions of the same IVD with different amounts of probe will capture varying proportions of target molecules from the same sample, and may give different diagnoses. Therefore it is vital to understand how much probe is present at an interface. Interface chemistry varies between different IVDs, thus in order to develop accurate and traceable benchmark measurement methods, standardised reference surfaces must first be developed.

The project achieved this by:

1. Developing flat reference interfaces and accurate, traceable methods to measure them:
    - a carbohydrate probe with silicon oxide surface and a traceable quantification of the functional group densities using traceable TXRF results to calibrate X-ray photoelectron spectroscopy (XPS) data.
    - a biotin probe attached to a gold surface (via a thiol linker) and a novel method to measure the amount of biomolecular probe using XPS data, in addition to a novel method to measure the thiol composition on the gold surfaces using SIMS data with an order of magnitude improvement in sensitivity over traditional methods
2. Developing the first reference nanoparticle interfaces and a novel method to measure the number of probe molecules attached to gold nanoparticles in aqueous suspension using LSPR and size measurement using traceable SAXS results. In addition, the project developed a novel method to measure the thickness and composition of organic coatings on gold nanoparticles and confirmed that these results agree with in-situ methods. These results, combined, represent a major advance in the metrology of core-shell nanoparticles for diagnostic devices. The achievements for the measurements of reference nanoparticle interfaces are also of wider importance to other fields in which core-shell nanoparticles are employed including photovoltaics, lighting and display technology and catalysis.

Further to this, a major project highlight was the achievement of the first successful inter-laboratory study of nanoparticle interface chemistry, in which consistent results were achieved across each participating laboratory. The collaboration demonstrated that reproducible interface measurements could be achieved in different locations, using the reference interfaces, if standardised preparation and data analysis procedures were followed.

3.2 Use innovative approaches to determine the orientation and structure of biomolecules at an interface and develop useful measurement approaches for the research, development and quality control of biomolecular interfaces in diagnostic devices.

3.2.1 Introduction

The amount of biomolecular probe is not always a useful guide to the functional performance of molecular diagnostic surfaces, as it is known that the activity of biomolecules is strongly affected by their structure and orientation. Therefore, in the context of diagnostic devices, there is not a straightforward relationship between surface concentration of a biomolecular probe and its activity.

There is a continual search for techniques and methods that may provide information on the spatial distribution, orientation and structure of biomolecular interfaces. In this project two approaches, as suggested in existing literature were evaluated: soft X-ray spectroscopy and SIMS. The former approach soft X-ray spectroscopy accesses molecular orientation information through linear dichroism. This means that the spectra for oriented molecules are different depending upon the incidence angle of polarised X-rays. SIMS, on the other hand, displays extraordinary sensitivity to the fine details of molecular distribution, structure and environment, and recent developments in ion beam technology has provided analysts with a powerful new tool for organic surface analysis: argon cluster ions. These argon cluster ions may be used to obtain mass spectra from surfaces which contain many more intact molecules. However, the key metrological question is whether one may interpret the data to obtain quantitative information.

3.2.2 Development of a new measurement device for investigation of liquids and solid/liquid interfaces.

Since both soft X-ray spectroscopy and SIMS operate only in ultra-high vacuum, an important question was whether the structure of the molecular layer changes once it is removed from the aqueous environment into the vacuum. To begin to addressing this important question, a novel measurement device was constructed by the project to permit soft X-ray spectroscopy directly at the solid-liquid interface.

An important output of the project was the development of a new measurement device (i.e. a liquid cell) for investigation of liquids and solid/liquid interfaces. This new measurement device enables investigations of biomolecules within a liquid or at the interface between an ultra-thin window and the liquid using soft-X-rays at Ultra-High Vacuum (UHV) conditions. The liquid cell was designed to facilitate the application of soft X-ray spectrometry for the *in-situ* analysis of biomolecular films at solid-liquid interfaces. The device was developed through close collaboration between REG(TUB) and PTB.

In X-ray spectrometry in the spectral range of soft X-rays, special requirements are placed on the test set-up as the experiments have to be performed under UHV conditions. In order to meet these requirements, vacuum-compatible measuring cells are used which have thin windows and are transparent or weakly absorbing for radiation. The liquid or the solid liquid interface located at the inner side of the window can be measured through the ultra-thin windows, and the material used for these windows is, for example, 150 mm thin Si_3N_4 and SiC layers. A schematic of such a window and a photograph of the developed liquid cell is shown in Figure 5(A). In addition, Figure 5(B) shows the typical experimental design for the characterization of solid-liquid interfaces and liquids under UHV conditions. The liquid cell consists of the body with the cavity, the pipes and the gaskets, a cap (which fixes the window), a pressure sensor and pressure regulation. The gaskets are designed like conflat gaskets in order to minimize the increase of pressure during sealing and presure control is essential to avoid catastrophic failure of the window. The liquid cell can be rinsed with a solution containing the analyte until the window surface is covered with the biomolecule under investigation.

Chemical characterization of the solid-liquid interface was performed by NEXAFS measurements. Organic substances, for example, are investigated as a function of the material selected for the window by means of NEXAFS analysis at the K edges of carbon and nitrogen. As proof of principle, a target-binding protein at a functionalized surface was first investigated. These measurements were carried out at the PTB beamline for undulator radiation in the soft X-ray range at the synchrotron radiation facility BESSY II. This beamline is well-characterized and the exciting photon flux is monitored by means of calibrated photo diodes. The emitted characteristic X-rays were recorded from the sample by calibrated energy-dispersive SDD detectors with both well-known detection efficiency and response behavior. This allows for a reference-free quantification of the mass deposition even in the picogram range involving soft X-ray excitation.

3.2.3 Soft X-ray spectroscopy at the solid-liquid interface.

The project also demonstrated the accessibility of liquids and liquid-solid interfaces for biomolecular applications using the newly developed liquid cell (from section 3.2.1). Using ultra-thin windows NEXAFS studies at the biochemically relevant carbon and nitrogen K-edges were performed. The back-side of the window was prepared as a functionalized surface using an epoxy-silane with an immobilized sugar and the sugar binding protein Concanavalin A (ConA) was detected as attached at the liquid-solid interface and in liquid.

Figure 5(C) shows the NEXAFS nitrogen K-edge spectra of a 10 mMol ConA solution in Phosphate Buffered Solution (PBS) and the ConA attached at the liquid-solid interface with PBS liquid. The characteristic N1s→π* O=C-N amide bond resonance at 401.2 eV, which is present in all proteins, can be clearly detected in the NEXAFS spectra. The graph shows additionally the spectrum of the pure SiC-window where no nitrogen cross-talk occured. For the Si_3N_4 window a small carbon contamination was found which overlaps with the characteristics of the ConA protein in the NEXAFS carbon K-edge spectrum.

Polarisation-dependent measurements of biomolecules and probes both in liquid and in aqueous media revealed no clear indication of molecular orientation. Thus, although such measurements are now feasible thanks to the development of the liquid cells in this project, no positive results of molecular orientation were demonstrated.

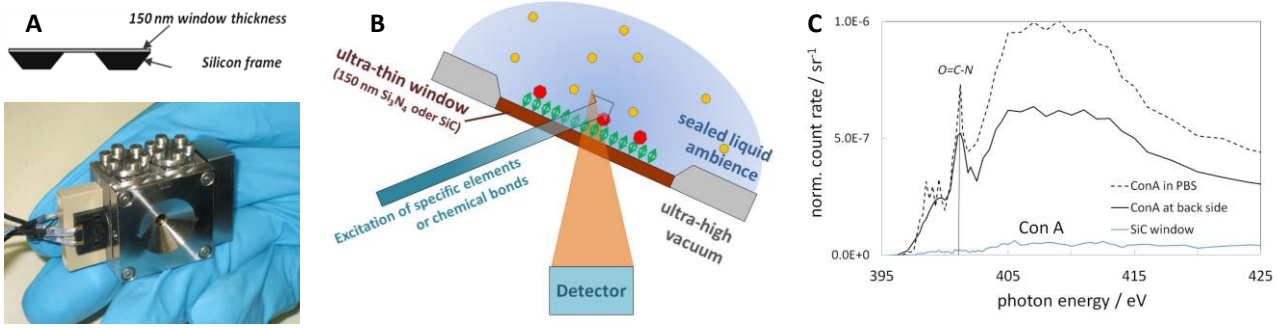

Figure 5. (A) Schematic of ultra-thin windows made from Si_3N_4 or SiC using a silicon wafer as frame and photograph of a liquid cell developed in the project. (B) Measurement principle to study liquids and solid-liquid interfaces in vacuum instrumentation. (C) NEXAFS nitrogen K-edge spectra of Concanavalin A (Con A) measured in PBS buffer solution and attached at the liquid- solid interface. The background spectrum of the SiC-window is also shown.

3.2.4 Argon cluster SIMS of reference surfaces.

The use of argon cluster ions to desorb and ionise molecular species from reference sensor surfaces was investigated by the project. The advantages of argon clusters over traditional single atomic ions and small metal clusters is that the resulting secondary ions from the surface have been shown to be less fragmented and therefore more representative of the surface chemistry. The project's investigation led to the remarkable finding that argon cluster ions generate very simple and useful mass spectra from the reference surfaces (from objective 1/section 3.1). The simplicity of the mass spectra are demonstrated in Figure 6(A), where the dominant peaks arise from a single gold atom (Au) with two thiol molecules attached (the biotin probe is indicated as B and the PEG diluent as P). If these secondary ions are formed in a simple process and the two types of thiol are intimately mixed on the gold surface then the relative intensities of these secondary ions should follow a binomial distribution. Figure 6(B) demonstrates that this is indeed the case, where the independent XPS data is used to calculate the predicted intensities shown as diamonds.

These results have profound implications. Firstly, it provides strong evidence that the biotin probes are randomly dispersed on the nanometre scale length across the reference surface, which is important for the interpretation of protein binding shown later. Secondly, the detection limit of this method is at least one order of magnitude better than XPS and enables the measurement of probe densities at biologically relevant levels. Thirdly, if the result can be generalised to other surfaces then this approach offers a new and powerful measurement technique for thiol-modified gold surfaces. Such surfaces are of major technological

importance with applications in electronics, adhesion, biology and medical diagnostics. Preliminary investigations within the project have indicated that the result can be generalised and that deviations from a binomial distribution may be used in the future to measure phase separation of thiols at a surface. Finally, if the secondary ions follow the binomial pattern they may be used to directly calculate the surface composition. The results of these calculations are shown in Figure 2 and Table 1 in section 3.1, where it can be seen that they compare favourably to more accurate X-ray based methods.

Details of the Argon cluster SIMS method were disseminated in a paper [Ray *et al*., Neutralized Chimeric Avidin Binding at a Reference Biosensor Surface] and a further manuscript extending the findings to the more general case of mixed thiols is in preparation.

Although it was hoped that the Argon cluster SIMS method could also provide information on the orientation of probe molecules, no evidence for such sensitivity could be found. Instead it was found that the most useful method in this regard was XPS. Detailed analysis of the data combined with knowledge of the probe structure demonstrated that the surface containing pure biotin probe was highly oriented with the biotin unit at the outermost surface.

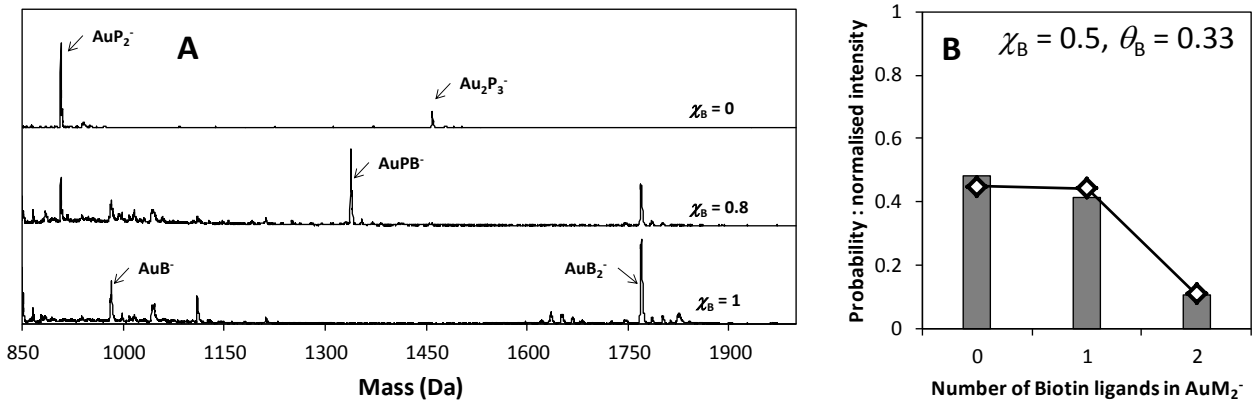

Figure 6. (A) Argon cluster SIMS spectra of three reference surfaces showing the three ions AuP_2, AuPB and AuB_2 used for measurement. (B) Demonstration that the relative intensities of these secondary ions (bars) closely matches the binomial prediction (diamonds) from the XPS surface composition, θ_B.

3.2.5 **Conclusions**

The amount of probe is not always the only guide to the functional performance of interfaces, as interfaces with poorly distributed (clumped) probes capture less target than surfaces with more evenly distributed probes. Probe molecules have active sites where they bind with target molecules, if probes are in the wrong orientation, or have lost the structural shape of their active site, they will not be able to bind with their targets. Therefore techniques that provide information on probe distribution, orientation and structure will further enhance diagnostic performance.

We developed proof-of-concept techniques for these measurements by testing two methods previously identified in the literature as possible approaches:

- We demonstrated the first use of soft X-ray spectroscopy to measure properties of the interface, and developed a novel measurement device to analyse probe molecule distribution, orientation and structure.
- Also for the first time, we used Secondary Ion Mass Spectrometry (SIMS) to measure both the distribution and structure of probes on flat interfaces.

The techniques developed represent a vital first step in the measurement of these additional probe properties, and present a direction for further research to incorporate the techniques into commercial devices.

3.3 Develop novel approaches for the measurement of interface structure that can be correlated with activity and binding efficiency.

3.3.1 Introduction

This objective forms the link between the physical measurements of biomolecular probe concentration and structure on a surface and the measurement of the attachment of the target biomolecule. The relationship between the signal from the IVD device and the amount of target attached to the surface is paramount. Using the reference surfaces developed in objective 1, the measurement of binding efficiency using different approaches was be tested and calibrated, leading to strategies that can aid the development of more efficient and reproducible IVD systems.

3.3.2 Target protein binding at a reference surface.

The binding of a target protein, neutralised chimeric avidin, at the biotin reference sensor was studied in the project using two methods: 1) quartz crystal microbalance (QCM) and 2) spectroscopic ellipsometry (SE). These 2 methods represented the two main types of direct detection used in diagnostic devices: i.e. acoustic methods (QCM) and optical methods (SE). The physics of SE and optical methods in general are better understood than acoustic methods/sensors as SE can provide a linear response to the amount of adsorbed target. However, the key metrological question is whether this may be reliably converted into an absolute amount of target, which relies upon an assumed refractive index for the target. The uncertainty associated with the refractive index of proteins is 20 % and a key advance in this project was to use XPS, with an uncertainty of 10 % to confirm that the correct refractive index was employed. By simultaneously analysing attachment of the target (neutralised chimeric avidin) with QCM and SE a non-linear response of acoustic sensors was demonstrated. QCM displayed high sensitivity at low target coverage, which declined as the target density increased. Therefore, at target coverages approaching a full layer of target the sensitivity of QCM to additional target attachment drops by an order of magnitude (compared to it's initial sensitivity). This is demonstrated by the black data in Figure 7(A), where the sensitivity of QCM is the slope of the curve and is steepest at the lowest target coverage, and these results may be adequately explained by considering the mass of water coupled to an isolated target molecule. In order to achieve approximately constant QCM sensitivity the reducing the surface density of probe molecules must be reduced so that the average spacing between probe molecules is larger than the diameter of the target molecules. In this case, this translates to a fractional coverage of <1 % biotin, which may only be measured by using the new argon cluster SIMS method developed in objective 2/sectioon 3.2. It is essential that the probe molecules do not form patches or islands and the the SIMS data provided evidence that neither patches or islands occurred. The red data in Figure 7(A), showing attachment of target to a surface with a probe coverage of 0.25 % biotin demonstrates this linear response.

An interesting finding in this work was that the mechanical response of the bound target was strongly affected by the density of probe. This was evident not in the frequency response shown in Figure 7(A), which was related to the amount of bound target, but in the dissipation response. The dissipation response is a measure of the energy loss and the data is shown in Figure 7(B). At low (0.25 %) probe coverage the data can be described by a model developed for loosely attached spherical particles, as shown by the dashed line. However at high (100 %) probe coverage the energy loss for attached target is much lower, indicative of a stiffer attachment. The transition occurs at ~1 % probe coverage, when the spacing between probe molecules is similar to the diameter of the target molecule. In this case, the biotin probe may attach to up to four sites on the neutralised chimeric avidin molecule and this change in response is due to multiple attachment of probes to a single target. This demonstrates, for the first time, that QCM can directly provide information on the mechanism of target attachment to a diagnostic surface. The analysis of this data was done through close collaboration between REG(Chalmers) and NPL.

Details of these results were disseminated in a paper [Ray *et al*., Neutralized Chimeric Avidin Binding at a Reference Biosensor Surface].

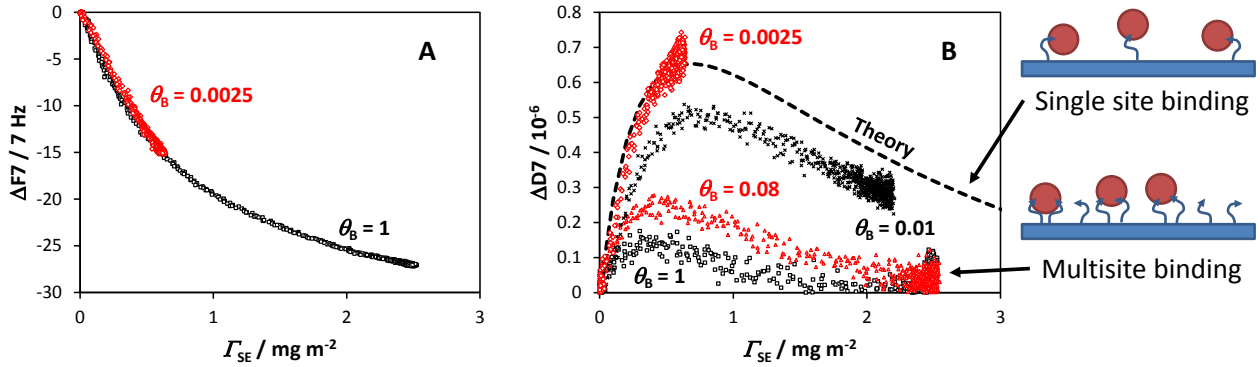

Figure 7. (A) Frequency response of QCM (ΔF7) as a function of the amount of target attached to reference sensor surfaces measured by SE and expressed as areic mass (Γ_{SE}). Two probe densities, 100% and 0.25% are shown. (B) Dissipation response of QCM (ΔD7) as a function of the amount of target bound (Γ_{SE}). Four probe densities, 100%, 8%, 1% and 0.25% are shown, the expected response is shown as a dashed line.

3.3.3 Target protein binding to reference nanoparticles.

The biotin probe reference surface described above was successfully transferred to nanoparticles and measurements of the ability to bind to the target were performed. Using normal avidin as the target, this resulted in the rapid agglomeration of nano particles due to the multiple binding sites for biotin on the avidin molecule which leads to particles sticking together. Whilst this is an excellent model of a number of diagnostic strategies, repreoducibility was found to be poor since the approach is very sensitive to the concentration of nanoparticles. Attention was therefore focussed upon molecules which displayed single-point binding and a form of streptavidin with only one active site was kindly provided by a project collaborator, the University of Oxford, UK.

Target attachment was performed with the form of streptavidin using DLS, centrifugal sedimentation and LSPR measurements. The results demonstrated that the particles remained well dispersed and provided good agreement on the amount of attached target, which was equivalent to 3.2 ± 0.5 nm thickness, or close to monolayer coverage. The change in surface charge (zeta-potential) of the particles also indicated the attachment of the target.

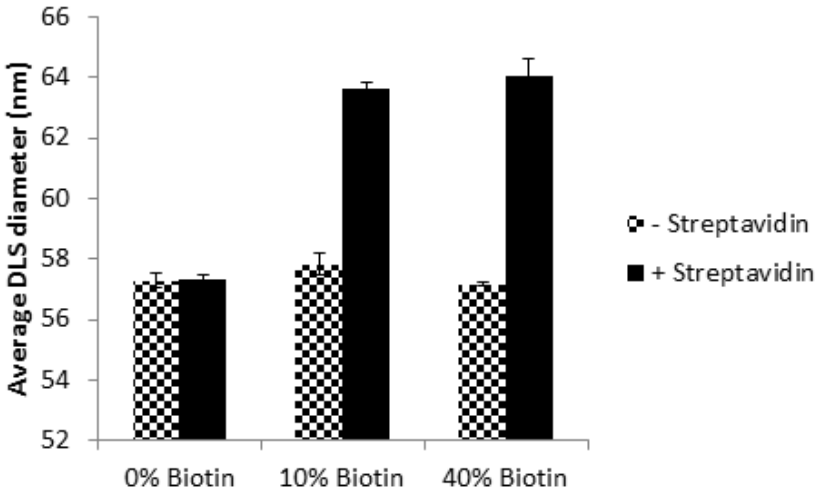

Figure 8. DLS measurement of streptavidin target attachment to reference nanoparticles as a function of probe density.

3.3.4 Numerical modelling of binding between probe and target molecules.

The binding mechanism between target and probe molecules on flat and nanoparticle references surfaces was numerically investigated using combined Poisson-Boltzmann (PB) and Brownian Dynamics (BD) models. The model, has been successfully adopted to study enzyme-substrate and protein-protein reactions, and was employed to compute the association constant and association rates of probe-target encounter complexes in a diffusional limited process and thus evaluate the binding affinity. The procedure for setting and running BD simulations and thus compute kinetic rate constants is outlined in the flow chart in Figure 9(A). Notably, each simulation starts with a randomly chosen position and orientation of proteins and is run until the proteins escape from a specific region. The post processing phase of the simulations enabled the computation of the kinetic rate constants. In order to compare the simulation and experimental results as a function of probe type, the zeta-potential profile of a gold spherical nanoparticle coated with a protein layers was experimentally measured at NPL and the results were then compared with the simulation data obtained at INRIM. The zeta-potential was derived by solving a modified Poisson-Boltzmann equation, where the simulated particle consists of a gold core with a coating bilayer composed of a thin citrate layer and an ion-penetrable layer of adsorbed IgG proteins with variable thickness, see Figure 9(B). In addition, in figure 9(C), simulated Zeta potential data of a soft particle including a hard core with a citrate layer and an ion-penetrable surface layer of adsorbed IgG proteins is shown along with the experimentally measured values. This shows excellent agreement, providing a level of validation of the computational/simulation method.

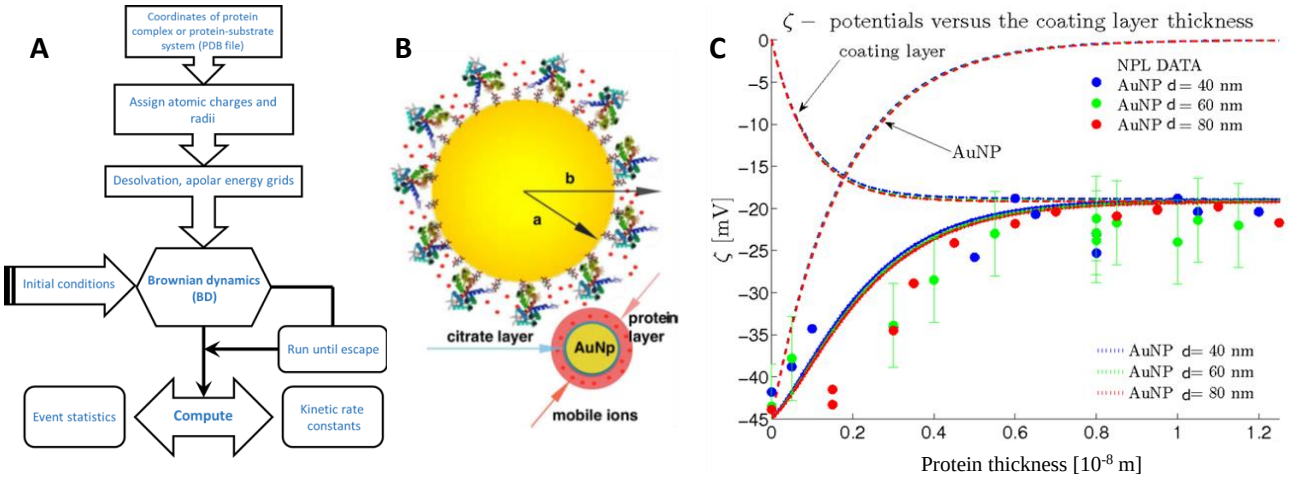

Figure 9. (A) Diagram showing the steps followed for the computation of diffusional encounter complex by BD simulation. (B) Schematic of a soft particle including a hard core with a citrate layer and an ion-penetrable surface layer of adsorbed IgG proteins. (C) Comparison between experimental and calculated zeta-potential values of a gold spherical nanoparticle coated with a protein layer.

3.3.5 Conclusions

In addition to understanding the properties of probe molecules at the interface, it is also vital to understand the efficiency by which the probes are binding with their target, to accurately assess the level of target molecules in a sample.

We studied the binding of avidin target molecules with the biotin reference surface, as avidin selectively and strongly binds with biotin, and is used routinely in IVDs. Two techniques were used. Ellipsometry measures changes in the speed of light at the interface caused by target binding. The quartz crystal microbalance (QCM) method measures changes in mass and stiffness at the interface caused by target binding.

- Simultaneous measurements with ellipsometry and QCM demonstrated that the sensitivity of the QCM method dropped as target density increased. Therefore accurate QCM measurements can only be performed if the probe molecules are separated by a specific minimum distance. This result demonstrates the importance of the reference surfaces in establishing the response of detection systems.

- The two methods revealed a novel approach to determine the type of binding between biotin and avidin targets – avidin bound by two biotin probes was significantly stiffer than avidin bound by one biotin probe. This binding was easily detected using QCM.

To study target binding of avidin with the reference nanoparticle surface, optical spectroscopy was used to detect changes in colour of nanoparticles as they bound with targets. This approach is potentially very accurate, but can be made uncertain if targets bind with multiple nanoparticles (agglomeration). To ensure measurement accuracy, agglomeration must be accounted for.

- We developed a method for measuring the number of target molecules attached to each nanoparticle by combining the optical spectroscopy with particle size measurement.

- We used high resolution sedimentation techniques to demonstrate that genetically modified avidin molecules with a single binding site did not produce agglomerates, but normal avidin molecules did form agglomerates.

A computational method was successfully implemented to describe the change in surface charge during protein attachment to nanoparticles.

3.4 Assess and evaluate the capabilities of new, emerging techniques and approaches to biomolecular sensing which enable multiplexed and label-free identification and quantification of bound targets.

3.4.1 Introduction

This objective assesses the capabilities of methods that can detect and measure multiple biomarkers simultaneously (i.e. multiplexing). Such methods are expected to provide a more certain diagnosis of a particular disease, or be able to identify more than one disease

Health conditions may produce multiple target molecules, and measuring more than one can provide a more accurate diagnosis. For example, in the treatment of heart attacks, three target molecules are measured to determine the time since the heart attack, and to treat the patient appropriately. In most situations, it is necessary to 'tag' target molecules with 'label' molecules that can be more easily detected. However, sometimes this is not possible, either because the target has not previously been identified, or the presence of the label changes the behavior of the target. In these cases a multiplexed method is preferable, which detects multiple targets without using label molecules.

3.4.2 Development of an optical waveguide device for ultrasensitive target detection.

REG(Chalmers) developed a novel optical waveguide device/chip capable of ultra-sensitive detection of molecules on surfaces. The device was evaluated with respect to it's optical properties and detection capabilities and the requirements for the device were the need to demonstrate highly efficient light in-coupling and have a surface which does not scatter light, as well as being biocompatible and having adaptable surface chemistry. These challenging requirements were met by the novel optical waveguide device/chip by ensuring that exacting standards were met in the manufacture of the waveguide in terms of very low surface roughness and refractive index control.

The novel optical waveguide chip's surface compatibility with standard glass-surface functionalization protocols, such as PLLgPEG, silane treatment and supported bilayer formation, was tested and evaluated. The general result was that the chip's surface seems to show similar surface properties as compared to normal silica (microscope glass slides and cover glass). This achievement was only possible through collaboration between REG(Chalmers) and SP, which provided REG(Chalmers) with the necessary expertise and equipment to measure surface chemistry and uniformity of coverage.

The setup of the novel optical waveguide device in a microscope is shown in Fig. 10(A), demonstrating how easily the chip can be used in combination with a standard microscope to achieve an evanescent illumination profile. The chip offers advantages over conventional total internal reflection fluorescence (TIRF) illumination schemes which include: a larger field of illumination, simple multi-wavelength excitation and a larger choice in excitation depth. Additionally the novel optical waveguide chip offers the possibility of detecting unlabeled entities, by directly monitoring up the scattered signal from the surface, which is not possible in TIRF.

The versatility of the novel optical waveguide chip with respect surface functionalization and with respect to detecting different types of nanoscopic material (metallic, dielectric, biological etc.), both label-free and using fluorescent labels, was evaluated and spherical gold nanoparticles (AuNPs) with nominal diameters down to 15 nm could be detected and imaged, as shown in Figure 10(B). Furthermore, the scattered intensity could be directly transformed into a measure of particle size using a Rayleigh scattering approximation as shown in the inset to Figure 10(B). Fluorescently labeled unilaminar lipid vesicles (1% PE-rhodamine labeled) extruded through 100 nm filter could also be detected both in fluorescence and in scattering as shown in Figure 10(C). Although the contrast in the fluorescence signal is higher than for the scattering signal for vesicles with diameter smaller than 80 nm, being able to detect direct scattering has the important advantage that the signal does not photo-bleach and it also removes the requirement of having to label the entity under study. Virus like particles (VLPs) and extracellular vesicles (exosomes) immobilised on the novel optical waveguide chips´ surfaces could also be detected both using fluorescence labeling and directly by scattering, most likely because VLPs and exosomes usually contain both membrane-bound proteins and internally confined proteins, lipids, and nucleic acids, which makes them easier to visualise by scattering.

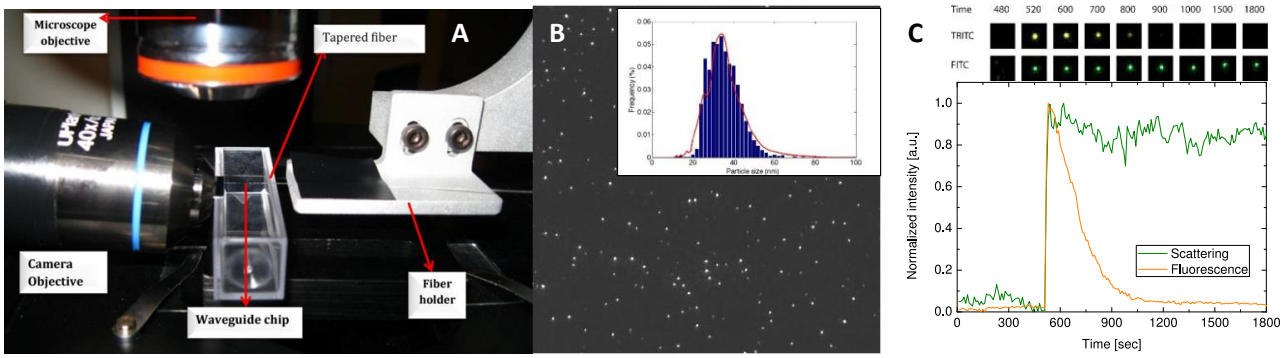

Figure 10. (A) Photograph of the novel waveguide device in practical operation. (B) 200x200 μm^2 bright field image of 30 nm in diameter gold nano particles absorbed in the sample-well of the waveguide chip. The inset shows the intensity distribution of the particles to the power 1/6 fitted to the size distribution obtained by nano-particle tracking analysis (red curve). (C)). Micrographs showing the time-evolution of the scattering (green, FITC) and fluorescence (yellow, TRITC) signals from a single 200 nm in diameter lipid vesicle containing fluorescent dye. The fluorescence signal decays with time due to photobleaching (orange line) while the scattering signal remains constant (green line).

3.4.3 Development of a method for multiplexed target detection using liposome binding and SIMS detection

By using SIMS for the detection of target-bound liposomes, it may, in principle, be possible to detect target molecules at surfaces in a highly multiplexed manner and with sensitivities down to the single-molecule level and spatial resolutions in the micrometre range. This offers a significant advantage over conventional fluorescence methods in which it is challenging to extend multiplexing beyond three targets due to limitations in fluorescent dye responses. However, several challenges are associated with the method using SIMS for the detection of target-bound liposomes and the purpose of this task was to investigate the possibilities to overcome these challenges and to evaluate the capability of the method with respect to sensitivity, target quantification and multiplexed analysis.

The major challenges of using SIMS for the detection of target-bound liposomes included preparation and characterisation of antibody-conjugated liposomes (so called immunoliposomes) that bind specifically to the antigen target molecules on the surface, as well as the preparation of model surfaces with controlled concentrations of target molecules. Furthermore, since the TOF-SIMS analysis is conducted in vacuum, a robust protocol had to be developed by the project for the transfer of the sample from the aqueous conditions under which the liposome binding takes place to the vacuum conditions of the TOF-SIMS analysis, without removing or displacing the surface-bound liposomes. Briefly, this transfer was accomplished by quenching the aqueous sample by rapid freezing in liquid propane followed by freeze drying in vacuum. These challenges were overcome by close collaboration between REG(Chalmers) and SP.

The possibility to detect individual liposomes with SIMS was demonstrated by the project in ion images showing the spatial distribution of the specific lipid ion signal from liposomes bound to the sample surface at low concentrations as shown in Figure 11(A). The observation of distinct spots representing individual liposomes highlights the potential of the SIMS approach for single-molecule target detection, assuming that one target molecule is sufficient to bind one liposome to the surface. Furthermore, increasing the target concentration on the surface results in an increasing number of detected liposomes on the surface, clearly demonstrating that the majority of the liposomes are bound to target molecules on the surface and, thus, reflect the target concentration. However, a relatively low number of liposomes were detected also on the surface without target molecules, as shown by the 0 % image in Figure 11(A). These are unspecifically bound liposomes (without target molecules) and produce a signal background that limits the sensitivity of the target concentration measurement. As the target concentration increases on the surface, the signal from individual liposomes starts to overlap and the liposomes can no longer be accurately counted in the images. Thus at the highest target concentrations, the lipid distribution is more or less homogeneous, indicating that saturation has occurred. Since the liposomes are considerably larger than the target molecules, saturation occurs well before the maximum target concentration is reached on the surface and using SIMS for the detection of target-bound liposomes is capable of quantifying the concentration of targets on the surface in the range 2x10^8 to 10^{10} targets/cm^2.

Multiplexing is the major advantage of using SIMS to detect the target-bound liposomes. By using different lipid compositions coupled to different antibodies, many different immunoliposomes/targets can be detected in parallel due to the very high specificity of the lipid signals in the TOF-SIMS mass spectra. In fact, the multiplexing capacity based on the liposome detection alone can be expected to be of the order of 100 or higher, suggesting that other factors are more likely to limit the multiplexing capacity in practice. The multiplexing capability of the SIMS method was demonstrated by the project by the parallel detection of amyloid-β (Aβ) and Tau on a model surface, Figure 11(B) shows a schematic of the approach. Surfaces with different concentration ratios of the two targets were prepared using biotin-avidin coupling chemistry and the results clearly show simultaneous and independent detection of liposomes bound to the Aβ and Tau targets, respectively, on the sample surface as shown in Figure 11(C) and associated spectrum in Figure 11(D). Even at the highest concentration of anti-Aβ liposomes, the anti-Tau liposomes were detected as distinct individual liposomes. This observation indicates that the lipids of the different liposomes are not mixed during the preparation for TOF-SIMS analysis, and suggests that individual liposomes should be possible to detect and identify in highly multiplexed measurements, even if the total concentration of liposomes is high on the surface.

In conclusion, the SIMS-based method for multiplexed target detection using immunoliposome binding was found to be promising, as demonstrated by the detection and quantification of target-bound liposomes at surfaces with sensitivities down to the single liposome level and by the simultaneous and independent detection of two different targets. However, technical challenges that limit the performance of the method and need further development include unspecific binding of immunoliposomes to the substrate surface and incomplete binding efficiency of the immunoliposomes to the targets. Furthermore, an inherent limitation of the technique is the maximum target concentration that can be quantified, which is determined by the maximum liposome concentration that can bind to the surface and is in the range 10^{10} targets/cm^2.

These results were disseminated in a paper [P. Sjövall *et al*, Liposome binding for multiplexed biomolecule detection and imaging using ToF-SIMS].

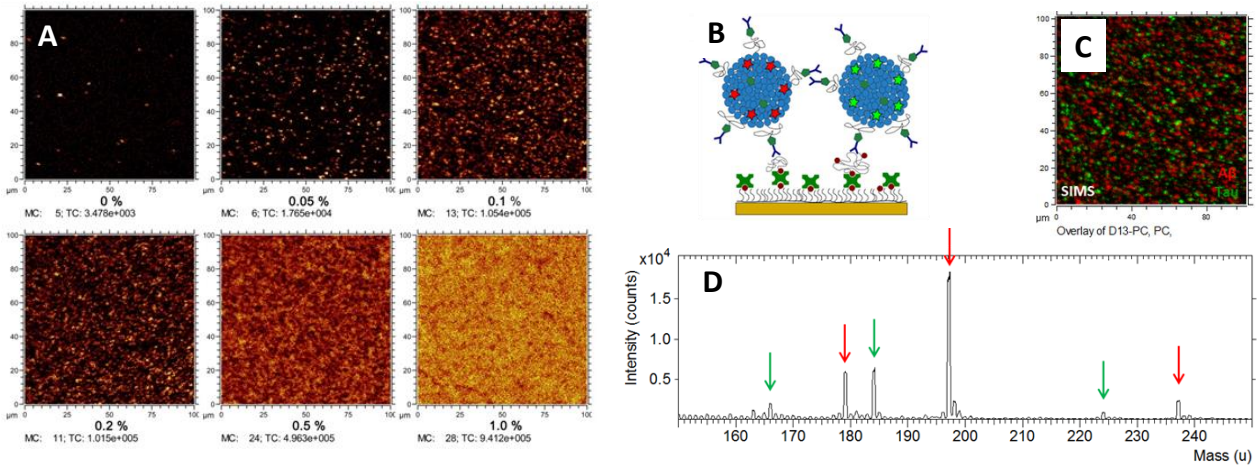

Figure 11. (A) TOF-SIMS images of antibiotin immunoliposomes bound to PLLgPEG surfaces with different relative concentrations of biotinylated PLLgPEG. Field of view 100 x 100 µm^2. (B) Schematic figure showing detection of amyloid-β and Tau on mixed surfaces using immunoliposome binding. (C) Overlay TOF-SIMS image showing anti-amyloid-β (red) and anti-tau immunoliposomes (green) on a model surface with amyloid-β/tau ratio 1:5 in the protein incubation solution. (D) TOF-SIMS spectrum of sample surface with bound liposomes showing lipid ion peaks from anti-amyloid-β (red arrows) and anti-Tau (green arrows) immunoliposomes

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3.4.4 Evaluation of ambient mass spectrometry imaging for direct, label-free detection of targets at surfaces

The project also investigated the capabilities of an ambient surface mass spectrometry, desorption electrospray ionisation (DESI) to directly identify targets at a diagnostic surface. The advantage of DESI over SIMS is that the sample may be analysed in ambient air, without the need to introduce it into ultra-high vacuum. This has significant advantages in terms of the speed of analysis and significantly reducing the

need for sample preparation. DESI operates by directing a stream of charged solvent at a surface from an electrospray source. This lifts the target from the surface in an aerosol and the aerosol is introduced directly into a mass spectrometer where residual solvent evaporates leaving the target in a charged state which may be mass analysed. When coupled to a high resolution mass spectrometer, such as an Orbitrap (as at NPL), the identification of target is facile from the accurate mass of the molecular ion. But, the open question is whether the technique is sufficiently sensitive to detect target molecules on typical diagnostic surfaces, such as microarrays. In collaboration with BAM and REG3(Charite) microarray slides of the saccharides GU03 and GU04 were obtained, both with and without bound ConA target. However, it proved impossible to obtain a DESI signal which could be assigned to either the probe or the target.

A more fundamental approach was taken to investigate the cause of this failure. In collaboration between REG4(UNOTT) and NPL microarray slides were generated with spots of known amount of a peptide (CGGGSHRSMDRYP, GenScript, USA, 98.9% purity, Mw = 1464.59 Da) and a low molecular weight protein (bovine insulin, Sigma-Aldrich, UK, Mw = 5733.49 Da) as model target species. Detection of these species was found to be possible and, through careful methods which ensured the complete consumption of each spot, a linear response was found above the detection floor of the Orbitrap spectrometer (DESI), see Figure 12. The detection limits were of the order of 50 to 100 fmol for the peptide and insulin respectively. However, for microarray operation the required detection limit is of the order of a few fmol, i.e. the DESI equipment needs to become 10 to 100 times more sensitive. Therefore it was concluded that DESI in its current form is unsuitable for microarray analysis and requires target bound to an area of ~1 cm^2 or larger for useful operation in multiplexed target detection.

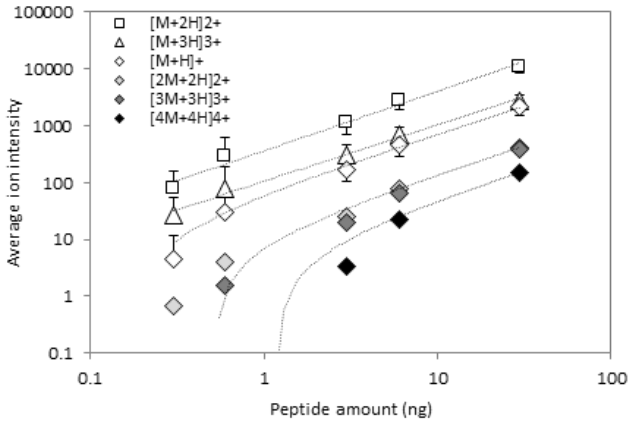

Figure 12. DESI ion intensities from the peptide model probe as a function of the amount of probe in each spot.

3.4.5 **Conclusions**

A novel optical waveguide device was developed and demonstrated, capable of ultra-sensitive detection of molecules on surfaces without using labels. The waveguide device uses light to cause target molecules bound by probes to fluoresce, so that they can be detected directly. This device offers major advantages over conventional methods in terms of ease of use, the adaptability to different illumination wavelengths, a larger field of view, and the ability to detect unlabelled targets directly through light scattering. Proof of principle has been demonstrated using fluorescently labelled liposomes and gold nanoparticles

The ability of secondary ion mass spectrometry to detect multiple target molecules using unique labels was also demonstrated for the first time. The use of unique molecular labels offers an almost unlimited potential for multiplexing, and the limitations of the method in terms of background signal and ability to resolve liposomes have ben identified and quantified. This demonstrates a dynamic range of approximately two orders of magnitude, which represents the major limitation of the method.

Additionally, label-free detection of targets on a diagnostic surface using ambient surface mass spectrometry was demonstrated. The detection limit for DESI was determined for model protein and peptide targets. This provides essential limitations on the use of the method in multiplexed diagnostic detection.

Multiple techniques were developed for multiplexed, label-free target identification, providing avenues for further research to refine the techniques for commercial exploitation.

4 Actual and potential impact

4.1.1 Dissemination activities

To promote the uptake of the outputs of this project, results have been shared through the publication of 15 papers in international journals (listed in the next section), and over 60 presentations delivered at international conferences and workshops, including five invited talks. The number of invited talks is testament to the fact that our project consortium is seen to be leading the European effort to provide measurement methods for biological interfaces. Stakeholder engagement was greatly enhanced by the consortium bringing together world-leading experts at two international workshops: the 2012 65th IUVSTA workshop on “Measuring molecular Adsorption at the Solid-liquid Interface”, and the 2015 “Nanoparticle concentration, chemistry and interfaces” workshop. Two press releases highlighted the work of the consortium, and resulted in an interview with the journal “The Analytical Scientist“. In addition, five training courses were organised during the project to share best practices amongst the consortium members and external stakeholders. BioSurf consortium members have also been consulted by several research groups, including the University of Washington, the Technical University of Vienna and the University of Manchester, on the best approaches to understand and measure nanoparticle surface chemistry.

In addition, this project will have a fundamental impact on standards through enabling the initiation of the first ever European standardisation activities for measuring the properties of interfaces. During the project, consortium partners participated in the Versailles Project on Advanced Materials and Standards (VAMAS) and ISO meetings: VAMAS TWA 2 “Surface Chemical Analysis”, ISO TC 201 “Surface Chemical Analysis” and ISO TC 229 “Nanotechnology”. Resulting in a request for an ISO TC 201 SC4 New Work Item on “Surface chemical analysis - Characterization of Glass substrates for biosensing applications”, arising from the work by BAM. As a result of an ISO TC 201 request, an inter-laboratory comparison was launched to use the reference gold nanoparticle interfaces to develop an ISO standard on the preparation of nanomaterials for surface chemical analysis.

4.1.2 Intermediate impacts

Through participating in the VAMAS study, the project shared our improved methods for measuring nanoparticle interface properties with 14 research organisations, 6 national laboratories (including the NMIs of Brazil, China and Korea) and 4 instrument manufacturers. These institutes perform measurements for the developers of IVD systems, and more accurate measurements will support the production of new and improved IVDs.

Two patent applications have been prepared by two academic partners. A researcher based at Technische Universität Berlin has filed an application for their experimental cell for soft X-ray fluid interface analysis (objective 2). A researcher based at Chalmers Tekniska Högskola AB has filed an application for their novel optical waveguide device, which has gained interest from both the public and private sector, including pharmaceutical company AstraZeneca (objective 4).

4.1.3 Wider long-term impacts

The reference interfaces and traceable methods developed in this project are expected to have a direct impact on the manufacture of IVDs in Europe. Almost all European manufacturers of IVDs, and private test laboratories offering clinical diagnosis services, are SMEs that lack the financial and technical infrastructure required to develop standardised measurement techniques for biomolecular interfaces. In contrast to Europe, National Measurement Institutes (NMIs) in North America and Asia already support manufacturers by providing such standardisation activities. With this project we have initiated a new area of activity for European NMIs, creating the foundational infrastructure from which high-performance IVDs can be developed and tested. The results of this project will allow European IVD manufacturers to produce devices that consistently meet the requirements of the EC IVD directive, and which will be competitive in European and international markets.

Specific, high-potential innovations the project developed include:

- The novel waveguide technique opens up new opportunities for carrying out measurements with both improved sensitivity and increased ease. The potential commercialization of the technique will likely have significant social impact as it will facilitate a more sensitive, simpler, faster and more precise detection of disease biomarkers, considerably improving diagnoses and the selection of successful treatments. To

demonstrate this, we successfully detected Amyloid-beta molecules, a biomarker for Alzheimer´s disease, using a gold nanoparticle interface.

- The possibility of monitoring scattered light from surface immobilized nanoparticles has raised an interest in using the waveguide device for nanoparticle size determination, which could have a considerable impact within the nanotechnology community because of the proposed EU definition of nanomaterials and their potential regulation. The waveguide device has also gained interest from the private sector, particularly from the pharmaceutical company AstraZeneca, which sees the waveguide as a possible tool for carrying out screening of drug candidates with extraordinarily high sensitivity.
- There has also been a considerable interest within the research community to use the waveguide device for studying the interaction of virus and bacteria with interfaces. Collaborations have been initiated with BOKU University in Vienna and the University of Göttingen, who want to use the device to study bacteria and the kinetics of platelet activation.

We anticipate the results of this project will be used by manufacturers to develop more accurate and reliable IVDs for a broader range of medical conditions. This will lead to a more competitive European IVD industry, will aid in the treatment of disease, and will ultimately provide a much needed means to help tackle the rise in public healthcare expenditure throughout Europe.

5 Website address and contact details

http://projects.npl.co.uk/HLT04-BioSurf/

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6 List of publications

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