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# Surface chemical analysis — Characterization of functional glass substrates for biosensing applications

*Analyse chimique des surfaces — Caractérisation de substrats de verre fonctionnels pour les applications de biodétection*



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# Contents

Page

# Foreword

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This document was prepared by Technical Committee ISO/TC 201, *Surface chemical analysis*.



# Introduction

Sensing devices based on immobilized biomolecules on solid substrates are a steadily growing market in personalized medicine and point of care (POC) diagnostics, which are becoming tremendously important for our society. Precise knowledge of these biointerfaces is a prerequisite for a reliable and proper functionality of such biosensing devices. This kind of knowledge includes surface composition, surface chemistry (e.g. functional groups, surface species), surface structure/morphology, in-depth compositional profiles and film thickness, which can be obtained by a thorough physico-chemical characterization using surface chemical analysis.

This report on surface chemical analysis of glass substrates for biosensors prepared by ISO/TC 201/WG 4 has been prepared in coordination with the overall characterization needs identified by experts in TC 201.

This document describes the information that can be obtained by the different analytical techniques and examines how this information can be used to understand and solve important questions and challenges in the biosensor production process.

With that focus, consideration of

— bulk composition;

— surface composition;

— cleanliness;

— wettability;

— reactivity; and

— stability



are relevant and (in an ideal case) should be known for each component, i.e. substrate, functional coating/layer and biomolecular probes, of a reliable biosensing device.

# Surface chemical analysis — Characterization of functional glass substrates for biosensing applications

## 1 Scope

This document gives examples of how methods of surface chemical analysis in the scope of ISO TC 201 are useful to characterize the nature of substrates used to produce biosensing devices. Successful characterization will give the opportunity for a better understanding of aspects of surface chemistries and reactions at each step of production influencing the overall performance of the final device, for example a microarray. The steps of preparation are the activation of the substrate by immobilization of linker molecules and the functionalization of the activated substrate with biomolecules required for specific biosensing, the so-called probes.

Herein, a focus is set on silane-based functionalization of glass slides, a critical production step for subsequent immobilization of probe molecules. Those probes are used for sensing of biological recognition events. The silanization process has been selected because it is one of the most popular in biosensor production today.

This document gives an overview of methods, strategies and guidance to identify possible sources of problems related to substrates, device production steps (cleaning, activation and chemical modification) and shelf-life (storage conditions and ageing). It is particularly relevant for surface chemical analysts characterizing glass-based biosensors, as well as developers or quality managers in the biosensing device production community. Based on quantitative and qualitative surface chemical analysis, strategies for identifying the cause of poor performance during device manufacturing can be developed and implemented. This document shows how far the light may shine today and possible starting points for more specific activities of ISO/TC 201 in the future, which end in standardized procedures for measurements.



No specific protocols on processing are discussed in this document. To learn more about protocols the reader is referred to specialized literature, see for example References [1] to [9].

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 18115-1:2013*Surface chemical analysis — Vocabulary — Part 1: General terms and terms used in spectroscopy*

ISO 18115-2:2013*Surface chemical analysis — Vocabulary — Part 2: Terms used in scanning-probe microscopy*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 18115-1:2013 and ISO 18115-2:2013 apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

## 4 Symbols and abbreviated terms

| | |
|---|---|
| AFM | atomic force microscopy |
| APDMES | 3-aminopropyldimethylethoxysilane |
| APS | 3-aminopropylsilane |
| APTES | 3-aminopropyltriethoxysilane |
| APTMS | 3-aminopropyltrimethoxysilane |
| FT-IR | Fourier transform infrared spectroscopy |
| LEIS | low energy ion scattering |
| MEIS | medium energy ion scattering |
| NEXAFS | near-edge X-ray absorption fine structure |
| PCA | principle-component analysis |
| SCA | surface chemical analysis |
| SFG | sum frequency generation spectroscopy |
| STM | scanning tunnelling microscope |
| ToF-SIMS | time-of-flight secondary ion mass spectrometry |
| TXRF | total reflection X-ray fluorescence |
| WCA | water contact angle |
| XPS | X-ray photoelectron spectroscopy |



## 5 Characterization of substrates for biosensors by surface chemical analysis

### 5.1 Introduction

Biosensing as a concept utilizes a biological or biomolecular recognition event that is transduced into a measurable signal (electrical, optical, chemical or thermal). Therefore in many biosensor devices, a biomolecular system acting as probe is immobilized to a solid support to capture the specific target (analyte) from the sample giving a biochemical reaction that is transformed into a signal[7][10–12].

A special type of biosensing application is the microarray, a high-throughput analytical tool for studying biological processes enabling quantitative and simultaneous analyses of a large number of biomolecular interactions in a very short time. A typical microarray consists of biomolecules arranged in single spots in a miniaturized, spatially defined fashion and attached to a solid surface (see Figure 1).

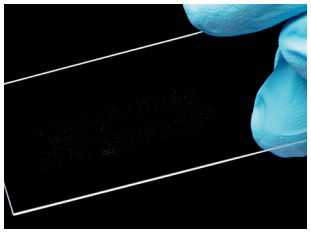


**Figure 1 — Example of a microarray with subarrays**

Microarrays are essential tools for high-throughput screening and detection in biochemical, bioanalytical, medical or diagnostic research and especially in the continuously growing field of omics (genomics, proteomics, glycomics and metallomics)[13–15]. However, there is still a need for approaches to sidestep current problems associated with the repeatability, reproducibility and quantification of DNA and peptide arrays by understanding the factors that contribute to unreliable performance. Analytical tools are required which may help to understand the origin of variability in array performance and mitigate such variability[16]. Substantial improvement in reliability and reproducibility of biosensors will support clinical acceptance of biosensor-based diagnostic devices.

Glass is one of the most frequently used substrates for biosensor and microarray fabrication because of its low price, availability, stability (against temperature, many chemicals and biological materials), low fluorescence, flatness, non-porosity and manifold possibilities of surface modifications.

Most glass substrates used in biosensor manufacturing are coated first with a functional silane layer. Then, these silane films are used directly or after activation for binding the probe molecules of interest. Careful quality control for the cleaning, pre-activation and silanization steps is mandatory, especially for identifying contaminants and proper characterization of the applied coatings[17–19].

In this report, well-known approaches of surface chemical analysis that can be used to control every single step of glass-based biosensor production are introduced to developers of biochips and quality managers in their production. Equally, surface chemical analysts who have a specific need to characterize glass-based biosensors are addressed by this report.

Routinely applied simple methods for surface analysis, such as water contact angle measurements[20], have an intrinsic limitation of giving only macroscopic summary information of the analysed surfaces. In contrast to that, a multi-method approach of surface analytical techniques can yield detailed information about surface chemistry, species and composition, and subsequently, a combination of vacuum-based surface characterization techniques bears great potential for identifying issues in manufacture and quality control.



## 5.2 Surface chemical analysis (SCA)

### 5.2.1 General

This subclause gives a short overview of surface characterization techniques currently covered by TC 201 which are fit for purpose. It is prepared to inform users from the biosensing community unfamiliar with those methods. For details on individual techniques the reader is referred to specific textbooks and other literature[21][29].

### 5.2.2 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is a powerful tool for surface chemical analysis, as it gives specific elemental as well as chemical information with only a few restrictions concerning the specimen.

In XPS the specimen is irradiated with X-rays under ultra-high vacuum (UHV) conditions. Due to the photoelectric effect the surface is emitting photoelectrons. The kinetic energy of the detected photoelectrons is characteristic of the individual constituent element acting as emitting atom. A quantitative elemental analysis of the probed surface layer is possible, except for H and He. Details of specific binding states of the emitting atoms are also possible because the binding energy of the emitted photoelectron is correlated with its binding situation.

A characteristic chemical shift is observed in many cases. By analysis of highly resolved spectra, quantitative information on binding states of the elements in the probed surface can be discovered. Coexisting chemical species in the sample can be often differentiated from each other because today most laboratory XP spectrometers are using monochromatized X-rays, which enable high energy resolution. Imaging photoelectron spectroscopy can also be carried out. In this case contrast results either from different elemental distributions or different chemical shifts for spectral components of a highly resolved core-level spectrum of one element. An ultimate lateral resolution of ~10 µm can be reached using recent instruments. Typical sensitivities in XPS are in the range of 0,1 at% to 1,0 at%[30].

With a typical Al Kα X-ray source the information depth is in the order of 10 nm and depends on the photoemission signal used[22][27].

Because modern XPS instruments are equipped with charge neutralizers, bare and functionalized glass slides can be analysed without any conductive coating of the samples.

### 5.2.3 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is secondary ion mass spectrometry (SIMS) using a time-of-flight (ToF) mass analyser. SIMS is based on primary ion bombardment of the sample in UHV with primary ion energies of some keV. During that process the energy of the primary ions is transferred by atomic collisions into the solid, causing a cascade of collisions. Since some of the energy is transferred back to the surface, atomic and molecular fragments – but also complete molecules (masses up to 10 000 u) – are ejected from the surface topmost layers, which can be detected if they were ionized. Fragment patterns in SIMS spectra can often be correlated with specific compounds present in the probed surface area. Careful analysis of the detected masses of desorbed secondary ions in SIMS spectra reveals details on elemental and molecular composition of the analysed surfaces.

For chemical analysis SIMS is operated in the "static mode", in which the material surface is sputtered at a sufficiently low rate that the original surface is insignificantly damaged during the analysis. Distribution images with lateral resolution in the sub-µm region are obtained if focused primary ion beams are used. SIMS is more surface sensitive than XPS because the detected secondary ions are emitted from the topmost surface (1-3 monolayers) and ppm sensitivities can be reached for selected elements.



ToF mass spectrometers are characterized by high transmission and sensitivity with a broad application range. Different operation modes are possible surface spectroscopy, imaging and depth profiling. Because modern ToF-SIMS instruments are equipped with charge neutralizers, bare and functionalized glass slides can be analysed without any conductive coating of the samples.



ToF-SIMS experiments usually result in huge data sets. Fragmentation often leads to hundreds of peaks in a spectrum. In most cases the relevance of a single peak is unknown, but sometimes characteristic fragment patterns are known and (quasi)molecular peaks are identified. Therefore, data reduction methods, for example principal-component analysis (PCA), are used to extract all relevant information. Scores and loadings plots delivered by PCA help to differentiate chemical states, to identify characteristic peaks and to retrieve semi-quantitative information[23–26].

### 5.2.4 Near-edge X-ray absorption fine structure (NEXAFS) spectroscopy

Near-edge X-ray absorption fine structure (NEXAFS) spectroscopy provides chemical state information that is complementary to XPS, concerning the chemical binding and quantity of molecules on a surface. In the electron yield mode NEXAFS spectroscopy has the same information depth as XPS. Saturated and unsaturated bonds in organic matter can be easily differentiated by NEXAFS spectroscopy.

Synchrotron light is polarized and utilizing angle-resolved NEXAFS spectroscopy is a tool to determine preferential orientations of molecules or specific functional groups on the surface in a similar way to what can be done in IR spectroscopy. In a NEXAFS experiment the energy of synchrotron light is stepwise varied across the absorption edge, for example for a K-edge of carbon or nitrogen. By doing so resonant transitions from a core level, for example C 1s or N 1s, into molecule-specific unoccupied molecular states, for example the LUMO, can be observed in the X-ray absorption spectra as $\sigma$ and $\pi$ resonances. These spectral features make NEXAFS spectroscopy a very useful method to characterize a sample on a molecular level, but due to the necessity of a synchrotron source it is not a routine surface analytical technique like XPS or ToF-SIMS[28][29].

### 5.2.5 Other methods

#### 5.2.5.1 General

In this document the main focus is on XPS, SIMS and NEXAFS, but there are complementary techniques that are often used in characterization of functional glass substrates for biosensor applications. These include spectroscopic ellipsometry, Fourier transform infrared spectroscopy (FT-IR), Raman, sum frequency generation spectroscopy (SFG), fluorescence spectroscopy, atomic force microscopy (AFM), scanning tunnelling microscope (STM), low energy ion scattering (LEIS), medium energy ion scattering (MEIS), total reflection X-ray fluorescence (TXRF) and water contact angle (WCA), to name only the most relevant ones. Some of them are less known to the community addressed by this document and are explained in some detail in this subclause.

#### 5.2.5.2 Ion scattering (LEIS and MEIS)[25][26][31][32]

LEIS delivers quantitative information on the atomic composition of the outermost atomic layer of a solid sample. In LEIS, a beam of low energy noble gas ions with a kinetic energy of a few keV is used to probe the surface. The energy of each backscattered ion is characteristic for the mass of the corresponding collision partner, which is here a surface atom. The energy of the backscattered ions is measured by an energy analyser and element analysis of the surface is enabled. Ions that are scattered from depths up to 10 nm can also be detected and lead to a signal that is characteristic for the particular in-depth composition of the respective elemental, and layer thickness can also be determined. Because the LEIS scattering regime includes only negligible sputtering, the chemistry of biosensing platforms can be studied and depth profiling is non-destructive.



MEIS basically uses the same approach as LEIS but uses ions of higher energies around 100 keV. MEIS is a technique that allows probing film composition with sub-nanometre depth resolution and is preferentially applied to oxide layers used in microelectronics. It can be easily used to study bare substrates as glass slides used for bio sensing devices. So far there is only one MEIS application in the literature where an organometallic film is studied[32].



#### 5.2.5.3 Spectroscopic ellipsometry

Ellipsometry is capable of measuring changes in film thickness with sub-nanometre precision, if there is sufficient knowledge of the refractive indices of the materials in the substrate and overlayer. For organic films on glass, the refractive indices of the two materials are very similar in the visible region, and therefore measurements of overlayer thickness are very challenging. Ellipsometry in regions of the infrared, where the organic film has significantly different optical properties to glass, shows excellent potential[26][28][33][35].

#### 5.2.5.4 Total reflection X-ray fluorescence

TXRF utilizes extremely low-angle X-ray excitation of flat surfaces of bare or functionalized glass slides to obtain the concentration of surface contaminants and elements in functional layers. Using total reflection conditions (incident angle of the X-ray beam typically as small as 0,05°), excitation is limited to the outermost surface of the sample. By using monochromatic synchrotron radiation in the soft X-ray range and an ultra-high vacuum setup including windowless detectors, even light elements on a substrate surface, for example carbon or nitrogen, can be excited effectively. That high experimental effort facilitates quantitative TXRF analyses of organic nanolayers on flat substrates[36–41].

Recently traceable TXRF has been shown to provide direct access to the mass-per-unit area of selected elements in a thin surface layer. The mass-per-unit area of nitrogen was determined for aminosilane functionalized Si wafer substrates, which can be used to determine the number of amino groups on the surface. Based on this, XPS was calibrated and absolute and traceable quantification of surface-bound organic molecules on silicon oxide surfaces by XPS enabled[42].

Methods such as fluorescence spectroscopy [43–45], vibration spectroscopy (FT-IR and Raman) [25][26][28][46], scanning probe microscopy (e.g. AFM, STM)[25][26][28] and water contact angle goniometry[28]

are widely used in the community addressed by this document and references to relevant reviews and textbooks are referenced in this subclause.

## 5.3 Characterization of microarray glass substrates

### 5.3.1 Glass surface composition

An important but often disregarded or ignored factor influencing the whole biosensing experiment is the chemical composition of the glass substrate. For example, glass with high contents of sodium and phosphates is very challenging. Non-bonding ions such as phosphates and alkali metal ions are known to catalyse Si-O bond breakage and redistribution. Additionally, contaminants from the bulk tend to segregate to the surface. Therefore, these unwanted species have to be removed before any further processing. Species can be identified and their successful removal verified by surface analysis[47].

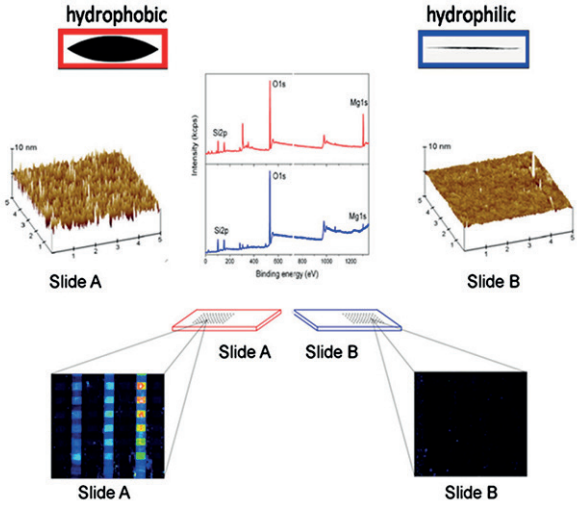
The elemental composition in regions close to the surface (within ca. 10 nm) of glass samples can be measured by XPS according to, for example, ISO 18118:2015. From that information it is possible to differentiate between different glass substrates, such as soda-lime, phosphate or borosilicate glasses[48][49].

At BAM Federal Institute for Materials Research and Testing, the surface composition of glass slides from different vendors used for microarray applications were compared using XPS. Silicon and oxygen were the major constituents in all the glass slides. Slides made of soda-lime silicate glass are characterized by significant amounts of Na, Mg and Ca. Borosilicate slides exhibit boron as one of the main constituents combined with only low amounts of sodium. In addition, minor amounts of aluminium, nitrogen and potassium were found on all slides. Samples made by the float glass process show characteristic tin peaks on one side. (For more details see Annex A, Table A.1.)



Alterations to the glass formulation of commercial soda-lime glass substrates may have dramatic effects on biosensor performance, as described by North et al.[49], who observed a significant change in antibody immobilization with glass composition. After a systematic screening of the bare glass slides using water contact angle measurements (WCA), fluorescence spectroscopy and XPS, the performance of microarrays expressed as antibody immobilization efficiencies was related to individual chemical compositions, specifically a variation of the magnesium content, and surface morphologies expressed as roughness of the used glass slides (see Figure 2). XPS was used to identify elemental disparities in the glass surface of different commercial soda-lime slides.






NOTE Slide A (red; high magnesium) and slide B (blue; low magnesium) by contact angle measurements (top level), XPS (centre), atomic force microscopy (middle level) and functional binding assay (lower level) reveal a significant correlation between magnesium content, surface roughness and bioimmobilization efficacy[7][49].

SOURCE Reproduced with permission from North, S., et al. Critical aspects of biointerface design and their impact on biosensor development. *Analytical and Bioanalytical Chemistry*, 2010. 397(3): 925–933.

**Figure 2 — Surface analysis of a soda-lime microscope glass slide**

### 5.3.2 Characterization of cleaned and pre-activated glass slides

Glass slide cleaning is another crucial step in biosensor production because its effectiveness determines the number of reactive silanol sites, which are necessary to immobilize silane linker molecules in the following step. Problems with glass cleaning procedures may contribute to variability in array performance in diagnostic applications.

Many different cleaning protocols for glass substrates are published and often the well-known methods for silicon substrates from the semiconductor industry are adopted[50–52]. However, many of them apply rather harsh conditions, such as strong acids and/or bases, peroxides or plasma, making these methods difficult to integrate in an industrial process where restrictions due to safety and waste disposal regulations apply. Moreover, the composition and especially the surface chemistry of glasses may be altered by these cleaning procedures[53].

For silicon substrates piranha treatment, using mixtures of aqueous hydrogen peroxide and sulfuric acid[47][51][54] is often sufficient to clean and activate the surfaces before silane coating[54–56], but much milder protocols have been reported, especially for glass substrates. Some of these are summarized in Table 1.



**Table 1 — Selection of different glass cleaning methods from various studies that proved to be efficient**



| Method | Conditions | Control method | Hazards | Equipment | Reference |
|---|---|---|---|---|---|
| 1 | a) HCl/MeOH(1:1), 0,5 h<br>b) $H_2SO_4$ conc. (0,5 h–2 h) | WCA, XPS, AFM, FT-IR | Acid | Standard labware | [18], [54], [57] |
| 2 | a) boiling in $Na_2S_2O_8$ (5 % aq.), 15 min.<br>b) Acetone, ultrasonic, 30 min. | XPS (residual carbon content), nano-indentation | Peroxide | Standard labware | [47], [58] |
| 3 | 10 % KOH (w/v) in MeOH, 2 h | XPS, WCA | Base | Standard labware | [49] |

For a more comprehensive overview of glass cleaning procedures and practical guidance, see References [18], [19], [47] and [54]. Unfortunately, there is no universally accepted cleaning and activation method or reagent fit for all purposes, but there are a few accepted methods, especially for glass surfaces, that can be applied[18][19][47][54]. All protocols are employed to create a clean surface with a large number of silanol functional groups on the surface, which are able to react in the following silanization step (see 4.4). Typically such a cleaned and activated silicon-based surface is characterized by a surface density of approximately $5 \times 10^{14}$ silanol groups per $cm^2$[57][59][60].

In each case the efficiency of the glass-cleaning protocol should be tested and validated independently by surface chemical analysis. For example, SIMS can be used to detect and even quantify silanol groups and contaminations, especially in the (sub)monolayer regime. A detailed study where commercially available (pre)cleaned glass slides are investigated in terms of the effectiveness of the applied cleaning protocols by XPS, WCA and SIMS is given in Annex A.

An important surface contamination with impact on the amount of reactive silanol groups on glass slides is ambient hydrocarbon accumulation. Smith presented a simple and straightforward method to estimate the thickness of the hydrocarbon contamination overlayer on samples handled in laboratory environments. The method is based on the amount of carbon measured by XPS[61].