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Sigla settore scientifico disciplinare: **BIO/10**

Surface chemistry evaluation in the design and fabrication  
of micro-devices for protein analysis

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# Abstract

In this Ph.D. thesis, the problem of the coating and adhesion of polymers onto selected surfaces has been investigated with the aim of designing micro-devices and/or microarrays for protein analysis. In the first part of the thesis, the micro-fabrication of a device for isoelectric focusing has been studied. The development of this device has required the covalent attachment of hydrogel plugs functionalized with pH buffering monomers on an Indium Tin Oxide (ITO) flat surface. The two ITO-coated slides are separated by silicon frames (both 3mm thick) stuck on the surface and the whole device was named as Micro Parallel Isoelectric focusing Device (MPID). The problem of the adhesion of the hydrogel plugs onto the ITO anode under the high vacuum conditions of the MALDI-TOF/TOF analysis has been solved, by properly selecting the percentage of monomers and related polymerization conditions.

The MPID fabricated in accord to previous observations did not afford a completely efficient isoelectric separation of the model protein mixture (commercial carbonic anhydrase). Furthermore, the proteins were absorbed onto plugs too tightly for a satisfactory, direct analysis by MALDI-TOF/TOF of the plugs.

At this stage of the Ph.D. program, the MPID project has been abandoned and the second part of the Ph.D. thesis has been directed towards the study of a functional coating onto different surfaces using a polymer that is the result of the co-polymerization of N,N-dimethylacrylamide (DMA), 3-(trimethoxysilyl)-propylmethacrylate (MAPS) and acryloyloxysuccinimide (NAS) [Copoly (DMA-NAS-MAPS)]. It has been shown that this copolymer is able to generate thin layer of nanometer size onto inorganic substrates like glass, silicon oxide, or gold, typically used for the development of microarrays or bio-sensors for immunoassays. Applications of silicon and gold surfaces coated by Copoly (DMA-NAS-MAPS) are presented and discussed.

# Introduction

Among the classic methods for protein analysis, electrophoresis is a simple, rapid and sensitive tool to evaluate the properties of proteins.<sup>1</sup>

In electrophoresis, the mobility of a molecule in an electric field is inversely proportional to molecular friction which is the result of its molecular size and shape, and directly proportional to the voltage and the charge of the molecule. Proteins could be resolved electrophoretically in a semi-solid matrix, usually made of polyacrylamide gel (gel electrophoresis) strictly on the basis of molecular weight if, at a set voltage, a way could be found to charge these molecules to the same degree and to the same sign. Under these conditions, takes place the SDS-PAGE technique (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), in which the mobility of the molecules would be simply inversely proportional to their size. The resolution of proteins can take place according to the fact that charged molecules can migrate through a matrix if surrounded by an electric field, usually provided by immersed electrodes. Proteins migration is determined by the overall charge which is influenced by the pH of the surrounding solution or medium that be reached dissolving proteins in a specific buffer.

The separation of the proteins takes place into a polyacrylamide gel cross-linked with bis acrylamide in variable percentage depending on the porosity needed. An electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive (+) electrode (anode). Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty (they encounter more resistance).

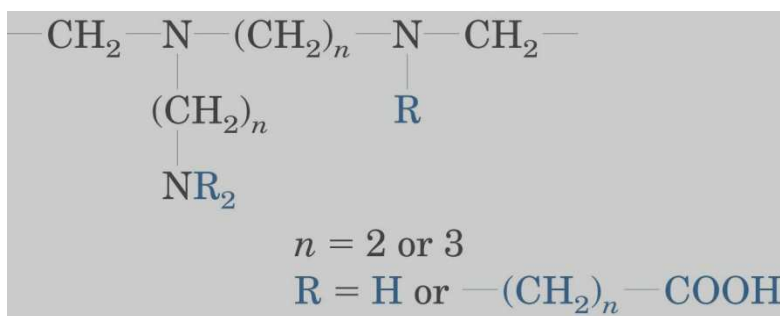
After a few hours, the proteins will have differentially migrated based on their size; smaller proteins will have traveled farther down the gel, while larger ones will have remained closer to the point of origin. The time depends on the voltage applied across the gel; protein migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. At the end of the migration the gel may be stained (most commonly with Coomassie Brilliant Blue R-250 or silver stain), allowing visualization of the separated proteins.

After staining, different proteins will appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel, in order to calibrate the gel and determine the approximate molecular mass of unknown proteins by comparing the distance relative to the marker.

## Isoelectric focusing: general principles

Isoelectric focusing (IEF) is an electrophoretic method in which proteins are separated on the bases of their isoionic points ( $pI$ s), usually in the range of pH 3-12.<sup>2</sup> Proteins are positively charged in solutions at pH values below their  $pI$  and negatively charged above their isoelectric point. When a protein is forced in a medium with linear pH gradient and subjected to an electric field it will initially move toward the electrode with the opposite charge; when the protein will arrive at the point in the pH gradient equaling its  $pI$ , being uncharged it stops migrating. In this way, proteins condense, or focus, into sharp bands in the pH gradient at their individual characteristic  $pI$  values.

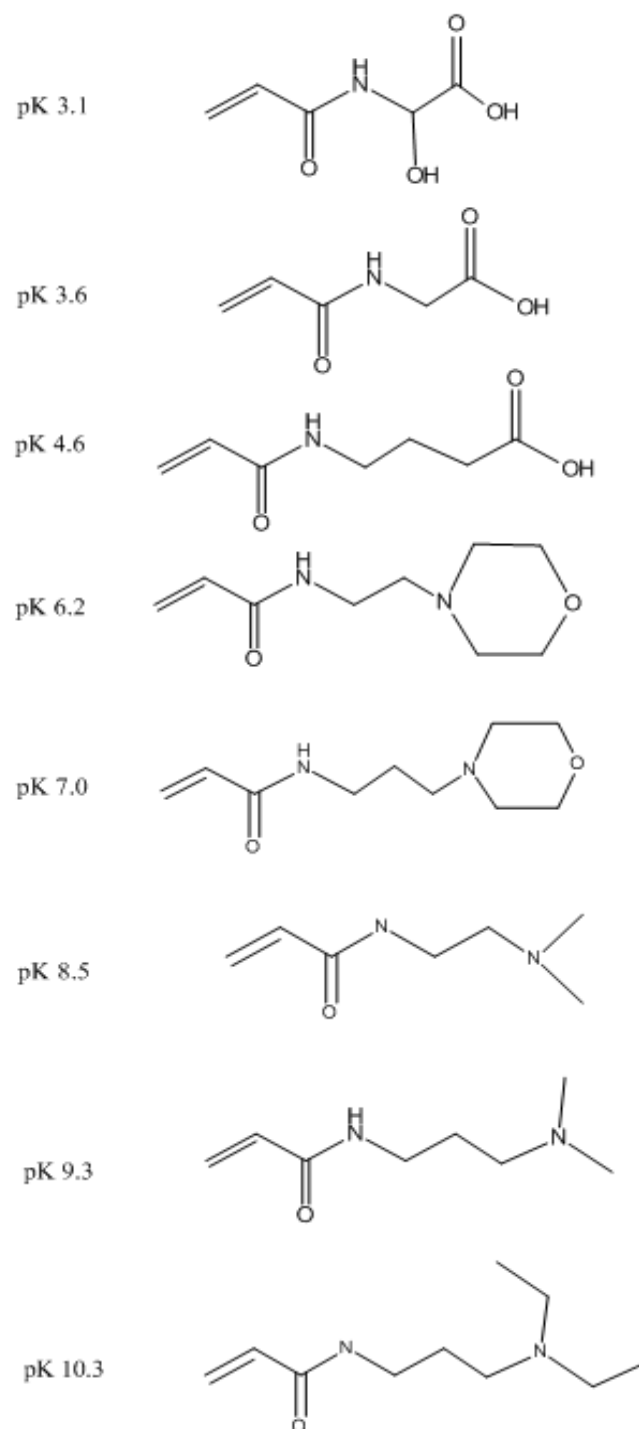
In isoelectric focusing, proteins can be loaded on polyacrylamide gel or acrylamide gel matrix copolymerized with the pH gradient, on the base of the technique chosen. In the first IEF approach the pH gradient is given by a mixture of ampholytes, typically isomers of aliphatic oligo-amino or oligo-carboxylic acids (Figure 1).



**Figure 1.** Structure of a polymeric ampholyte

In the second approach named Immobilized pH Gradient gel (IPG gel) the pH gradient is obtained by the continuous change in the ratio of immobilized pH monomers in the co-polymer. The IPG gel is made by co-polymerization of analogues of acrilamide  $\text{CH}_2=\text{CH-CO-NH}_2$  (Immobilines) that bear a carboxylic or an amino group [ $\text{CH}_2=\text{CH-CO-NH-R}$ , where R is a carboxylic or amino group, Figure 2].<sup>3</sup>

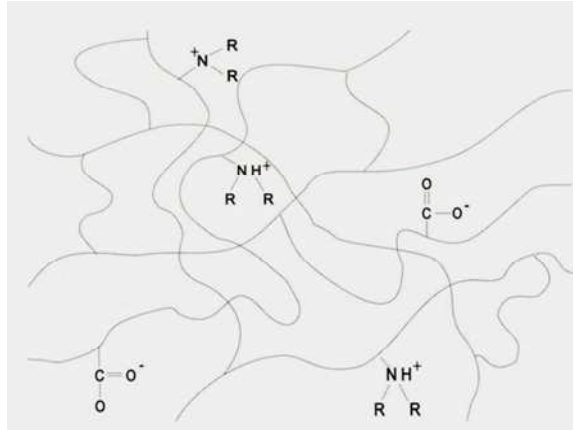




**Figure 2.** Structure of Immobilines

Figure 3 shows a schematic representation of an Immobilized pH Gradient gel (IPG gel).

### Schematic drawing of a IPG



**Figure 3.** Schematic representation of an Immobilized pH Gradient gel (IPG gel).

The method is applied particularly in the study of proteins, which separate based on their relative content of acidic and basic residues, whose value is represented by the pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated. In fact, isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01. In Figure 4, a gel electrophoresis run in a pH gradient (without SDS) and an electrophoretic chamber for monodimensional IEF are presented.



**a**

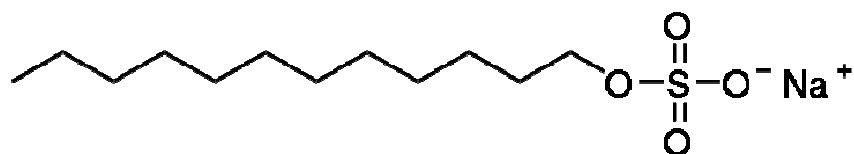
**b**

**Figure 4.** (a) Electrophoretic run in a pH gradient (without SDS); (b) an electrophoretic chamber for mono-dimensional IEF

Isoelectric focusing is the first step in two-dimensional gel electrophoresis (2D-gel electrophoresis) in which proteins are first separated by their pI and then further separated by molecular weight through SDS PAGE.

If the proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point (isoelectric focusing, IEF) in native polyacrylamide gel electrophoresis (native PAGE), they will remain in their native state and will be separated in the second dimension in the electric field following their mass and the mass of their complexes respectively.

In 2D-gel electrophoresis after completion of the first dimension IEF, the complexes are destroyed by applying the denaturing Sodium Dodecyl Sulphate (SDS, Figure 5), an anionic denaturing detergent that breaks hydrogen bonds and hydrophobic interactions, and destroys secondary and tertiary structures of proteins.



**Figure 5.** Chemical structure of SDS (sodium dodecyl sulfate).

This process is termed SDS-PAGE in which the coating of the protein in SDS confers a negative charge to the protein that can migrate in the second dimension. Here, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel proportionally to their mass-to-charge ratio, the proteins' progress being slowed by frictional forces. The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.

The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are Coomassie Brilliant Blue or silver staining. In the second case, a silver colloid is applied to the gel. The silver binds to cysteine groups within the protein and later darkened by exposure to ultra-violet light. The amount of silver can be related to the darkness, and therefore the amount of protein at a given location on the gel. This measurement can only give approximate amounts, but is adequate for most purposes.

Historically, the use of 2-DE is strictly linked to the proteomic analysis.<sup>4</sup> Proteomics, is the large-scale study of proteins and particularly of their structures and functions.<sup>5</sup>

## **Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry technique coupled with 2D gel electrophoresis**

The identification of the visualized proteins has been a significant challenge before the advent of the 2D gel electrophoresis. However, the potential of this technique for proteome analysis was somehow limited by the large amount of time (usually several days) needed to develop the 2D electrophoresis. Furthermore, proteins analyzed are in non-native conditions but are denatured by the SDS treatment. The 2-D electrophoresis remained an essentially descriptive technique, until two revolutionary MS methods for analysis of large biomolecules, electrospray ionization (ESI)<sup>6</sup> and matrix-assisted laser desorption/ionization (MALDI)<sup>7</sup> were introduced and have become the major ionization methods for the analysis of biological molecules.

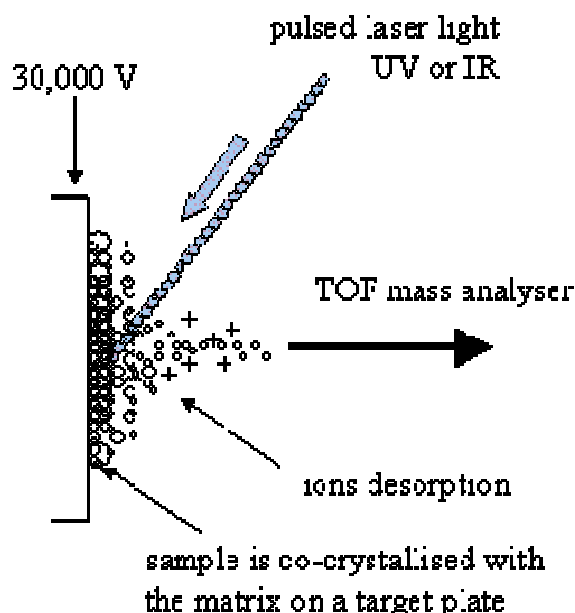
In 2002 the inventors of these techniques were awarded of the Nobel Prize in Chemistry.

Electrospray ionization (ESI) is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionized. ESI is the ion source of choice to couple liquid chromatography with mass spectrometry. The analysis can be performed online, by feeding the liquid eluting from the LC column directly to an electrospray, or offline, by collecting fractions to be later analyzed in a classical nanoelectrospray-mass spectrometry setup.<sup>8</sup>

From a practical point of view, the liquid containing the analyte(s) of interest is dispersed by electrospray into a fine aerosol. Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds such as methanol or acetonitrile). To decrease the initial droplet size, compounds that increase the conductivity (e.g. acetic acid) are customarily added to the solution. Large-flow electrosprays can benefit from additional nebulization by an inert gas such as nitrogen or carbon dioxide. The aerosol is sampled into the first vacuum stage of a mass spectrometer through a capillary, which can be heated to aid further solvent evaporation from the charged droplets. The solvent evaporates from a charged droplet until it becomes unstable, then deforms and emits charged jets in a process known as Coulomb fission.

The ions observed by mass spectrometry may be quasi-molecular ions created by the addition of a proton (a hydrogen ion)  $\{[M + H]^+\}$  or of another cation such as sodium ion  $\{[M + Na]^+\}$  or the removal of a proton  $\{[M - H]^-\}$ . Multiply charged ions such as  $[M + nH]^{n+}$  are often observed. For large macromolecules, there can be many charge states, resulting in a characteristic charge state envelope.

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique that allows the analysis of biopolymers such as DNA, proteins, peptides and polysaccharides and large organic molecules (such as polymers, dendrimers and other macromolecules, which tend to be fragile and fragmented when ionized by more conventional ionization methods). MALDI is a two step process. In the first, desorption is triggered by a UV laser beam and laser light is absorbed heavily by matrix material, with consequent ablation of upper layer of the matrix. A hot plume produced during the ablation contains many species: neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters and nanodroplets. The second step is ionization (protonation or deprotonation) of analyte by some of the ablated species. In Figure 6, a schematic representation of laser light application on target for MALDI TOF analysis is represented. The matrix consists of crystallized molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid),  $\alpha$ -cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid (DHB). A solution of one of these molecules is made, often in a mixture of highly purified water and an organic solvent (normally acetonitrile or ethanol). Trifluoroacetic acid may also be added. A good example of a matrix-solution would be 20 mg/mL sinapinic acid in acetonitrile: water: trifluoroacetic acid (50:50:0.1).



**Figure 6.** Schematic representation of laser light application on target for MALDI TOF analysis

Compound	Other Names	Solvent	Wavelength (nm)	Applications
<b>2,5-dihydroxy benzoic acid</b>	DHB, Gentisic acid	acetonitrile, water, methanol, acetone, chloroform	337, 355, 266	peptides, nucleotides, oligonucleotides, oligosaccharides
<b>3,5-dimethoxy-4-hydroxycinnamic acid</b>	sinapic acid; sinapinic acid; SA	acetonitrile, water, acetone, chloroform	337, 355, 266	peptides, proteins, lipids
<b>4-hydroxy-3-methoxycinnamic acid</b>	ferulic acid	acetonitrile, water, propanol	337, 355, 266	proteins
<b><math>\alpha</math>-cyano-4-hydroxycinnamic acid</b>	CHCA	acetonitrile, water, ethanol, acetone	337, 355	peptides, lipids, nucleotides
<b>Picolinic acid<sup>l</sup></b>	PA	Ethanol	266	oligonucleotides
<b>3-hydroxy picolinic acid</b>	HPA	Ethanol	337, 355	oligonucleotides

**Table 1.** Matrix solutions and their applications.

The rapid rise of MALDI, coupled with advances in MS instrumentation sensitivity and automation, have provided a foundation for high throughput approaches to study the complex and diverse properties of proteomes.<sup>9</sup> A more recent technique related to MALDI is the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS).<sup>10</sup>

The application of this MS technique to protein analysis is well documented.<sup>11,12</sup>

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## Chapter 1

### **The first PhD project: a micro-device for IEF analysis of proteins coupled with MALDI-TOF/TOF detection.**

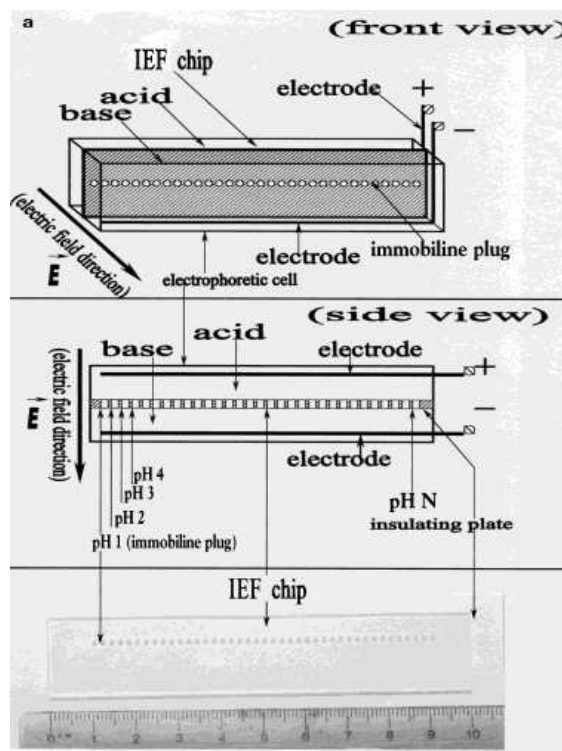
Assay miniaturization and integrated sample processing have proven to be useful in chemistry and life sciences providing much faster analyses, with sensitivity similar to conventional methods.<sup>1</sup> The advantages of microfluidic devices dedicated to protein analysis has been recently discussed in terms of the micro-device performance, taking into account two important features specifically related to miniaturisation, namely reduction of sample volume and time-to-result.<sup>2</sup>

For the realization of a lab-on-the-chip devoted to any biochemical analysis complex mixture of different species have to be separated. Fast separation and stationary spatial distribution of different fractions is highly desirable for a bio-analytical chip. IEF is the most convenient method to meet these objectives since it gives stationary distribution of different species.<sup>3</sup>

An interesting solution to accelerate IEF separation of proteins in a micro-device has been proposed by Zilberstein and al.<sup>4</sup>

These authors have demonstrated that the use of standard elements (immobilines, immobiline membranes, multicompartiment electrolyzer, *etc*) for IEF, arranged in parallel resulted in a significant acceleration of separation processes. This parallel isoelectric ruler-like device was then coupled to a fast 2-D (IEF and  $M_r$  separation) analysis of protein mixtures.

Zilberstein's technique relies upon a device constituted by a dielectric membrane with channels filled by immobiline gels of various pH. These channels are conducting since they are filled with polyacrylamide gel. The set of buffering monomers (immobilines) is covalently linked to the gel matrix by copolymerization. The different combinations of immobilines create different values of pH. The applied electric field is perpendicular to the chip plane, *i.e.* the pH value changes perpendicularly to the electric field direction. The system is defined as a parallel isoelectric device since all chambers operate simultaneously in parallel and the technique is named parallel isoelectric focusing (PID).



**Figure 1.** The parallel isoelectric ruler-like device (PID) according to Zilberstein et al <sup>4</sup>

## 1.1 Separation of marker proteins by Zilberstein's PID

The Zilberstein's separation chip (PID) had a ruler-like form with 41 holes that overlay two pH units. The chip was made from polymethylmethacrylate and had a thickness of 0.2 cm. Holes of 1 mm diameter were filled with immobiline gels with discrete pH values. Immobiline gels were prepared from immobiline acrylamide buffer (Amersham Biosciences) with pK 3.6, pK 4.6, pK 6.2, pK 7.0, pK 8.5, or pK 9.3.<sup>5</sup>

The gel solution was polymerized at room temperature for between 15 and 30 min after mixing. After polymerization, chips were washed thoroughly twice over a 30 min period with weak agitation. Chips were assembled with electrodes by using rubber gaskets as spacers. The electrophoretic cell was shaken for better mixing of electrolytes and for more intensive solution convection. Clamps were used to keep the electrodes together. The distance between electrodes was 0.5 cm.

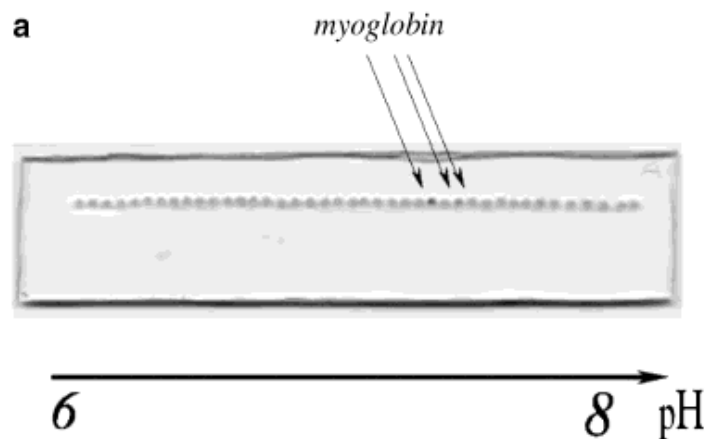
The electrolytes Tris (1 mM base) as the catholyte and HEPES (1 mM acid) as the anolyte were used for the pH 6.0–8.0 interval. HEPES (1 mM base) as catholyte and glutamic acid (1 mM acid) as anolyte were used for the pH 4.0–6.0 interval. The volume of running buffer used was 750 mL. The focusing was performed with increasing voltage. The starting electrical conditions were 50 V for 2 min followed by 100 V for 2 min, 200 V for 2 min, 300 V for 24 min for the pH 4–6 chip. For

the pH 6–8 chip for 2 min, 300 V for 3 min, 700 V for 2 min, 1000 V for 3 min, and 1200 V for 20 min was used.

The proteins were dissolved in ultra pure water to a final concentration of 1  $\mu\text{g}/\text{L}$ . Protein mixture (10–50  $\mu\text{g}$  corresponding to 10–50  $\mu\text{g}$  protein) was put into electrolyte and mixed.

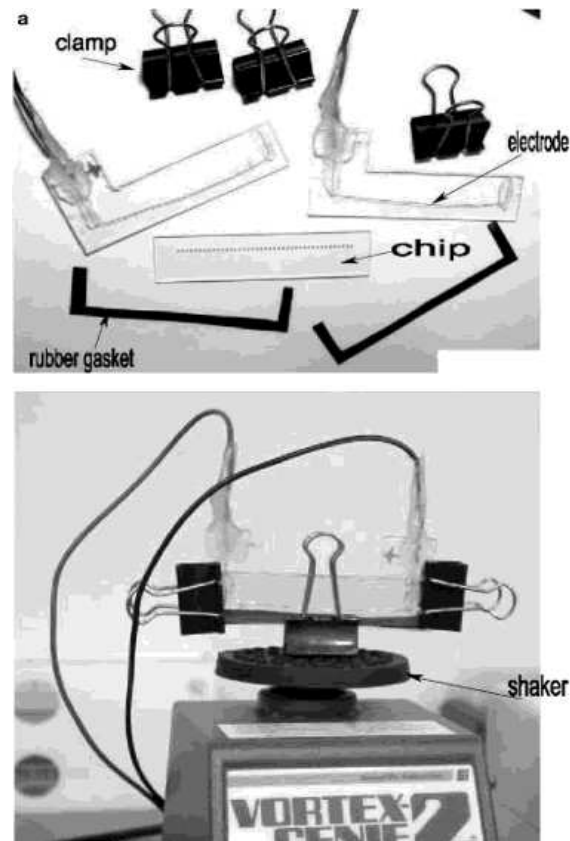
IEF standards and a mixture of nine natural proteins with  $pI$ s ranging from 4.45 to 9.6 were used to demonstrate separation of the protein mixture. For the acid pH range the proteins phycocyanin C (3 bands;  $pI$  4.45, 4.65, and 4.75),  $\beta$ -lactoglobulin B ( $pI$  5.1), and bovine carbonic anhydrase ( $pI$  6.0) were used. For the pH 6.0–8.0 interval the proteins human carbonic anhydrase ( $pI$  6.5), equine myoglobin (2 bands;  $pI$  6.8 and 7.0), human hemoglobin A ( $pI$  7.1), human hemoglobin C ( $pI$  7.5), and lentil lectin (3 bands;  $pI$  7.80, 8.00, and 8.20) were used. Bio-Rad IEF standards (1.3  $\mu\text{L}$ ) were analyzed by IEF.

After 30 min, three red-brown spots (natural color of myoglobin from horse heart) were observed on the chip (Fig. ). After IEF, three myoglobin fractions were observed after  $M_r$  separation and Coomassie staining ( $pI$  7.35, 7.45, and 7.50) of the slab gel.



**Figure 2.** Myoglobin focalization on PID.

Finally, the real ruler-like PID for parallel IEF analysis is shown in the Figure 3.



**Figure 3.** Real ruler-like PID for parallel IEF

One of the major limitation of the device proposed by Zilberstein is the analysis of the proteins separated by the PID, that consists in a classical 2D gel electrophoresis coupled with MALDI analysis.

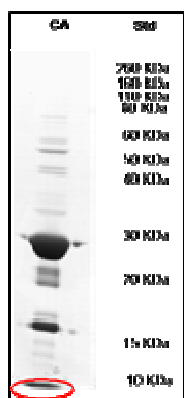
Within this research framework, for the first project of this PhD thesis we have evaluated the possibility to build an isoelectric focusing (IEF) micro-device utilizing a polymeric material compatible with MALDI-TOF/TOF analysis. For this reason, starting from the same concept of parallel isoelectric focusing, we have evaluated a micro-device for parallel isoelectric focusing (MPID) that should be able to perform the electrophoretic separation directly on a conductive substrate compatible with MALDI analysis.

The final target of this part of the PhD project was to create a pH gradient on a conductive surface by spotting little drops containing few  $\mu\text{l}$  of polymeric solution with precise and discrete pH values, via robotic spotter deposition, with the aim to achieve a fast IEF separation of proteins. The polymeric layer should present good properties of adhesion onto the conductive surface in order to allow a direct analysis of proteins accumulated at different pHs by MALDI TOF/TOF that include high vacuum operational conditions.

## 1.2 Selection of the model protein mixture for MPID analysis and 2DE/MALDI-TOF/TOF determination of its composition.

For our analytical purposes, we have selected carbonic anhydrase (CA) from bovine erythrocyte as a model protein. CA has been chosen on the base of its molecular weight that is in the range of the medium size proteins (30 kDa) and the commercial availability from Sigma-Aldrich (USA). Bovine CA has been also analyzed by Zilberstein's PID<sup>4</sup> as a protein with slightly acidic isoionic point (pI 6) and three spots of CA were observed (pI 6.00, 6.05, and 6.10).

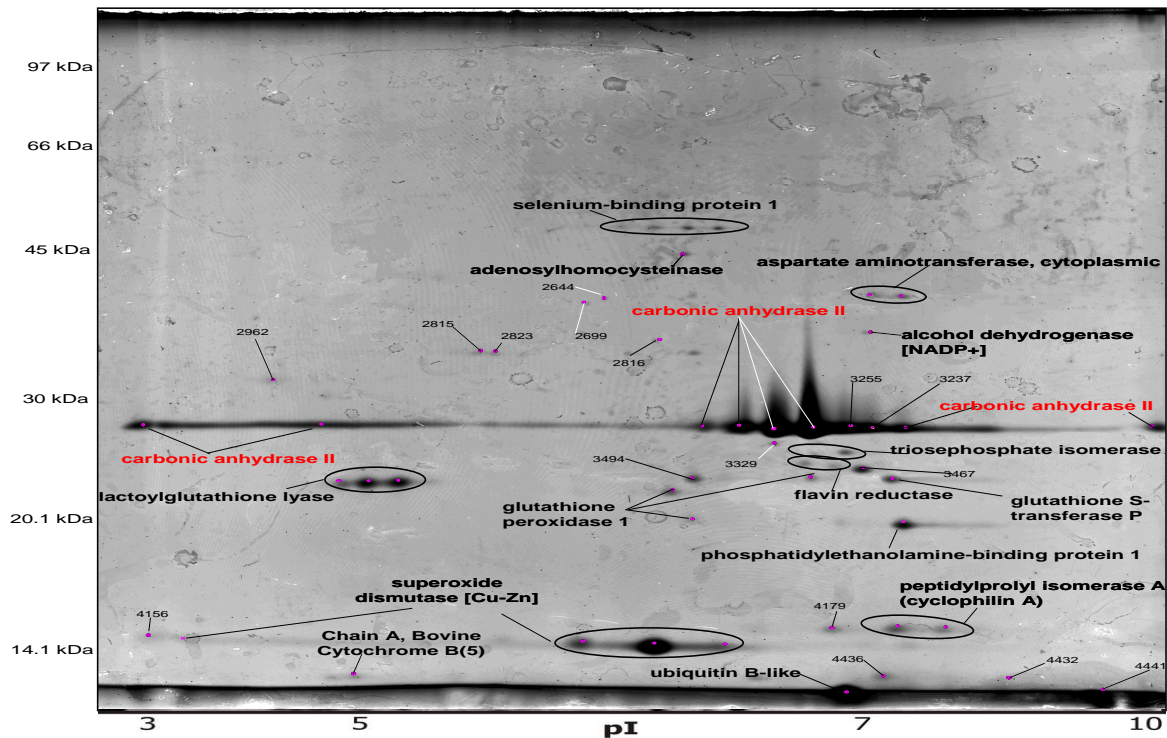
CA has been analyzed at first by a classical SDS-PAGE for the evaluation of the average molecular weight of the isoforms present in the commercial sample. From the SDS-PAGE electrophoretic analysis reported in Figure 4 the average MW of CA was confirmed for the predominant protein (30 kDa), but a few additional spots were observed.



**Figure 4.** SDS-PAGE of CA from Sigma Aldrich.

A preparative 2D electrophoresis of CA, followed by gel picking, protein digestion and MALDI or ESI MS/MS allowed the identification of the mixture components. The 2D gel electrophoresis has been conducted in collaboration with the Laboratory of Proteomics and mass spectrometry of the Department of Biomedical Sciences for Health, Università degli Studi di Milano.

Main proteins and contaminants present in the commercial sample of CA analysed are indicated in Figure 5. Details of the 2D gel analysis are reported in Materials and Methods.



**Figure 5.** Carbonic anhydrase 2D gel electrophoresis

The preparative bi-dimensional gel electrophoresis of CA results in a complex set of different protein focalized in different zones of the gel surface, thus showing that the commercial preparation was a mixture of proteins. The major part of the sample is composed of several isoforms of CA that focalize within pH 6.5 and 7.5 and Superoxide Dismutase (SOD) which focalizes at pH 3 and 6. Minor amounts of other proteins like selenium binding protein, ubiquitin B like, peptidylpropyl isomerise A and other, appear in 2D-cromatogram.

In any event, taking advantage of the known impurities present in the commercial sample of CA, we decided to adopt this a mix of known proteins as our model protein mixture for the IEF separation of our MPID.

## Material and Methods

Carbonic anhydrase (CA) lyophilized powder,  $\geq 2,500$  units/mg protein from bovine erythrocyte was purchased from Sigma-Aldrich (USA).

### SDS-PAGE of CA.

The analysis has been conducted by the pre-cast NuPAGE (Invitrogen) system, 12% of polyacrylamide with NuPAGE MOPS SDS Running Buffer (MOPS 50 mM, Tris Base 50 mM, SDS 0,1% e EDTA 1 mM)

The protein has been negatively charged by lithium dodecyl sulfate (LDS) and dithiothreitol (DTT) has been used as reducing agent.

### Preparative 2D gel electrophoresis and relative MALDI-TOF analysis.

The 2D gel electrophoresis and MALDI-TOF analysis has been carried out in collaboration with the Laboratory of Proteomics and mass spectrometry of the Department of Biomedical Sciences for Health, Università degli studi di Milano.

The experimental procedure was as described by Fania *et al.*<sup>6</sup> with the following modifications:

- CA was dissolved in lysis buffer and diluted in a rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 65mM DTT, 0.5% IPG buffer pH 3.5–9.5 and BBF in traces), then added to a final volume of 350  $\mu$ L each strip.
- Samples were separated on 3 strips 18 cm for the I dimension, pH 3–10 non-linear (NL) gradient IPG strips (GE Healthcare), to separate 100  $\mu$ g of sample each one. IEF has been carried out at 60000 VhT (total volts hour)
- The II dimension was carried out on 12% SDS-PAGE after reduction and alkylation of the strips.
- Application of Deep Purple coloring agent according to the GE Healthcare protocol. Visualization of the spots with Typhoon 9200 (GE Healthcare).
- Spots of interest were excised from gel using the Ettan spot picker robotic system (GE Healthcare), de-stained in 50% methanol/50mM ammonium bicarbonate and incubated with

30 mL of 4 ng/mL trypsin (Promega) dissolved in 10mM ammonium bicarbonate for 16 h at 37° C.

- Released peptides were subjected to reverse phase chromatography (Zip-Tip C18 micro, Millipore), eluted with 50% acetonitrile/1% formic acid. An aliquot of 0.35 mL of peptides mixture was spotted onto the sample plate of a MALDI MS 1000 (MALDI ultraflex III Bruker Daltonics) (GE Healthcare) mass spectrometer.
- An equal volume of 10 mg/mL  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA) matrix dissolved in 70% acetonitrile/30% 50mM citric acid, was applied and spots were air dried at room temperature. MS proceeded at an accelerating voltage of 20 kV and spectra were externally calibrated using Peptide Mix 4 calibration mixture (Laserbio Labs); 256 laser shots were taken per spectrum.
- Proteins were identified by MASCOT, which utilizes a probabilistic scoring algorithm with *other mammalia* entries in the NCBIInr database.



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## Chapter 2

### The design of the micro-device: the electrophoretic chamber

The project of the first part of this Ph.D thesis is aimed at the fabrication of a miniaturized solid-phase parallel isoelectric focusing device (MPID) on the base of the theoretical and practical considerations previously explained in Chapter 1 and here detailed in specific.

In the design of the electrophoretic chamber, spots or plugs of conductive polymers isoelectric at different pHs should be patterned on a conductive surface compatible with MALDI TOF/TOF analysis. Commercially available, ionogenic monomers known as acrylamido buffers (Immobiline) mixed with monomers such as acrylamide and N,N'-methylene-bis-acrylamide, able to form an hydrogel after polymerization, are currently used to generate an immobilized pH gradient in isoelectric focusing.<sup>1</sup> The matrix is formed by properly mixing certain amounts of couples of acrylamido buffer (Immobiline) solutions, chosen on the base of the pH needed. The ratio of buffering and titrating monomer is calculated by means of a customized software, commercially available and termed Buffer Workshop di MB Design and Solution Mountain View CA.

This program takes into account Henderson-Hasselbalch equation that links the pH with the pK of the ionogenic monomers as well as more complex formula that allow the prediction of buffering power and ionic strength generated upon mixing several buffering species titrated at a given pH.

In other words by simply adding the pK values of the Immobiline chosen and the pH needed the software gives back detailed information regarding concentration of Immobiline to be used, ionic strength and buffer power of the solution so obtained.

In Table 1, an example of couples of Immobilines mixed to obtain different pH plugs are reported.

pH	pK 3.6 (mM)	pK4.6 (mM)	pK 6.2 (mM)	pK 7.00 (mM)	pK 8.5 (mM)
4		24.40			5.40
4.5		22.20			10.89
4.8		23.16	15.90		
5		21.00	16.75		
5.5		24.05	25.28		
6	13.17		19.80		
6.3	10.48		21.30		
6.4	9.80		22.60		
6.6	8.60		26.65		
7	9.72			17.75	
7.2	9.80			22.65	
7.4	8.59			26.63	

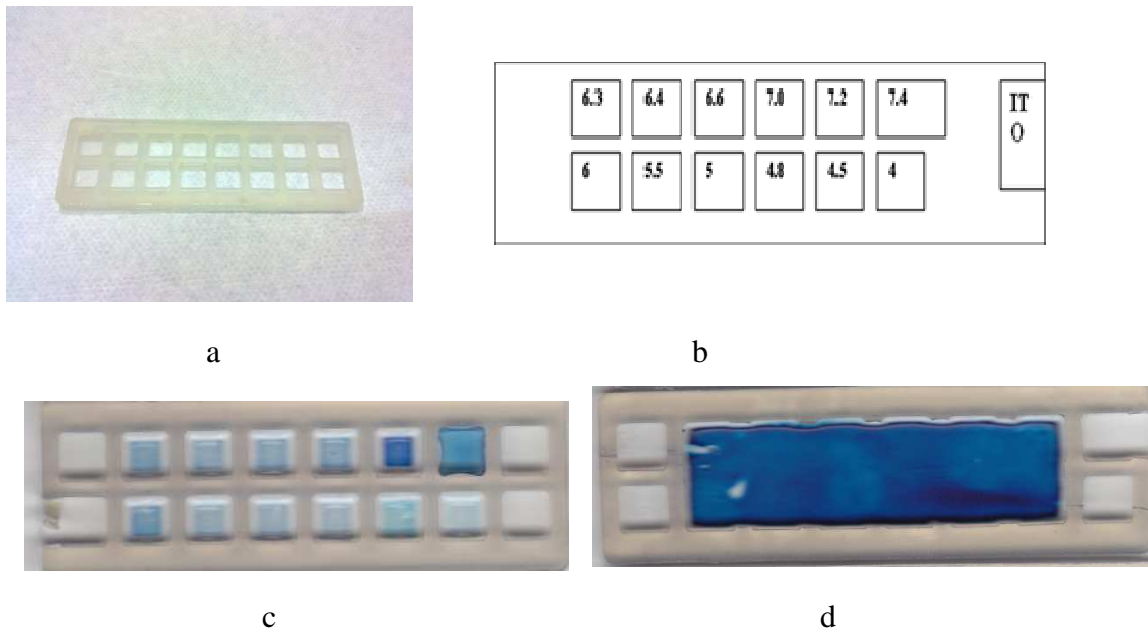
**Table 1:** Combination of different pK values of buffering Immobiline from commercial Sigma 200mM stock solutions.

The Immobiline copolymer so obtained should be bound to a conductive surface (here-after named substrates) previously derivatized with a chemically versatile coating capable of inducing the copolymerization with the acrylamido monomers responsible of the immobilized pH.

Among commercially available conductive surfaces, glass slides coated with an Indium Tin Oxide layer (ITO) have been selected, due to their commercial availability, price and chemical versatility.

The preparation of the micro device starts working on two ITO slides that should be activated for the next step, i.e. coating by the polyacrilamide gel.

The electrophoretic chamber is then built up superimposing the two polymer coated ITO slides, one with hydrogel spots of different pH (up to 12 spots 6mm x 6mm each) (Figure 1a) and one coated by one hydrogel layer at pH 12 (Figure 1b) and separated by two frames, 3mm thick each one.



**Figure 1.** (a) Silicon frame utilized for the part of the chamber containing different polymeric spots at different pH; (b) pH at different plugs (c) and (d) upper vision of the silicon frames stuck on the ITO glass slides after the polymerization. Plugs are colored in blue to put in evidence the presence of the hydrogels.

The micro-device, filled with a buffer containing the model protein CA, will be connected to a power supply. The idea behind the separation is to electrophoretically attract negatively charged proteins on the surface where the plugs at different pHs are located. During the capturing phase, a potential of 2V is applied for a period of 40 seconds, that was established according to some theoretical considerations based on a simulation software.

The polarity between the ITO plates should be switched at fixed time intervals to first promote the migration of the proteins from the solution into the conductive polymer plugs and remove from the plugs the proteins that are not isoelectric to the plug itself. The ultimate goal is to accumulate in each plug only the proteins that are isoelectric at the given pH of the conductive gel. In a second step, an electrophoretic wash is introduced by changing the polarity of the electrodes. In this way, proteins that are captured on plugs of pH higher than their pI could be removed and only isoelectric or near isoelectric proteins could be trapped in their respective plugs.

## 2.1 ITO surfaces: chemical activation and preparation of the substrate for the hydrogel polymerization.

Each slide consists of glass, of the dimension of a typical microscope glass slide, coated with a thin micro layer of indium tin oxide (ITO, or tin-doped indium oxide) that is a solid solution of indium(III) oxide ( $\text{In}_2\text{O}_3$ ) and tin(IV) oxide ( $\text{SnO}_2$ ), typically 90%  $\text{In}_2\text{O}_3$ , 10%  $\text{SnO}_2$  by weight, commercially available from Bruker Daltonics.

In Figure 2 a picture of an ITO glass slide is reported.



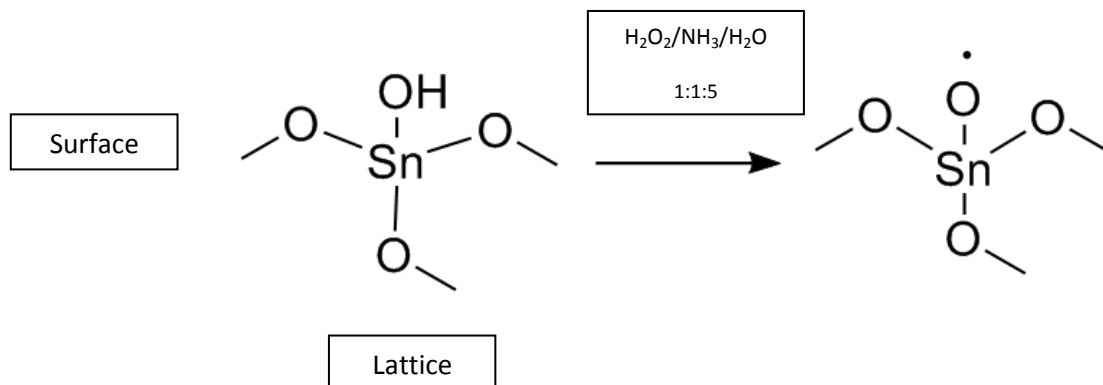
**Figure 2.** ITO glass slide

Indium tin oxide is one of the most widely used transparent conducting oxides because of its two main properties, its electrical conductivity and optical transparency, as well as the ease with which it can be deposited as a thin film.<sup>2</sup>

As with all transparent conducting films, a compromise must be made between conductivity and transparency, since increasing the thickness and increasing the concentration of charge carriers will increase the material's conductivity, but decrease its transparency. Thin films of indium tin oxide are most commonly deposited on surfaces by electron beam evaporation, physical vapor deposition, or a range of sputter deposition techniques. ITO is often used to make transparent conductive coatings for displays such as liquid crystal displays, flat panel displays, plasma displays, touch panels, and electronic ink applications. Thin films of ITO are also used in organic light-emitting diodes, solar cells, antistatic coatings and EMI shieldings. In organic light-emitting diodes, ITO is used as the anode (hole injection layer).

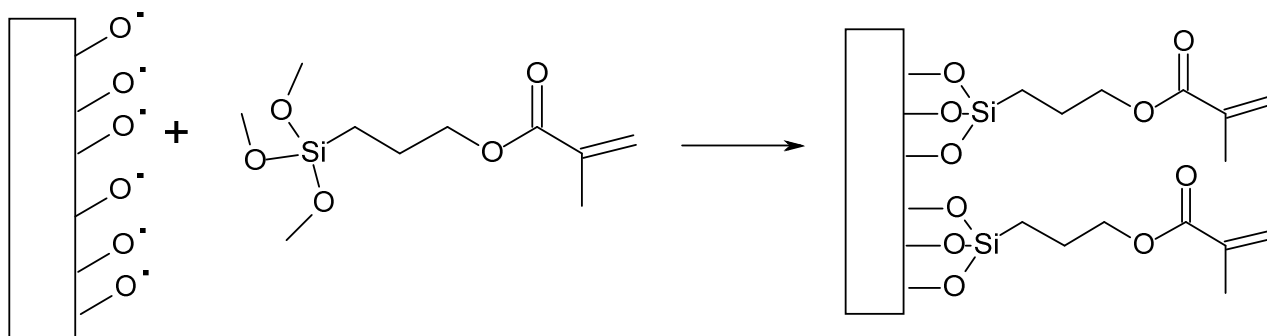
To work on ITO glass slides as support for polymerizations it necessary to activate the surface via proper oxidizing treatment<sup>3</sup> within a choice of plasma oxygen, ozone under UV irradiation, hydrogen peroxide, aqua regia, or nitric acid.

We decided to work with an oxidizing mixture of  $\text{H}_2\text{O}_2/\text{NH}_3/\text{H}_2\text{O}$  in the ratio 1:1:5 immersing the slides in this solution for 1 h at 70 °C. The activation of the surface gives rise to a set of oxygen radicals, as shown in Figure 3.



**Figure 3.** Schematic representation of the oxidizing treatment of the tin oxide layer present on the surface of the glass slides.

The so formed radical species are now available for the reaction with the silanizing agent constituted by methacryloilsilyl trimethoxysilane. By this process, a set of acrylic moieties are anchored to the surface and available for the successive copolymerization with the mix of acrilamido buffers, acrylic monomers and N,N'-methylene-bis-acrylamide (Figure 4).



**Figure 4.** Schematic representation of the reaction between the activated ITO surface and the organosilane methacryloilsilyl trimethoxysilane.

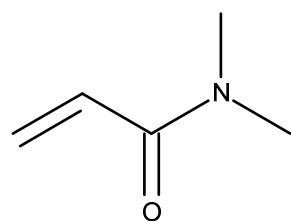
## 2.2 The polyacrilamide gel matrix: chemical composition of the hydrogel.

After activation and organosilanization process, the ITO surface is ready for the polymerization of the hydrogel with specific pH to be anchored on it. A typical hydrogel used in electrophoresis is a polyacrylamide<sup>4</sup> gel formed by the polymerization of the monomer acrylamide crosslinked by N,N'-methylene-bis-acrylamide (abbreviated BIS).

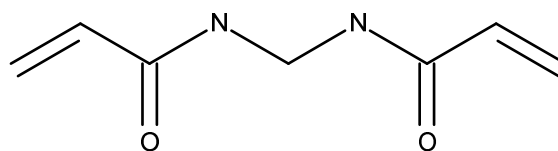
Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves or when mixed together. Another way to start the polymerization is by mixing with the monomer solution photoactive compounds able to generate radicals after UV light exposure. An example of photoinitiator molecule is the commercially available Irgacure moieties, provided by BASF.

The distinct advantage of acrylamide gel systems is that the initial concentrations of acrylamide and BIS control the hardness and degree of crosslinking of the gel. The hardness of a gel in turn controls the friction that macromolecules experience as they move through the gel in an electric field, and therefore affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix. The choice of the hardness of the gel depends on the type of sample to analyze. The chemical structure of the monomers responsible of the hardness of the gel are shown in Figure 5.

To obtain hydrogel with a specific pH it is necessary to add to the reaction mix a set of couples of reactive acrylamido buffer monomers, able to copolymerize with the acrylamide and N,N'-methylene bis-acrylamide monomers.



Dimethylacrilamide

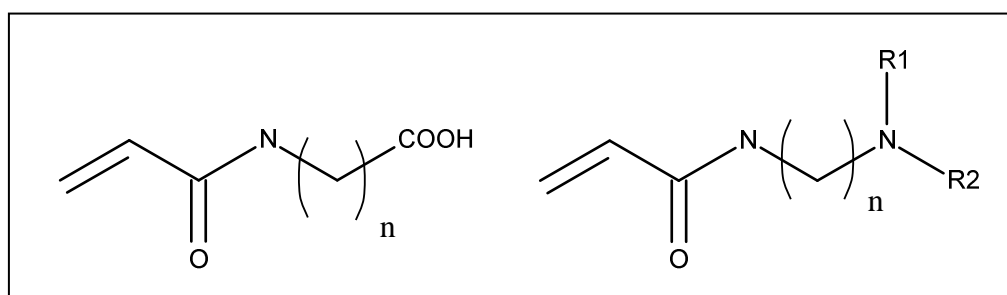


N,N-Methylene bisacrylamide

**Figure 5.** Monomers participating at the copolymerization of the hydrogel

Crosslinked poly-dimethylacrylamide (pDMA) containing the opportune amount of Immobiline buffer solutions is covalently grafted to the activated ITO surface after a UV-assisted polymerization initiated by the photoreactive compound Irgacure 2959 as explained in Material and Methods.

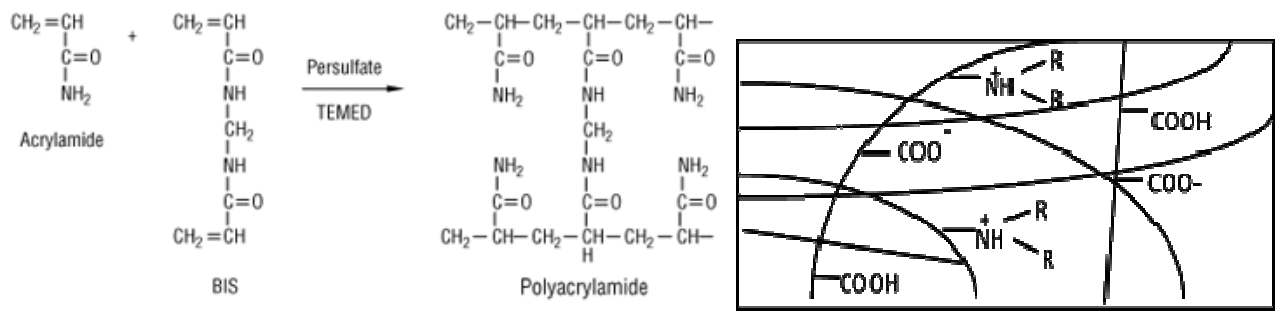
The chemical structure of the components of an Immobiline acrylamido buffer is reported in Figure 6.



**Figure 6.** General chemical structure of components of an Immobiline acrylamido buffers

A scheme of the polymerization process and a schematic representation of the hydrogel matrix with immobilized Immobiline buffering moieties is reported in Figure 7





**a**

**b**

**Figure 7.** (a) Polymerization process and (b) a schematic representation of the hydrogel matrix resulting from the copolymerization of Immobilines.

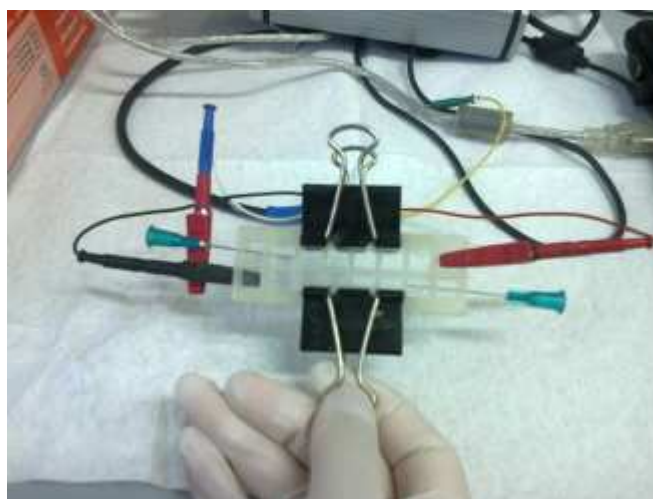
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# Chapter 3

## Results and Discussion

### 3.1 The micro-fabrication process

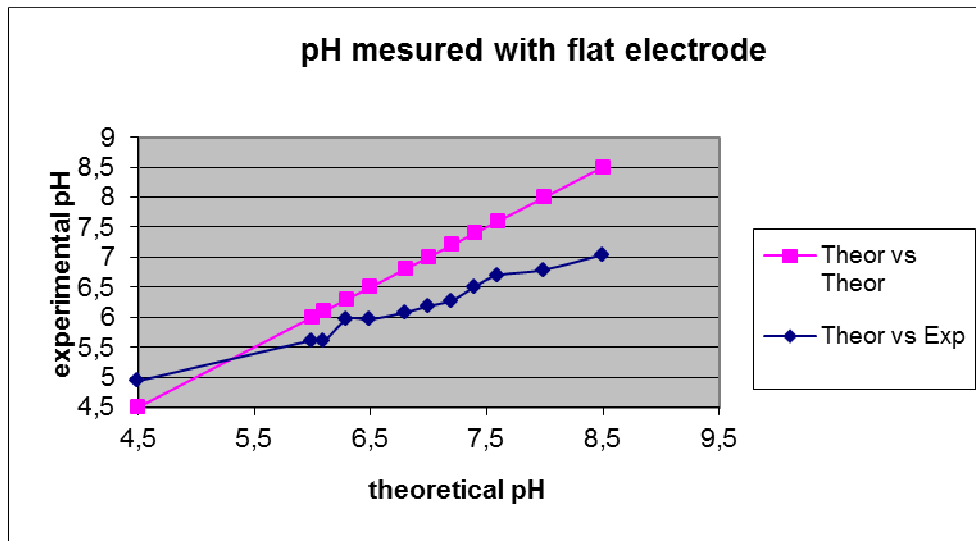


The micro-fabrication process of our MPID should face several practical problems related to the formation of a pH gradient on the conductive ITO-glass surface. The pH gradient was supposed to be realized by a robotic spotter deposition of drops containing few  $\mu\text{L}$  of polymeric solution with precise and discrete pH values. The polymeric material used to build up the micro-IEF chamber should add here onto ITO-glass substrate and be compatible with the high-vacuum conditions of MALDI TOF/TOF direct analysis of proteins separated and accumulated at different.

Each compartment of the silicon frame stuck on the ITO slide is filled with a volume of  $60\mu\text{L}$  of monomer solution containing dimethylacrylamide, acrylamido buffers in differ ratios, depending on the desired pH, NN, methylenebisacrylamide (bisA). The total monomer concentration expresses as w/v ranges from 4% to 8% depending on the hardness of the gel needed. The concentration of bisA is 3% of the monomer moles. To the monomers mixture the photoinitiator Irgacure 2959 (2mM) was added. After 15 minutes of exposure to UV light the slides are washed three times by direct immersion in water for 20 minutes under agitation.

Initially, it was controlled that the practical pH of the plugs corresponded to the expected value according to the Buffer Workshop program.

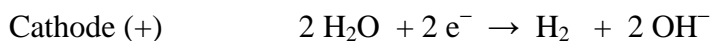
The effective correspondence between the theoretical pH and the experimental one was checked using a pHmeter equipped with a flat electrode that was in contact with the surface of the plug. In this way, the pH of the plug surface, after the polymerization and before the electrophoretic run was measured and results are collected in Figure 1.



**Figure 1.** Theoretical pH of the plugs plotted against experimentally measured value

### 3.2 The electrophoretic run in MPID

The observed variation of the pH value of the gel plugs with respect to the theoretically expected could be explained by electrolytic effects that may occur in aqueous solution, since there is a great productions of ions  $H^+$  and  $OH^-$  respectively on the anode and on the cathode.



On the basis of the above electrolytic effect, a few modifications of the original MPID were considered.

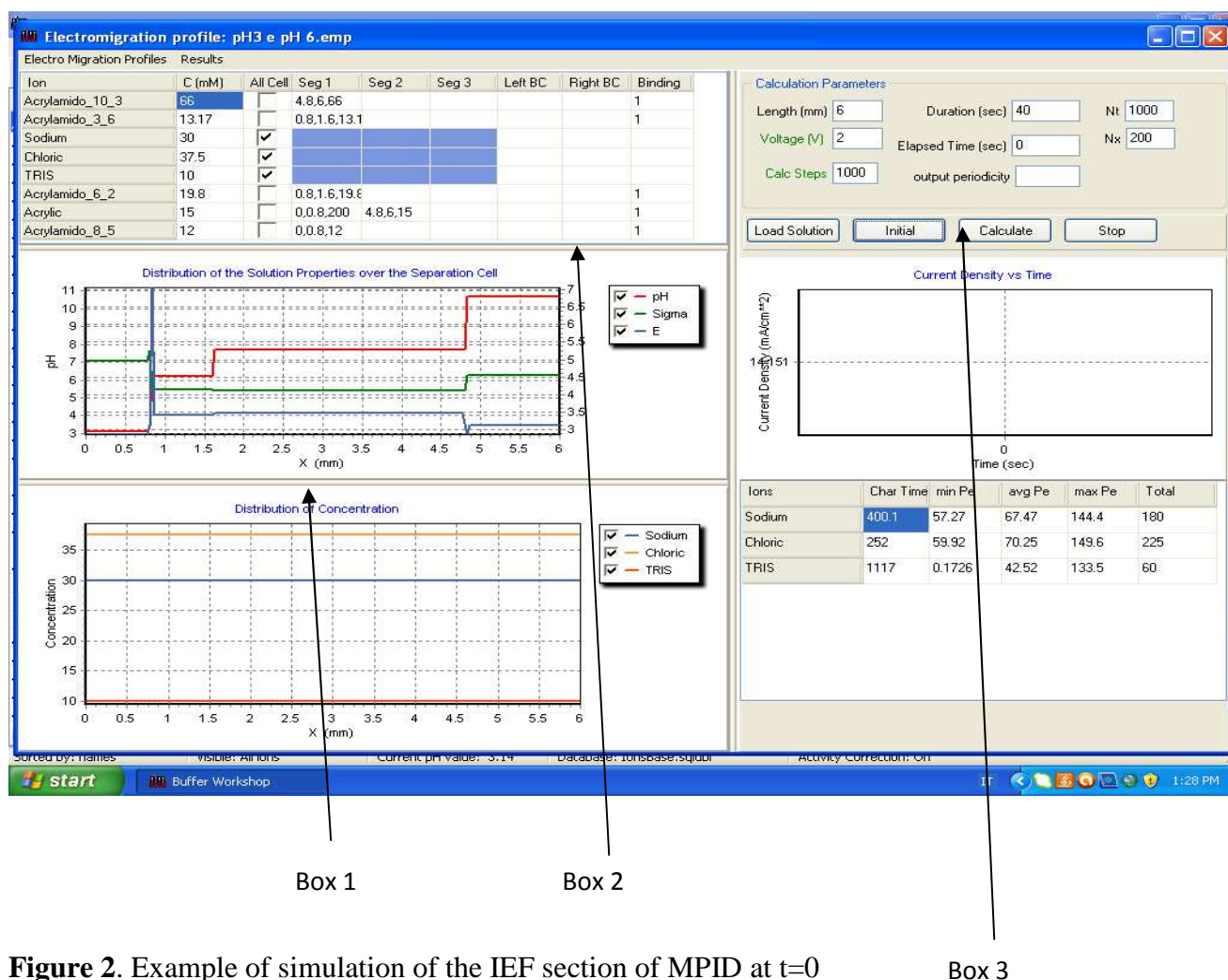
In order to prevent acidification and basification of the hydrogels grafted to the electrode surface, it is necessary to project the gel plug with the lower part, in direct contact with the electrode, with a buffering capacity higher than the upper one. In this way, the IEF section of MPID should be protected against acidification effect of the anode and kept at a constant and controlled pH, necessary condition for the best focalization of the protein on its surface.

The pH variations on the surface of the electrodes during the electrophoretic run could be also related to the composition of the buffer solution or the amount of added background electrolytes such as NaCl.

In order to evaluate the effect of all above parameters on the electric field strength distribution, conductivity and pH in the electrophoretic chamber, a customized software has been created *ad hoc* by Dr. Michael Bello at MB Design & Solutions, Mountain View, CA, USA and developed into a product available at [www.BufferWorkshop.com](http://www.BufferWorkshop.com).

The electrophoretic chamber of MPID presented the following characteristics: the gel plug on the anode is 1.6 mm thick divided in two zones. From the electrode up to 0.8 mm the gel's pH is 3 with a buffer power of 30, from 0.8 mm up to 1.6mm the pH is 6 with a buffer power of 12. The cathode is composed by a monolayer 1.2 mm thick ( from 4.8 mm up to 6 mm) of hydrogel with buffer power 30 and pH 12. It is not divided in two parts because the PID is projected to collect the proteins only on the anode, by dissolving the model protein CA in a solution of Incubation Buffer 10 mM with a pH of 7.6 (Material and Methods). This pH is higher than the isoelectric point of the protein, so the protein dissolved and negatively charged should migrate only to the anode. The function of the gel on the cathode is only to protect the solution from basification. During the simulation it is possible to see the online change and migration of all the components added in Box

1 for the time set up in Box 3. The simulation of the initial situation before the electrophoretic run is represented in Figure 2 (t=0)

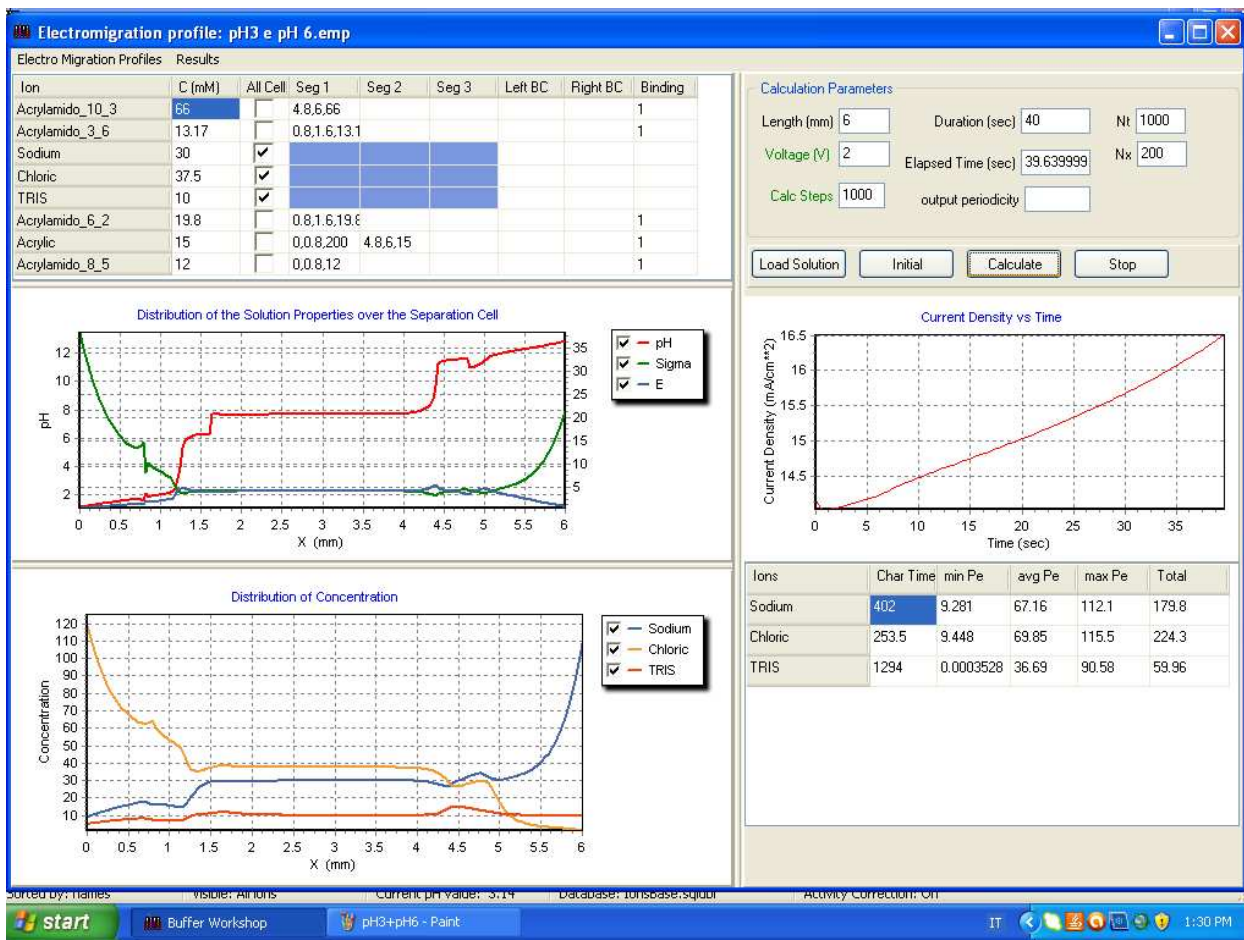


**Figure 2.** Example of simulation of the IEF section of MPID at t=0

Box 3

In Box 1 of Figure 2 a schematic representation of one of the 12 gel plugs with a distance between the electrodes of 6 mm is shown. The x axis reports the thickness of the device (distance between the two electrodes) and the y axis reports the pH of the plug. The left part represent the anode and the right one the cathode. In Box 2 the concentrations of all the reagents and their position (in mm) inside the cell are reported. In Box 3 is reported the set up screen for the calculation parameters such as voltage, duration of the run, distance between the electrodes (length).

In Figure 3 the change and migration of the components set up as in Figure 2 are shown for a time t=40 sec, that is the time programmed for an IEF run of MPID at 2 V.



**Figure 3.** Example of simulation at t=40 sec

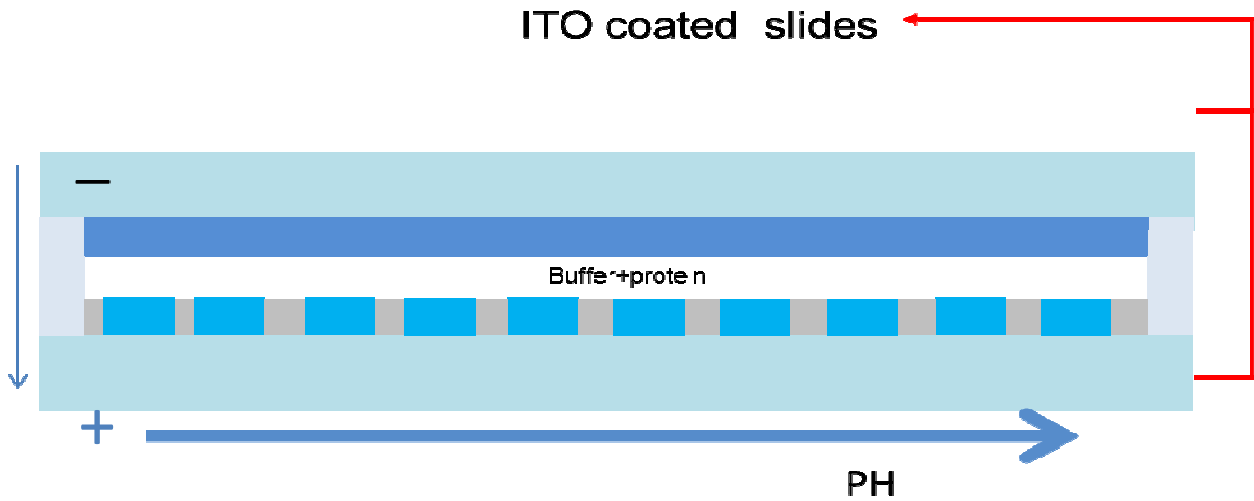
As it is clear from the picture, after 40 seconds the part of the gel in direct contact with the electrode is subjected to pH change, whereas the upper part, that is distant 1.3 up to 1.6 mm from the anode is still at pH 6 as it was at t=0.

This simulation furnished a first indication on the conditions to be selected for analysis lasting 40 seconds at 2V. According to the above results of the simulation it has been possible to develop the PID with the following conditions.

**Anode:** Double layer of hydrogel with pH 3 and buffer power 30 in the lower part of the gel with a thickness of 0.8 mm and upper part of the gel with a thickness of 0.8 mm with a buffer power of 12 and discrete pH on the base of the needs. The total thickness of the plug is 1.6 mm in a surface of 6\*6 mm, corresponding to a total volume of 57.6  $\mu$ l for each plug.

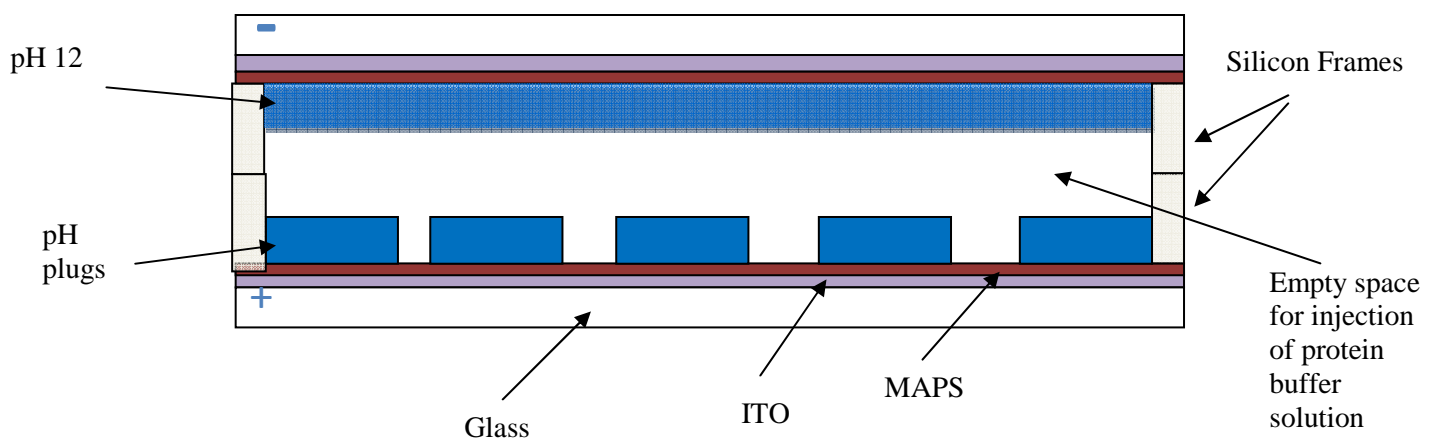
**Cathode:** Monolayer at pH 12 with Buffer Power 30 with a thickness of 1.2 mm in an area of 65\*20 mm corresponding to a volume of 1560  $\mu$ l

A scheme of the electrophoretic chamber of MPID is presented in the Figure 4.



**Figure 4.** Scheme of the electrophoretic chamber of MPID.

A schematic representation of the lateral section of the iso-electric focusing part of MPID is shown in Figure 5.



**Figure 5.** Lateral section of a schematic representation of MPID.



### 3.3 MPID electrophoretic run coupled with MALDI TOF/TOF analysis

In a typical electrophoretic run with MPID, the model protein CA has been dissolved in concentration 0.28 mg/ml in Incubation buffer ( Tris/HCl 10 mM pH 7.6, NaCl 30mM, Tween 20 0,02% v/v, BSA 1% w/v)

As earlier stated, one of the major aim of this Ph.D. project was to couple the electrophoretic run directly to MALDI TOF/TOF analysis. On a classical MALDI analysis of a protein, the matrix solution is mixed with the protein sample. A mixture of water and organic solvent allows both hydrophobic and hydrophilic molecules to dissolve into the solution. This solution is spotted onto a MALDI plate , usually a metal plate designed for this purpose Figure 6.



**Figure 6.** Sample target for a MALDI mass spectrometer.

The solvents vaporize, leaving only the recrystallized matrix, but now with analyte molecules embedded into MALDI crystals (co-crystallization). The analysis is carried in deep vacuum ( $10^{-9}$  bar) and, therefore, for our direct analysis of the plugs that absorb proteins at pI, an important parameter to be evaluated is the resistance of the hydrogel during the high vacuum dehydration process.

In this respect several composition of the hydrogel mix comprising various type of neutral monomers in different percentages have been tested, in particular N-hydroxyethylacrylamide a monomer that goes under the trade name of Duramide (DUR) and dimethylacrylamide (DMA) both cross-linked with NN, methylenebisacrylamide (BisA) in different percentages were evaluated.

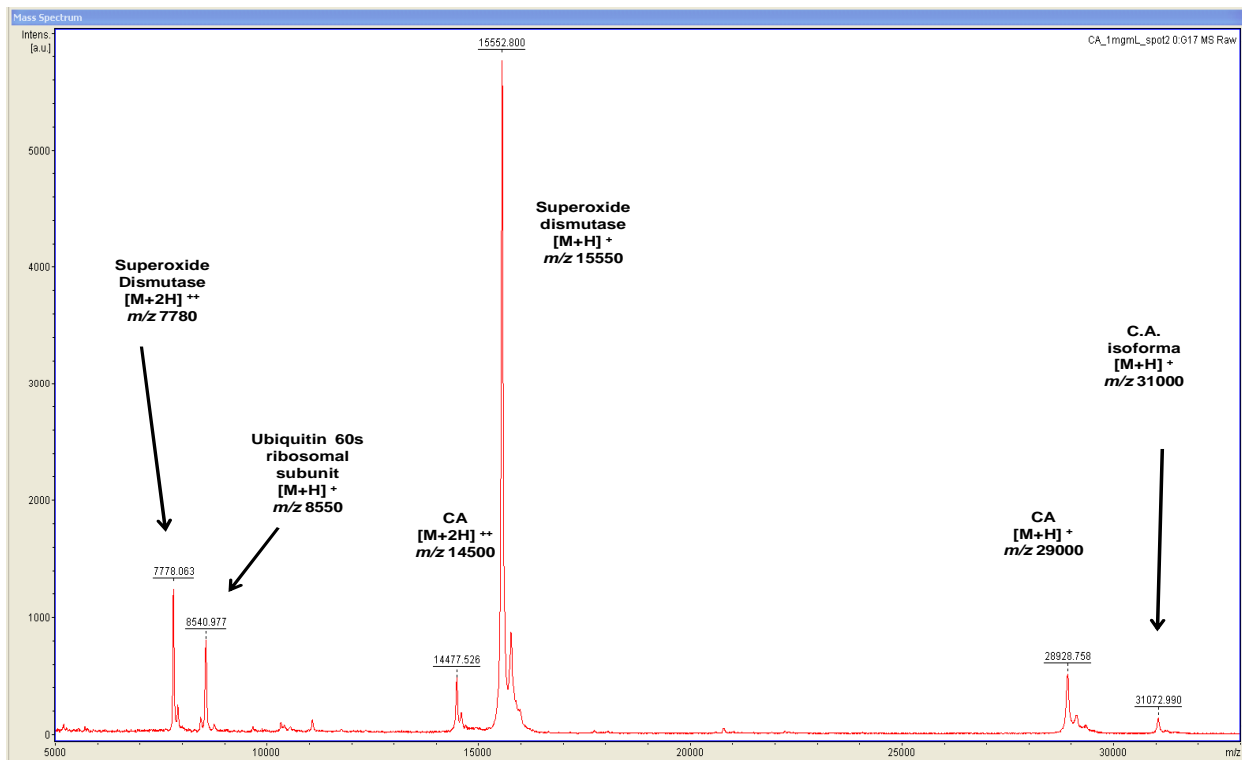
The results obtained according to the conditions here described are reported in Graphs 1 and 2 presenting pH on the abscissa axis and in ordinate the Intensity expressed in arbitrary units (a.u.).

Time-of-flight mass spectrometry is a method of mass spectrometry in which ions are accelerated by an electric field of known strength. This acceleration results in an ion having the same kinetic energy as any other ion that has the same charge. The velocity of the ion depends on the mass-to-charge ratio. The time that it subsequently takes for the particle to reach a detector at a known distance is measured. This time (*time of flight*) will depend on the mass-to-charge ratio of the particle (heavier particles reach lower speeds). From this time and the known experimental parameters one can find the mass-to-charge ratio of the ion. The elapsed time from the instant a particle leaves a source to the instant it reaches a detector. In the tandem mass spectrometry method MALDI-TOF/TOF two time-of-flight mass spectrometers are used consecutively [Medzihradszky, K. F. et al. *Anal. Chem.* **2000**, 72, 552-558].

To record full spectrum of precursor (parent) ions TOF/TOF operates in MS mode. In this mode, the energy of the pulse laser is chosen slightly above the onset of MALDI for specific matrix in use to ensure the compromise between an ion yield for all the parent ions and reduced fragmentation of the same ions. When operating in a tandem (MS/MS) mode, the laser energy is increased considerably above MALDI threshold. The first TOF mass spectrometer (basically, a flight tube which ends up with the timed ion selector) isolates precursor ions of choice using a velocity filter and the second TOF-MS (that includes the post accelerator, flight tube, ion mirror, and the ion detector) analyzes the fragment ions. Fragment ions in MALDI TOF/TOF result from decay of precursor ions vibrationally excited above their dissociation level in MALDI source (post source decay).

The measurement of the time of flight of a protein, that is also related to the molecular weight of the analyte, does not allow a quantitative determination of the amount. Nonetheless the intensity of the signal is a good indication of the amount of protein released by the plug polymer.

In Figure 7 is reported the MALDI TOF/TOF analysis of the commercial CA utilized.

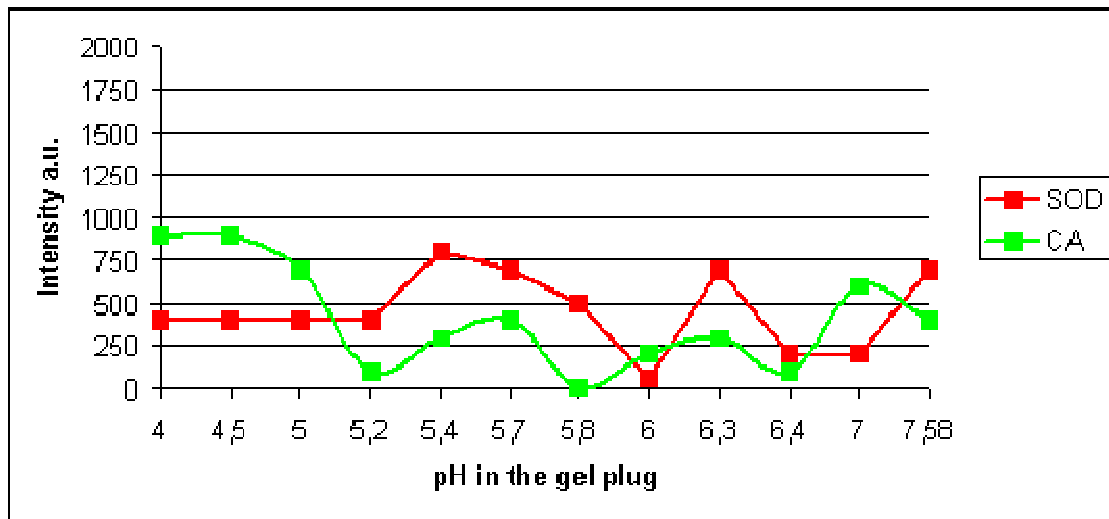


**Figure 7:** MALDI TOF/TOF analysis of CA

From the MALDI-TOF-TOF analysis related to the preparative 2D-gel electrophoresis (Chapter 1.2), the main components of the commercial CA preparation are CA isoforms. Superoxide Dismutase (SOD) represents only a minor amount of the protein content. This result is not in agreement with the MALDI TOF/TOF analysis of the commercial CA. In fact, the SOD signal is higher than the CA and this can be explained in a higher volatility of SOD that has a molecular weight half of the CA one.

Figures 8 and Figure 9 show the results of the direct MALDI-TOF-TOF analysis after a MPID IEF run. The relative amounts of CA and SOD are presented graphically as a function of pH (abscissa) and time of flight-derived intensity of the signal (ordinate axis in arbitrary units).

In Figure 8, the copolymer used is the result of 8% DUR crosslinked with 5% BisA.



**Figure 8.** MALDI-TOF/TOF analysis of model CA with MPID (hydrogel composed of 8% DUR crosslinked with 5% BisA)

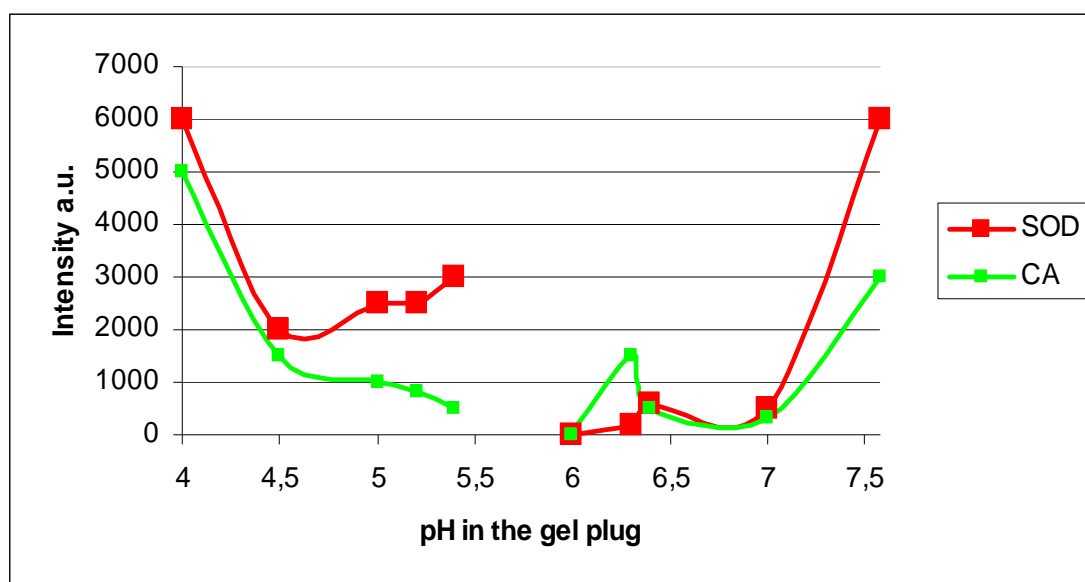
In Figure 8 the graphic shows the presence of carbonic anhydrase (CA) and superoxide dismutase (SOD) in each pH plug. It is interesting to observe, as previously proposed, that the intensity of SOD is higher than CA, probably because SOD molecular weight is lower than the CA one and, consequently, the volatility of SOD in MALDI analysis is higher than the one of CA.

Another important observation is related to the distribution of the two proteins in the plugs of MPID. Since the expected pI of CA and SOD are 6.4 and 5.8, respectively, as reported in Table 1 for the isoforms mainly found with the MALDI analysis of the commercial sample utilized, the course of the electrophoretic run presented in Figure 8 indicates that the IEF-separation by MPID is not successful.

m/z	IDENTIFICATION	Mw	pI
31000	N.D C.A. Isoform		
29000	Carbonic Anhydrase 2	29096	6.4
15550	Superoxide Dismutase Bovin	15844	5.8
8550	Ubiquitin-60s ribosomal protein bovin	7-9 kDa	5.2-5.7

**Table 1.** Characteristics of the proteins present in the commercial sample of CA

In Figure 9, the direct MALDI-TOF-TOF analysis after another MPID-IEF run is presented. In this case, the copolymer used is the result of 8% DMA crosslinked with 5% BisA.



**Figure 9.** MALDI-TOF-TOF analysis of model CA with MPID (hydrogel composed of 8% DMA crosslinked with 5% BisA).

In Figure 9, the MALDI-TOF-TOF analysis of the plugs made by the hydrogel 8% DMA crosslinked with 5% BisA confirms that the intensity of SOD is higher than CA, but the intensity of both proteins is higher than the one achieved with the previous hydrogel (8% DMA crosslinked with 5% BisA). This is an important information about the influence of the chemical structure of the hydrogel on time of flight of absorbed proteins.

Furthermore, the comparison between Figure 8 and Figure 9 shows that the amount of protein collected by the gel of DMA is higher than the one made of DUR but that adhesion of the DMA gel on the surface is lower. In fact, the intensity of the two proteins is not reported for pH values between 5.4 and 6.0 in Figure 9 because of the detachment of the gels observed during the deep vacuum ( up to  $10^{-9}$  bar) phase necessary in MALDI TOF/TOF analysis.

In any event, the course of the electrophoretic run presented in Figure 9 suggests that the IEF-separation by MPID of the two proteins is not yet successful.

### 3.4 Adhesion of the polymeric gel onto ITO surface

The observed lack of adhesion of the hydrogel made of 8% DMA crosslinked with 5% BisA was a disappointing event, since the hydrogel had shown a good response to the maldi-tof-tof analysis after the mpiid-ief running conditions. For this reason, we decided to set up a screening of different percentage of monomers with the aim to select a monomer mix that could confer a higher adhesion on the surface and prevent detachment of the gel during the deep vacuum phase, keeping intact, as possible, the protein desorption by the polymer.

The results of this screening are reported in Table 2 that details percentages and type of monomers tested together with the adhesion strenght obtained after dehydration of the hydrogels.

Ingredients	% Monomer	% bisA of co-monomer moles	Adesion on polymer coated ITO surface after dehydration
DMA/bisA	6 %	3%	+/-
DMA/bisA	8%	5%	+/-
DMA/bisA	8%	3%	--
DMA/bisA	4%	3%	++
DUR/bisA	8%	5%	+/-
DUR/bisA	8%	3%	--

**Table 2.** Percentage of monomers and degree of adhesion of hydrogels on ITO surface are reported.

In Figure 10 (siamo sempre nel Capitolo 3) two examples of hydrogel plugs with good adhesion and bad adhesion to the ITO surface are reported. They are obtained respectively with DMA 4% - BisA 3% (left glass slide) and DMA 8% - bisA 5% (right glass slide).

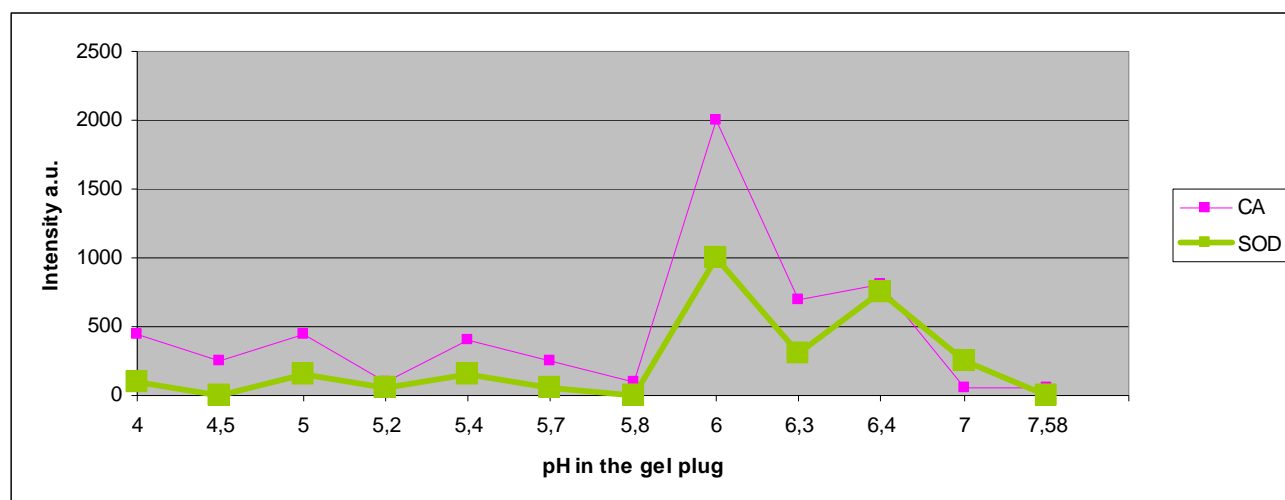
The picture has been taken after removal of the silicon frame for a better visualization.

The picture of the left glass slide shows little yellowish drops on the dehydrated hydrogels, because it has been taken after the preparation of the plugs for the MALDI TOF/TOF analysis. The little spots represent the area where the matrix solution of sinapinic acid/acetonitrile/trifluoroacetic acid (see material and methods) has been deposited to obtain the protein extraction from the surface.



**Figure 10.** Hydrogel plugs with good adhesion (left, DMA 4% - BisA 3%) and bad adhesion (right, DMA 8% - bisA 5%) to the ITO surface.

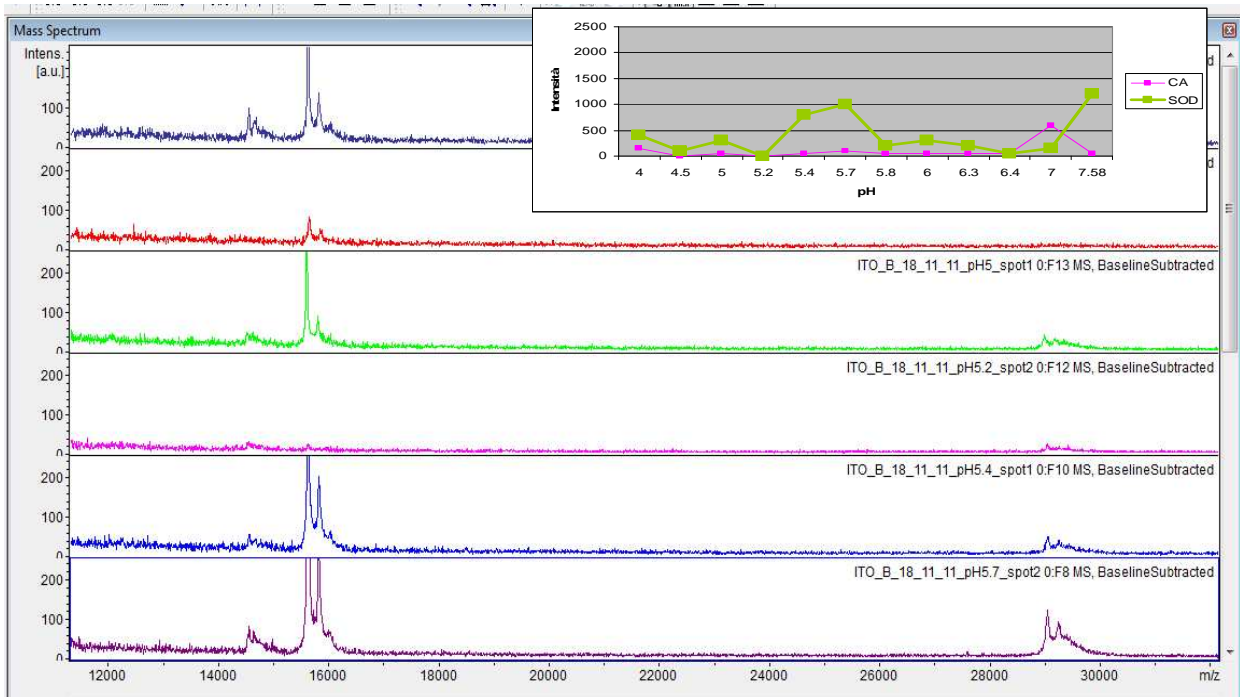
After screening the percentages and type of monomers we decided to proceed working with a gel matrix made of a 4% DMA cross-linked with 3% BisA. However, the MALDI TOF/TOF analysis of commercial CA after a MPID run revealed that the amount of protein collected in the plugs was reduced. This is presented in Figure 11, that shows, as for previous Figures A and B, a higher signal for SOD.



**Figure 11.** MALDI TOF/TOF analysis of model CA with MPID (hydrogel composed of 4% DMA crosslinked with 3 % BisA).



The Figure 12 reports the MALDI spectra of the plugs at different pH values, from which the lower intensity of the peaks is quite evident.



**Figure 12.** MALDI-TOF-TOF spectra of MPID plugs after IEF run with a of 4% DMA crosslinked with 3 % BisA hydrogel

# Material and Methods

## Polyacrilamide gel for MPID preparation

All the reagents has been purchased from Sigma Aldrich.

All the liquid monomers have been previously purified by filtration on alumina to remove the polymerization inhibitor.

In Table 3 are reported the volume in  $\mu\text{l}$  of the ingredients for a total of 1 ml polymerization mix for the upper part of the gel plugs, at specific pH and buffer power 12.

1 ml of polymerization mix for each pH has been prepared as follow:

- 200  $\mu\text{L}$  of the photo initiator Irgacure 2959, from a 10 mM stock solution
- 80  $\mu\text{L}$  of monomer stock solution as reported in Table 2
- a volume of 200 mM stock solutions of specific Immobiline buffer for each pH as reported in Table 1.
- water up to a final volume of 1ml as reported in Table 3

pH	Im pk 3.6	Im 4.6	Im 6.2	Im 7.00	Im 8.5	H <sub>2</sub> O
4		122			27	571
4,3		102,6			38	579,4
4,5		111			54,45	554,6
4,8		115,78	79,5			604,2
5		105	83,75			615
5,5		120,24	126,4			599,8
6	65,845		99			555,2
6,3	52,4		106,5			561,1
6,4	48,975		113			558
6,6	43		133,25			543,8
7	48,6			88,75		582,7
7,2	48,94			113,25		557,8
7,4	42,95			133,15		543,9

**Table 3.** Volumes in  $\mu\text{L}$  of couples of 200 mM stock solutions of Immobiline buffers according to the Buffer Workshop software and volume of water needed to obtain a final volume of 1 mL.

DMA/bisA stock solution has been prepared by mixing 3% moles of bis-Acrylamide per DMA or DUR moles, as reported in Table 4, for the preparation of 4 ml stock solution in 50% water.

		Bis Acrylamide	H2O
DMA/bisA(3%)	2 ml	90 mg	2 ml
DUR/bisA(3%)	2.298 gr	90 mg	2 ml

**Table 4.** Preparation of monomer stock solutions

The lower part of the gel plugs has been prepared as follow :

- Acrylic Acid 200 mM
- Acrilamido buffer pKa 8.5 12 mM
- Glicerol 20%
- Irgacure 2959 2mM
- DMA/bisA (3%) 8%
- Water

The pH hydrogel have been prepared by adding into the silicon frame's holes a lower part of polymeric mix containing glycerol at pH 3 and the upper one at specific pH without glycerol, so to create a discontinuous gradient of viscosity, then exposed to UV light for 15 minutes to carry out the photo polymerization.

The basic gel present on the cathode has been done by photo polymerization under UV light for 15 minutes of the polymeric mix here reported :

- Acrylic Acid 15 mM
- Acrilamido buffer pKa 10.3, 66 mM
- Irgacure 2959 2mM
- DMA/bisA (3%) 8%
- Water

## **MALDI matrix preparation**

Sinapinic acid was prepared at a concentration of 20 mg/mL in 50/0.25 (% v/v) acetonitrile/TFA. 0.8  $\mu$ L of the sinapinic acid matrix have been deposited directly on the gel surface.

## **MALDI TOF/TOF data acquisition on ITO surface**

All the mass spectra were acquired in linear positive mode with a UltrafleXtreme Mass Spectrometer (Bruker Daltonics) equipped with a Smartbeam laser (Nd:YAG/355nm), operating at 1kHz frequency and controlled by FlexControl 3.3 software (Bruker Daltonics). The source was set to an accelerating voltage of 25 kV with an extraction voltage of 23.3 kV and a lens voltage of 6.5 kV. Pulsed ion extraction was 450 ns and laser fluency was set at 60%, with a medium laser diameter focus. Data for each spectrum were acquired by summing up 200 shots in the mass range of 2 to 40 thousands  $m/z$ . External calibration was performed before each experiment with a mixture of standard proteins.

## Conclusions of the first Ph.D. project

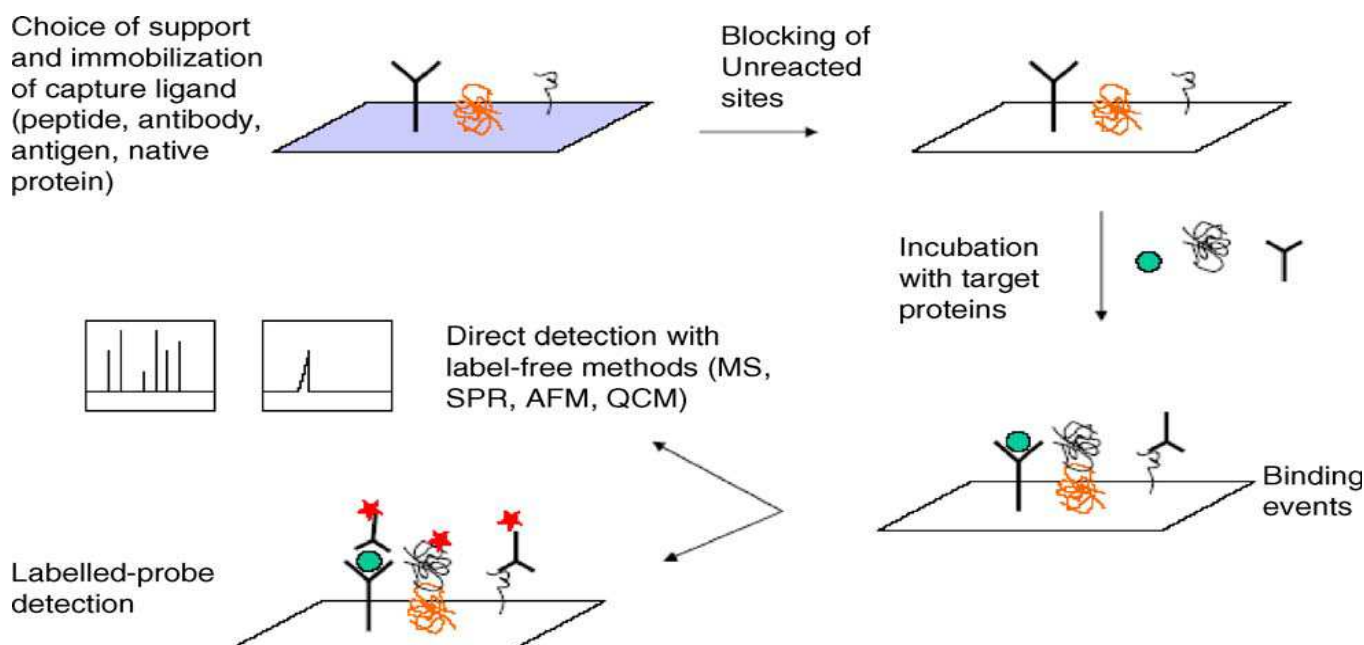
The results obtained from the preliminary approach to the fabrication of a new MPID suggest that, depending on the nature of the polymeric mixture, a direct analysis by MALDI-TOF/TOF is feasible. However, for this MS technique the chemical nature of the monomers cross-linked to form the hydrogel, their composition, and the structure of the copolymer play a fundamental role for what concerns the amount of absorbed proteins that have to be revealed. The monomer composition of the hydrogel may pose problems also for the adhesion of the hydrogel plugs onto the ITO surface. In our case, the problem was solved by varying the percentage of monomers, but a diminished detection of the protein was observed. Therefore, although a MALDI-TOF/TOF direct analysis has not been satisfactorily achieved, the solution of these problems could open a new perspective to the application of the MALDI-TOF/TOF technique to the direct analysis of protein mixtures. On the other hand, a IEF separation of the proteins has not been satisfactorily achieved with the micro-device fabricated in this PhD project. This unsatisfactory result can be attributed to morphological and structural defects due to the micro-fabrication process that consists of different steps, i.e. polymerization, adhesion of the silicon frame on the ITO slide, and adhesion of the silicon frame to the gel. Other problems that should be solved are related to the intensity of the current entering into the plugs during the run. This study involves the morphology of the plugs, the presence of the silicon frame, the adhesion of the gel on it, and concentration of salts inside and outside the plugs. It was considered that this research should face too many problems related to the MPID fabrication or even re-start from the beginning considering a conductive substrate different from ITO-coated glass slides, other hydrogels and/or other copolymerization processes. At this stage of the PhD project, it was concluded that the Ph.D. thesis should be addressed more conveniently to another aspect of surface chemistry, specifically the one related to the fabrication of micro-arrays for protein analysis, a field of research where the research group at the ICRM-CNR had already achieved several interesting results.

## Chapter 4

### The second Ph.D. project: the copolymer (DMA-NAS-MAPS) and its bio-analytical applications.

#### Introduction to the project: protein microarrays

Classical protein analysis (electrophoresis, ELISA, liquid chromatography) are time consuming technologies with limited automation.<sup>1</sup> On the contrary protein and peptide microarray are a inexpensive and high-throughput technology able to analyze hundreds of protein or peptides at the same time. A general scheme of a typical protein array experiment is provided in Figure 1 a large set of capture ligands (proteins, antibodies, or peptides) is arrayed on a solid support. After washing and blocking surface unreacted sites, the array is probed by incubation with a sample containing (among a variety of unrelated molecules) the counterparts of the molecular recognition events under study. If an interaction occurs, a signal is revealed on the surface by a variety of detection techniques. By scanning the entire array a large number of binding events are detected in parallel.<sup>2</sup>



**Figure 1.** General scheme of a typical protein microarray experiment. MS: mass spectrometry, SPR: surface plasmon resonance, AFM:atomic force microscopy, QCM: quartz crystal microbalance.

Three types of protein microarrays are currently used to study the biochemical activities of proteins: analytical microarrays, functional microarrays, and reverse phase microarrays.

*Analytical microarrays* are typically used to profile a complex mixture of proteins in order to measure binding affinities, specificities, and protein expression levels of the proteins in the mixture. In this technique, a library of antibodies, aptamers, or affibodies is arrayed on a glass microscope slide. The array is then probed with a protein solution. Antibody microarrays are the most common analytical microarray.<sup>3</sup> These types of microarrays can be used to monitor differential expression profiles and for clinical diagnostics. Examples include profiling responses to environmental stress and healthy versus disease tissues.<sup>4</sup>

*Functional protein microarrays* differ from analytical arrays in that functional protein arrays are composed of arrays containing full-length functional proteins or protein domains. These protein chips are used to study the biochemical activities of an entire proteome in a single experiment. They are used to study numerous protein interactions, such as protein-protein, protein-DNA, protein-RNA, protein-phospholipid, and protein-small molecule interactions.<sup>5,6</sup>

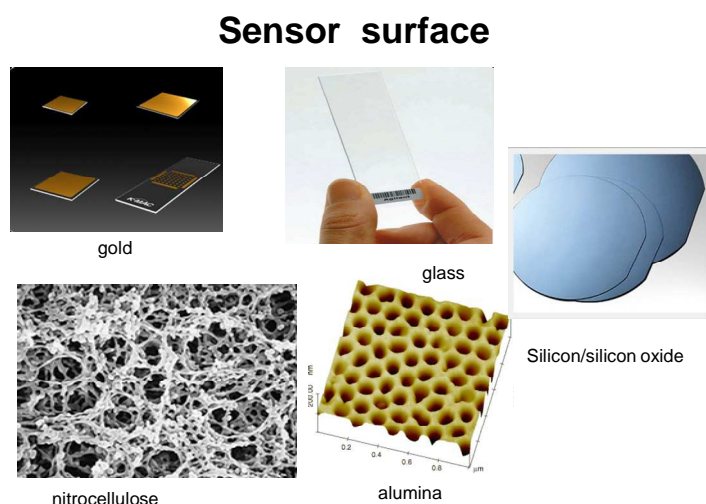
A third type of protein microarray, related to analytical microarrays, is known as a *reverse phase protein microarray* (RPA). In RPA, cells are isolated from various tissues of interest and are lysed. The lysate is arrayed onto a nitrocellulose slide using a contact pin microarrayer. The slides are then probed with antibodies against the target protein of interest, and the antibodies are typically detected with chemiluminescent, fluorescent, or colorimetric assays. Reference peptides are printed on the slides to allow for protein quantification of the sample lysates.

RPAs allow for the determination of the presence of altered proteins that may be the result of disease. Specifically, post-translational modifications, which are typically altered as a result of disease, can be detected using RPAs.<sup>7</sup> Once it is determined which protein pathway may be dysfunctional in the cell, a specific therapy can be determined to target the dysfunctional protein pathway and treat the disease of interest.

Despite the several advantages many problems hinder the spread of microarray for analytical purposes. Some of these problems, such as the development of appropriate strategies of immobilization,<sup>8</sup> the lack of reproducibility<sup>9</sup> and difficulty processing the large amount of data produced by the analysis,<sup>10</sup> are under current, active investigation.

## 4.1 Surface chemistry of protein immobilization

The realization of the microarray involves the use of a rigid planar substrate, such as glass, or silicon/silicon oxide slides, or gold (Figure 2) on which probes are deposited and immobilized in the form of spots with a diameter of 200  $\mu\text{m}$  approx.



**Figure 2.** Examples of microarray surfaces

The purpose of proteins or peptides microarray is the study of molecular interactions that exist between two partners: one contained in a liquid sample and the other immobilized on a solid support.

The surfaces typically used to immobilize the DNA are not always suitable for protein due to differences between the two classes of bio molecules.<sup>11</sup> The surface used in protein microarray must provide a high bonding capacity and must retain the biological activity of the ligand. In fact, proteins tend to lose their native conformation when immobilized on a support, using the internal hydrophobic chains to form hydrophobic bonds with the solid surface.<sup>12</sup> The surface must allow the accessibility of the ligand for interaction with the partner and the protein-substrate interaction reduces the accessibility of the target, leading to false negatives. This is particularly important for peptides microarray due to the small molecular mass of ligands. In this case, the microarray must present a low degree of non-specific interactions and this is extremely difficult to obtain when the sample is a complex mixture of thousands of molecules as in the case of a serum.<sup>11</sup>

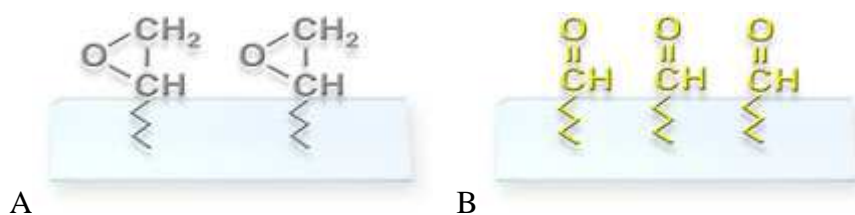
The mechanisms by which the probe protein bind to the surface can be summarized as:



- Electrostatic interactions / hydrophobic
- Physical entrapment
- Covalent bonds
- Bio-recognition-oriented.

The easiest way to bind proteins is through surface adsorption. This approach has been used in ELISA and Western blot for many years and is based on the adsorption of macromolecules through electrostatic forces on a charged surface (slide coated with poly-lysine <sup>13</sup>) or through hydrophobic interactions with membranes (nitrocellulose or PVDF) . The slides coated with nitrocellulose show an excellent binding capacity and a long-term stability of the probes spotted. The adsorption may also take place on a three-dimensional porous material. The membranes of polypropylene (PP) modified with polyaniline (PANI) show an adhesion mechanism that combines electrostatic and hydrophobic interactions and demonstrate high affinity and compatibility for different proteins. <sup>14</sup> In spite of its simplicity, the method of adsorption presents many disadvantages. In fact, the adsorbed protein can be removed by washing under stringent conditions, the level of background is usually high due to a nonspecific adsorption-desorption of proteins and also the proteins adsorbed on hydrophobic surfaces tend to denaturation. <sup>11</sup>

Another approach to promote the binding is based on the presence of covalent bonds between proteins or peptides and the substrate. The covalent attachment mechanism requires the presence of reactive groups on the support (usually electrophilic groups such as epoxides, <sup>15</sup> aldehyde <sup>16</sup>) (Figure 3) capable of reacting with nucleophilic groups (amines, thiols, hydroxyls) present on the ligand protein.



**Figure 3.** Scheme of a surface with reactive groups: (a) epoxides and (b) aldehydes

The functional groups on the surface are introduced by modification of the glass with organosilanes such as 3-glycidoxypropyltrimethoxysilane (GOPS) or 3-aminopropyltriethoxysilane (APTES). Alternatively they can be inserted into molecular architectures much more complex such as those provided by polymer coatings. The organosilanes can provide chemical groups able to bind the ligands(GOPS) or react with a bifunctional ligand bearing the desired reactive group. An alternative route to immobilize the proteins is the use of a matrix that traps the protein. This mechanism does not lead to a covalent bond of molecules with the surface but is based on the physical capturing of proteins in polyacrylamide gels <sup>17</sup> or agarose. <sup>18</sup> The three-dimensional structure of these substrates generally increases the binding capacity and does not disturb the potential sites or functional regulatory domains of the protein. Furthermore, the aqueous environment of the gel reduces the denaturation of proteins. However, the structure of the gel can be a barrier for the diffusion and the events of recognition may thus require a longer incubation time.

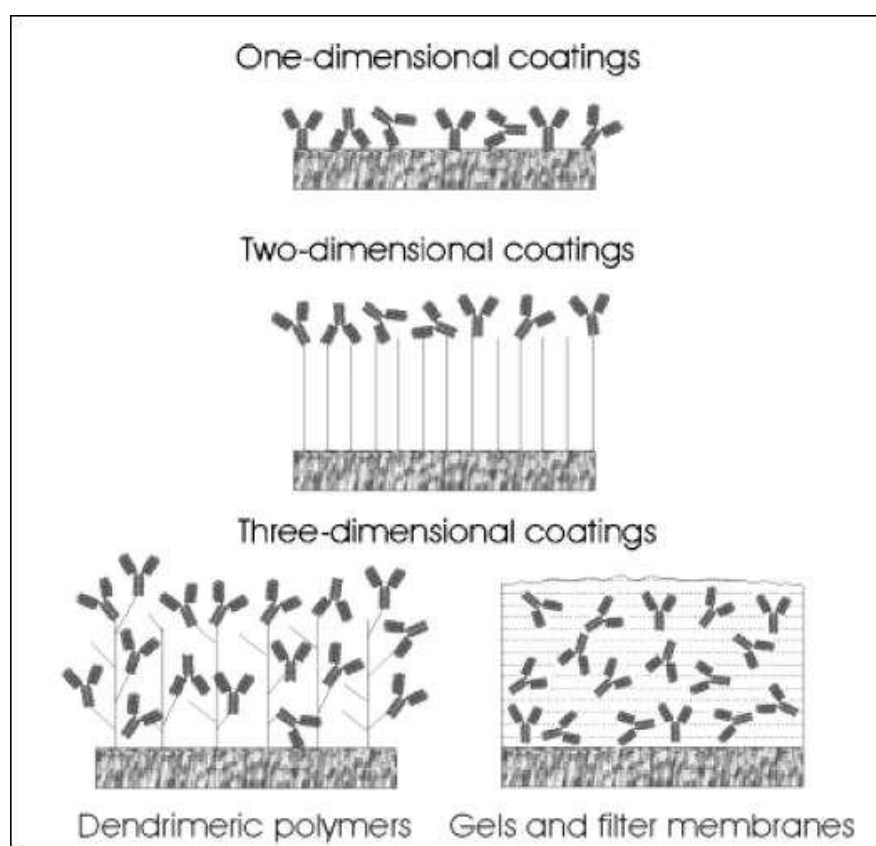
Whether the attack occurs by adsorption, physical entrapment or for formation of covalent bonds, the immobilization of the protein takes place with a random or non-specific orientation. In addition to the non-specific methods, the probes can be labeled and immobilized to favor specific interactions between the marker and non-covalent immobilized molecules. The surface immobilization is typically mediated by an event of molecular recognition such as that of biotin-avidin interaction, or between slides coated with nickel and proteins labeled with histidine, glutathione and glutathione-S-transferase (GST).

In addition to the attachment mechanism (adsorption, physical entrapment in the gel, covalent bonds or molecular orientation), the molecular architecture of the coating plays an important role and should be considered.

One of the most important components in developing a successful biosensing application in the use of an appropriate surface chemistry. Many biosensing assays implement investigation of target molecules in solution interacting with probe molecules that are immobilized on a solid support, hence the term “solid phase assays.” A biosensor with a high degree of sensitivity and a large dynamic range cannot be successfully implemented in a biological assay without a successful strategy of biomolecule immobilization, because surface chemistry greatly affects the affinity and the specificity of the target as well as the background noise caused by non-specific binding. There has been a tremendous effort to develop superior surface chemistry as solid phase assays became powerful tools for parallel investigation. Cretich *et al.* outlines the criteria for successful surface chemistry: <sup>19</sup>

- 1) The surface chemistry must allow a good control of capture probe density with homogeneous morphology for optimal binding capacity of the ligands.
- 2) The biological activity of the probes must be retained upon immobilization.
- 3) The probes must be accessible to the target of interest.
- 4) The surface must display a low degree of non-specific interactions.

Surface chemistries for solid phase assays can be categorized into 1-D, 2-D or 3-D groups based on their architectures as shown in Figure 4.<sup>20</sup>



**Figure 4.** Graphical representation of various forms of surface chemistry for solid phase assays. Figure is reproduced from reference 20.

Complex 3-D structures with polymeric matrix or gel and filter membranes are becoming increasingly popular for fabricating protein microarrays. Hydrogels coated surfaces are developed to maximize the loading capacity and the solution-like environment; however, these techniques are not versatile because the gel like structures minimize the diffusion of the target of interest.<sup>20</sup> This problem is

exacerbated when the target protein is in low concentration as the assay requires a prolonged incubation time.

## **4.2 Detection methods**

The strategies of detection for protein microarrays are generally classified<sup>21</sup> as:

1. Label-free methods (without the need of labeling the target), which include mass spectrometry (MS) (MALDI imaging), surface plasmon resonance (SPR), ellipsometry, interferometry (SRIB), scanning microscopy and Atomic Force Microscope (AFM).
2. Methods with labeled probes using fluorescence, chemiluminescence, electro-chemiluminescence and radioactivity.

## **4.3 Label- free detection methods**

In this class of detection methods one of the most used techniques is the SELDI-TOF-MS (surface enhanced laser desorption ionization - time of flight - mass spectrometry). It is based on the use of a chip that has a selection of proteins immobilized on a matrix. After the interaction of these proteins in solution take place the ionization of the molecules and the analysis using an analyzer which separates them on the basis of mass / charge ratio. The detector will then provide a spectrum of signals.<sup>22</sup>

Another technique commonly used for microarray detection is surface plasmon resonance (SPR). It is a spectroscopic method that measures the change of the thickness or the refractive index of biomaterials at the interface between the metal surface and the environment. Usually this is a thin layer of gold (50-100 nm) located on a glass slide. In SPR, proteins are immobilized on the gold surface, which is the target, and the change in the reflection of the incident light indicates the amount of captured target molecule on the surface in real time. The angle at which gives the minimum intensity of the reflected light, known as "SPR angle", is directly related to the amount of bio molecule bound on the gold surface.

Surface plasmon resonance imaging (SPRI) is another detection technique in which the entire surface of the biochip is illuminated with a broad beam of polarized monochromatic light. The rays of light reflected from each spot are simultaneously captured by a charge-coupled device (CCD). The camera continuously monitors the changes that occur on the surface and provides kinetic data in real time. The SPRI combines the advantages of the SPR (kinetic analysis and measures of affinity) with the capacity highthroughput microarray. The ellipsometry instead is a technique based

on the state of polarization of the reflected light, which is altered for changes in the dielectric properties or refractive index of the sample on the surface.<sup>23</sup> The technique combines the ellipsometer with the microscope and CCD camera, by measuring the total content of protein on the solid surface without concern of protein function. Other label free techniques are interferometric techniques that include spectral reflectance imaging biosensor (SRIB), dual-channel biosensor, on-chip interferometric backscatter detection (OCIBD), porous silicon-based optical interferometric biosensor, high-speed interferometric detection on the biological compact disc and spinning disc interferometry.

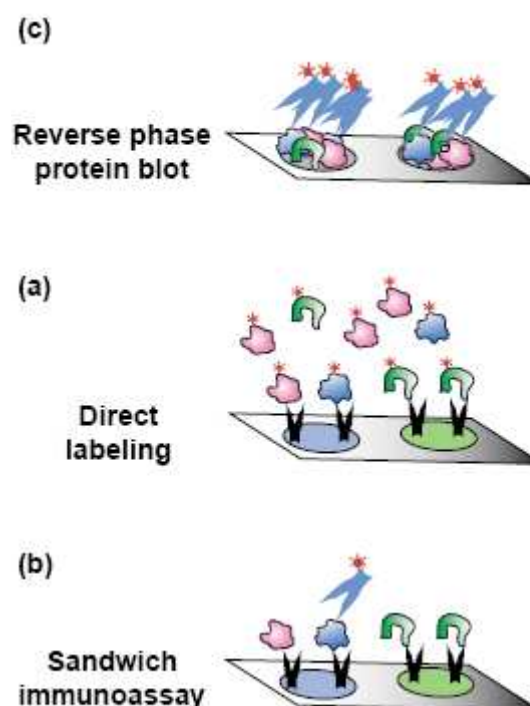
The principle which is the basis of interferometry is the transformation of the phase differences of the front of the light intensity fluctuations observed in known as interference fringes. A recently introduced interferometric technique is the spectral reflectance imaging biosensor (SRIB) in which, the changes in the optical index, as a result of the binding of biological material on the surface of the microarray, are detected using the interference of visible light. This technique monitors directly the interactions between molecules.

Atomic force microscopy (AFM) determines the changes in height of the immobilized protein caused by the binding with the target. The deflection of an array of cantilevers with a resolution of picometers can also be measured to probe molecular interactions. The microcantilevers are strips of silicon fixed at one end, with the probe (antibody or protein) bound on the surface. When an analyte binds to the microcantilever, the bending as a result of stress on the surface is measured by the deviation of an optical signal or through a change of electrical resistance in a thin piezoelectric film on the cantilever. Alternatively change in the mechanical resonance frequency can also be measured. The label-free techniques are promising methods for the characterization of antigen-antibody interactions, or more generally, protein-protein interactions, and for the characterization of binding events on the surface. They do not require marking of bio molecules, which can affect protein activity. However label free detection methods, often require sophisticated instruments not always available in all clinical laboratories.

#### 4.4 Detection methods with labeled probes

Lable detection methods evolve directly from the most widely used immunoassays, radiological assays or ELISA. They can be distinguished as shown in Figure 5: <sup>25</sup>

- reverse phase (when a mixture of proteins is immobilized and the detection is obtained using labeled molecules such as antibodies);
- direct (when antibodies are immobilized and are assayed with labeled proteins);
- sandwich (when a first antibody immobilized acts as a capturing agent for the protein tested that is revealed through the recognition with a labeled secondary antibody).



**Figure 5.** Revelation methods with labeled probes for in protein microarrays from reference 25.

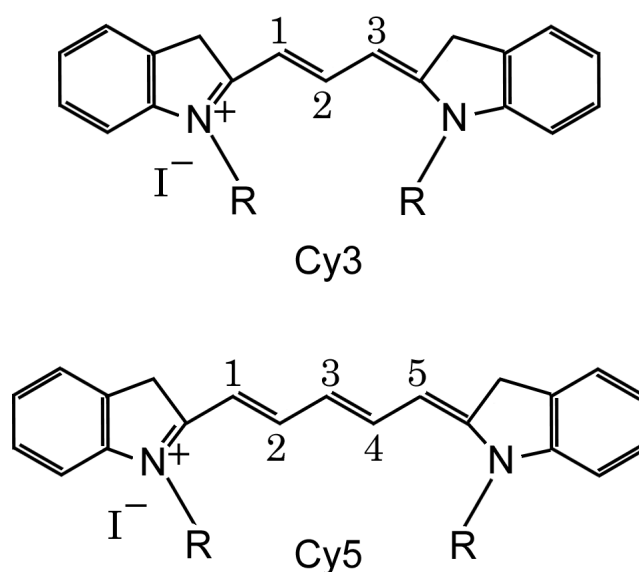
In the reverse phase approach, each spot in the array contains an individual sample of the test. Therefore, an array can contain many different sera from patients or cell lysates containing a complex mixture of proteins. The array is then incubated with a labeled protein (usually an antibody) allowing the comparison of a single analyte in different samples. In the direct approach, a known ligand (antibody) is immobilized on the surface and is assayed by a complex mixture of labeled proteins. The sample can be a cell lysate or a serum in which multiple analytes are measured simultaneously. With a two colors approach different pharmacological treatments or protein expression profiles can be compared.<sup>26</sup> The "sandwich" assays are based on immobilized antibodies to capture the protein of interest, while

a second labeled antibody, directed against a different epitope of the captured protein is used for the detection. This approach requires two different antibodies, each with affinity for different epitopes of the same protein of interest. Although the need of two antibodies often complicates the design of the array, the "sandwich" method is widely used as it increases the specificity of the antibodies array. Main problems of the methods that use labeled probes are the production of antibodies and the quantitative labeling of antibodies / antigens. Label detection methods are based on fluorescence, chemiluminescence, electrochemiluminescence, and radioactivity detection.

#### 4.4.1 Fluorescence

Fluorescence represent one of the methods of choice for the detection in microarray.<sup>27</sup> There are many fluorophores, in different formulations, with well-defined excitation and emission spectra. The labeling methods are based on the use of fluorochromes such as fluorescein, cyanine dyes, rhodamine, acridines, phycobiliproteins and BODIPY. The choice of the fluorescent probe depends on the type of sample, the emission spectrum and the substrate. Not all substrates are compatible with fluorescence detection due to auto fluorescence of the material on which the array is deposited. Furthermore, the sample may possess components that interfere with the fluorophores as in the case of flavoproteins that emit light in the same region of the fluorescein. Recombinant proteins with green fluorescence and red have been used to study protein-protein interactions.<sup>28</sup>

The Cy3 and Cy5 fluorescent molecules belong to the family of cyanine dyes and are among the most used dyes for the detection of fluorescent proteins (Figure 6).



**Figure 6.** Chemical structure of cyanines Cy3 and Cy5

Various structures are designated Cy3 and Cy5 in the literature. The R groups do not have to be identical and usually correspond to short aliphatic chains one or both of which ends in a highly reactive moiety such as N-hydroxysuccinimide or maleimide. The cyanins are usually synthesized with reactive groups on either one or both of the nitrogen side chains so that they can be chemically linked to either nucleic acids or protein molecules. Labeling is done for visualization and quantification purposes. They are used in a wide variety of biological applications including comparative genomic hybridization and in gene chips, which are used in transcriptomics. They are also used to label proteins and nucleic acid for various studies including proteomics and RNA localization.

The scanners actually use different laser emission wavelengths (typically 532 nm and 635 nm) and filter wavelengths (550-600 nm and 655-695 nm, Figure 7) to avoid background contamination. They are thus able to easily distinguish between two samples when one sample has been labeled with Cy3 and the other labeled with Cy5. They are also able to quantify the amount of labeling in either sample.

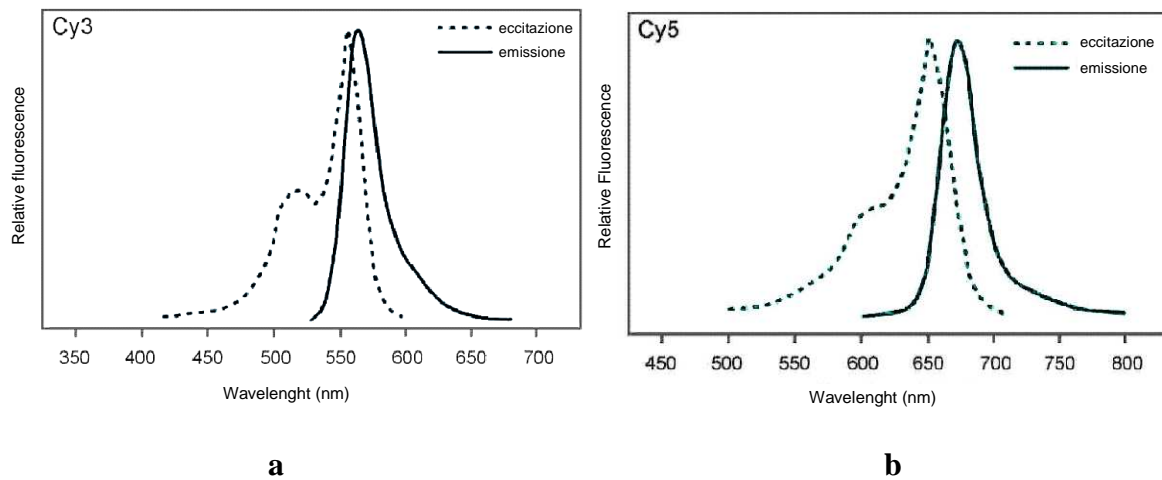
Dye	Absorbance Max	Emission Max	Quantum yield in PBS buffer	Molecular weight (Da)
Cy3	550 nm	570 nm	0.04	766
Cy5	649 nm	670 nm	0.28	792

**Figure 7.** Spectral characteristics of dyes Cy3 and Cy5

The technique of revelation with two colors, with Cy3 and Cy5, has been successfully applied to compare protein levels of two different samples. The diagrams of revelation multicolored (Figure 8) are useful when the measurements of the microarray are not based on absolute but on relative quantifications. The simultaneous detection of species allows a direct comparison of the intensity of



fluorescence in different samples without variation within the chip. In this assay, the two differently labeled samples compete for binding to the same antibody immobilized target.



**Figure 8.** Absorption and emission spectra of cyanine 3 and 5. The cyanine 3 (Cy3) absorbs at a wavelength of 550 nm and emits at 570 nm (a). The absorption peak of Cy5 is at 650 nm, while the emission is at 670 nm (b)

#### 4.4.2 Chromogenic detection

The chromogens are substrates for enzymatic reactions that generate a colored insoluble product. The chromogenic detection of protein microarrays produces permanent signals easily detectable. The enzymes most commonly used are alkaline phosphatase and horseradish peroxidase. These enzymes act on colorless substrates, chemically generating stable colored products. For example, the blue spots, generated from 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP / NBT) and by the system of the alkaline phosphatase, were used for the proteomic profile of cancer cells on nitrocellulose coated slides. <sup>29</sup>

#### 4.4.3 Chemiluminescence

Chemiluminescence is the luminescence generated by chemical reactions and generally used in microarray for the detection of proteins recognized by a secondary antibody labeled with alkaline phosphatase or horseradish peroxidase. The enzymatic oxidation of substrates such as luminol produces an emission of prolonged light that is captured by a charge-coupled device (CCD) camera. Although highly sensitive chemiluminescence has a limited dynamic range. <sup>30</sup>

## References (Chapter 4)

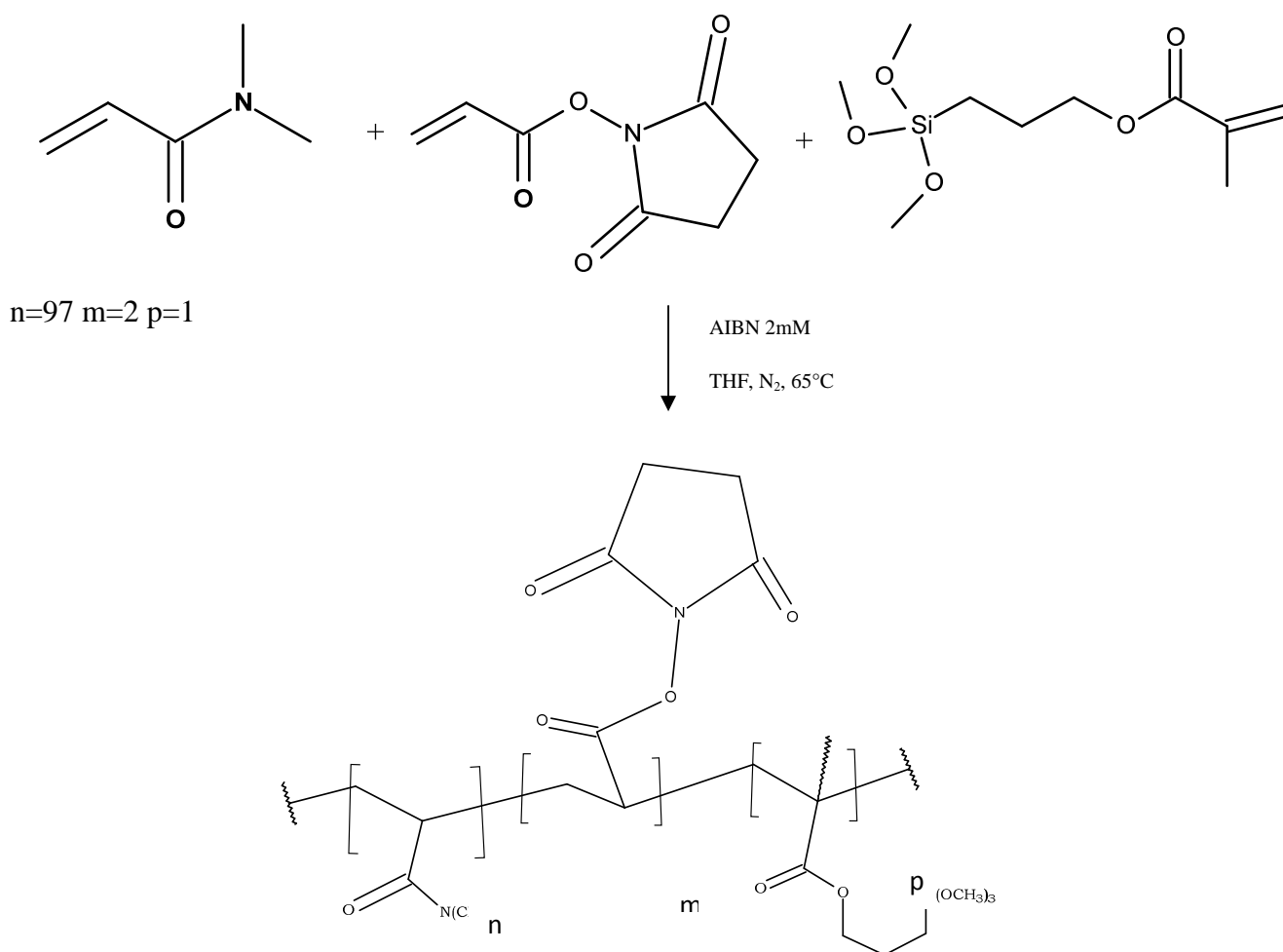
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## Chapter 5.

### The copolymer Copoly (DMA-NAS-MAPS)

In the research group where my PhD thesis has taken place (Institute of Chemistry and Molecular Recognition, National Research Council, Milan, Italy) a new 3-D polymeric matrix for immobilizing DNA or protein on different surfaces has been recently developed.<sup>1-3</sup> This polymeric matrix is the result of a copolymerization process of three monomers, N,N- dimethylacrylamide (DMA), acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)-propyl methacrylate (MAPS). The copolymerization takes place in the presence of the radical initiator R,R'-azoisobutyronitrile (AIBN) and the copolymer so obtained has been named as Copoly (DMA-NAS-MAPS) (Figure 1).



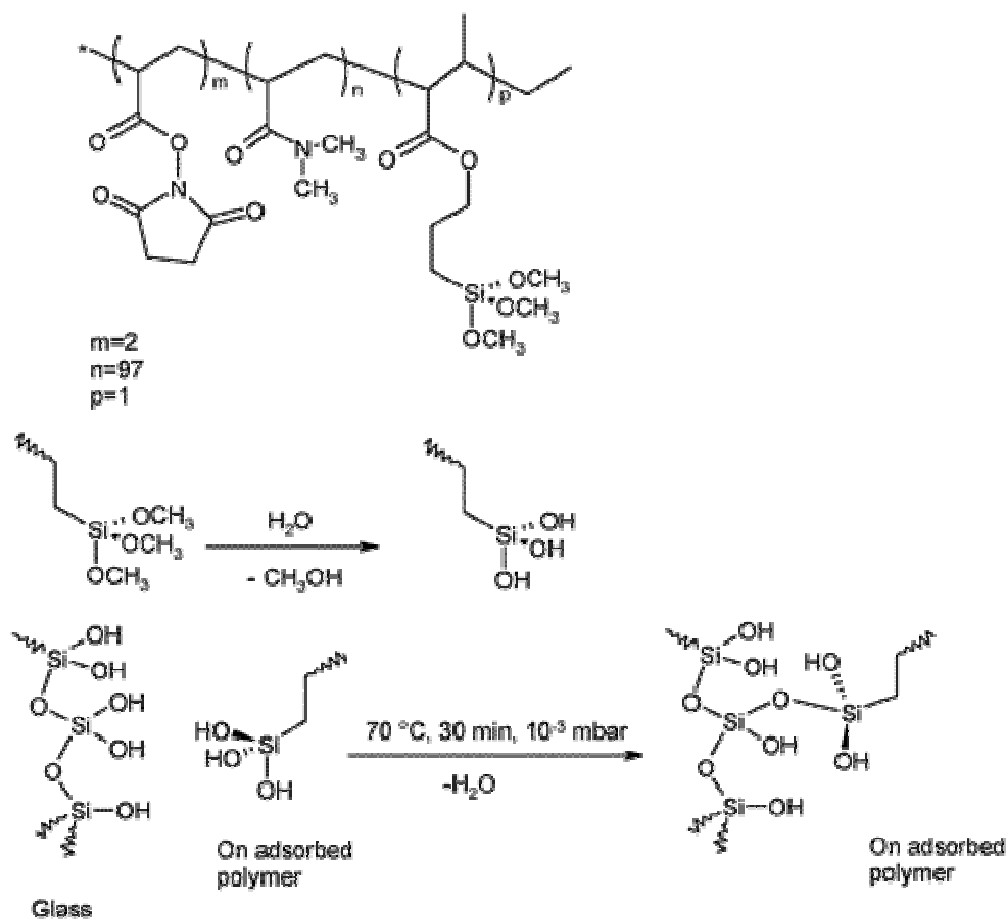
**Figure 1.** Scheme of the synthesis of copoly (DMA-NAS-MAPS)

Copoly(DMA-NAS-MAPS), is a ter-copolymer constituted by 97% of bound dimethylacrylamide (DMA) , while the second monomer, acryloyloxysuccinimide (NAS is present as 2% of the whole polymer . The third constituent, is 3-(trimethoxysilyl)propyl methacrylate (MAPS) (1%).

The major polymer constituent, responsible for glass self-adsorption, is dimethylacrylamide, while the second monomer, acryloyloxysuccinimide, is responsible for covalent binding of proteins and modified DNA molecules. The third constituent, 3-(trimethoxysilyl)propyl methacrylate, increases the strength of the binding of the polymer with the glass. Copoly(DMA-NAS-MAPS) has been fully characterized and its preparation standardized.<sup>1</sup>

When dissolved in an aqueous solution, Copoly(DMA-NAS-MAPS) quickly adsorbs onto a glass surface, typically in 5-30 min, generating a layer that is stable in an aqueous buffer containing various additives (SDS, urea, salts) even at high temperatures. The procedure requires the immersion of the glass slide into the polymer dilute solution. The only pretreatment required for the glass is the alkaline activation of surface silanols, which maximizes the number of hydrogen bonds with carbonyl groups on the polymer. The layer of the adsorbed copolymer on the glass surface has a double function: to block the glass surface sites by making hydrogen bonds and to control the interactions with the external medium by bearing specific chemical functions.

The copoly(DMA-NAS-MAPS) film showed that this coating is suitable for application in DNA microarrays, because of its ability to form more stable and thicker films. Indeed, the silane functionalities pending from the backbone induce, upon adsorption, silylation of the silanol groups on the glass surface, thus increasing the strength of the binding with the glass (Figure 2). This results in the formation of a layer with increased stability.



**Figure 2.** Copoly (DMA-NAS-MAPS). Molecular structure of copoly (DMA-NAS-MAPS) and its interactions to the glass surface.

### 5.1 Copoly (DMA-NAS-MAPS) on glass surfaces for DNA microarray assays

Copoly (DMA-NAS-MAPS) was developed for DNA microarray assays on microscope glass slide<sup>4</sup> The study was aimed to find a new method to covalently attach target molecules onto the surface of glass substrates such as microwell plates, beads, tubes, and microscope slides, for hybridization assays with fluorescent targets. The innovative concept introduced by the work is to physically adsorb onto non-derivatized glass surfaces a functional copolymer, able to graft amino-modified DNA molecules. Glass is one of the most widely used substrates for the immobilization of biomolecules and several methods are currently available to graft epoxy, amino, aldehyde, or other functional groups onto the glass surface. However, successful and reproducible deposition of a monolayer on a glass surface requires a strict control of operative parameters. The surface must be cleaned to remove contamination, to create surface attachment sites, and to control surface roughness. Numerous cleaning methods exist for glass substrates and these include gas plasmas, as

well as combinations of acids, bases, and organic solvents that are allowed to react at different temperatures. Surfaces can also be smoothed or roughened using various techniques such as chemical deposition, grinding, polishing, and chemical etching.<sup>5</sup>

For regioselective immobilization by either the 3' or 5' end of short oligonucleotides, polymeric coatings have been developed based on polyacrylamide or poly(dimethylacrylamide) gels.

However these coatings require glass silanization and careful control of operative parameters.<sup>6</sup> Pirri et al. have described a method for the covalent attachment of target molecules onto the surface of a glass substrate by the adsorption of copolymers with one of the monomers interacting directly with the glass surface and playing the role of an anchor. Another monomer goes in the solution and binds to the DNA target molecule. The new polymer forms a highly hydrophilic coating with accessible functionalities to which modified DNA can be covalently grafted. The coating was achieved by combining the adsorptive properties of poly(dimethylacrylamide) onto glass with a reaction between the silanol groups and electrophilic groups, pending from the polymer backbone. Copolymer composition was optimized to ensure an optimal combination of properties such as the following:

- (1) complete and uniform surface coverage of the surface with an ultrathin film
- (2) a hydrophilic surface having minimum nonspecific attraction for biomolecules
- (3) sufficient stability for use as the substrate for DNA microarray experiments
- (4) ease and reproducibility of the coating process.

The polymer, has been obtained by radical copolymerization of N,Ndimethylacrylamide, N-acryloyloxysuccinimide, and 3-(trimethoxysilyl) propyl methacrylate as reported in the Figure 1. The most significant characteristic is that it self-adsorbs onto the glass surface very quickly, typically in 5-30 min. In a typical glass slide coating, after a surface treatment with 1 M NaOH for 30 min and 1 M HCl for 1 h, to activate the surface, washing with water and drying, pretreated glass slides were immersed for 30 min in a solution of the polymer (0.2-1% w/v in a saturated water solution of ammonium sulfate (20% w/v). The slides were then washed extensively with water and dried under vacuum at 80 °C.

The glass coating procedure is fast, inexpensive, robust, and reliable, and it does not require time-consuming glass pre-treatments.

As an achievement, slides coated with copoly(DMA-NAS-MAPS) were profitably used as substrates for the preparation of low-density DNA microarrays prepared dissolving 5'-amine-modified oligonucleotides (100 nM/mL stock solution) in 150 mM sodium phosphate buffer pH 8.5. Solutions of oligonucleotides at different concentrations were printed on coated slides to form microarrays using an Arrayt SpotBot spotter from Telechem. Printed slides were placed in an uncovered storage box placed in a sealed chamber, saturated with NaCl, and incubated at room temperature from 4 h to overnight.

The density and the thickness of the films were evaluated by X-ray reflectivity measurements whereas the extent of reaction of functional groups with DNA molecules was determined by a functional test. The experiments indicate that half of the active groups present on the surface reacts with oligonucleotide probes.

The structure of the polymeric film deposited on the surface was characterized by means of X-ray reflectivity (XRR), which is a surface-sensitive technique that provides information on mass density, thickness, and roughness of very thin films that are deposited on flat substrates. This measurement is based on the specular reflection of X-rays from planar surfaces. The reflected intensities show fringes that depend on the film thickness, and different modulation lengths correspond to the existence of different layers. The critical angle of total reflection is related to the mass density.<sup>8</sup>

XRR measurements highlighted the presence of two polymeric layers of different thickness. The chains close to the wall, condensed with themselves and with the surface silanols, form a layer of 3.45 nm with a density of 1.8 g/cm<sup>3</sup>. The layer at the surface-air interface, made by less condensed chains, has a thickness of 1.8 nm and a density of 1.12 g/cm<sup>3</sup>. These are thickness values of the dry film. However, it is reasonable to assume that the film, on hydration, swells 10–15 times due to the highly hydrophilic nature of the copolymer.<sup>9</sup>

From the thickness and the mass density of the films, obtained by XRR analysis, it was possible to calculate the density of active esters on the surfaces. For Copoly(DMA-NAS-MAPS) the number of active esters per square centimeter was found to be  $8.9 \times 10^{13}$ . This value was calculated by taking into account film thickness and mass density and the molar fraction of NAS in the polymer. Following the same procedure, in the case of Copoly(DMA-NAS), a density of  $2.8 \times 10^{13}$  molecules of NAS/cm<sup>2</sup> was calculated. The number of functional groups per surface unit obtained from XRR data are in good agreement with those obtained by the oligonucleotide binding assay. Indeed, the functional test indicates that about half of the active groups present on the surface,  $4 \times 10^{13}$ /cm<sup>2</sup> for



Copoly(DMA-NAS-MAPS) reacts with the oligo probes, which indicates an excellent accessibility of surface functional groups.

## 5.2 Copoly (DMA-NAS-MAPS) and protein microarrays

Protein microarrays are more challenging to prepare than are DNA chips<sup>10</sup> because several technical hurdles hamper their application. The surfaces typically used with DNA are not easily adaptable to proteins, owing to the biophysical differences between the two classes of bioanalytes.<sup>11</sup>

In fact, arrayed proteins must be immobilized in a native conformation to maintain their biological function. Unfortunately, proteins tend to unfold when immobilized onto a support so as to allow internal hydrophobic side chains to form hydrophobic bonds with the solid surface.<sup>12</sup> The accessibility of the protein is also of crucial importance to achieve proper recognition during hybridization; protein–substrate interactions reduce the accessibility of the target, leading to false negative results.

Another important requirement of the surface is to provide a low unspecific background because unwanted adsorption of proteins leads to false positive results. The presence of an aspecific background is one of the most severe problems in antibody microarrays.<sup>13</sup> The achievement of a low degree of unspecific binding is extremely difficult when the protein sample is a complex mixture of thousands of molecules.<sup>14</sup>

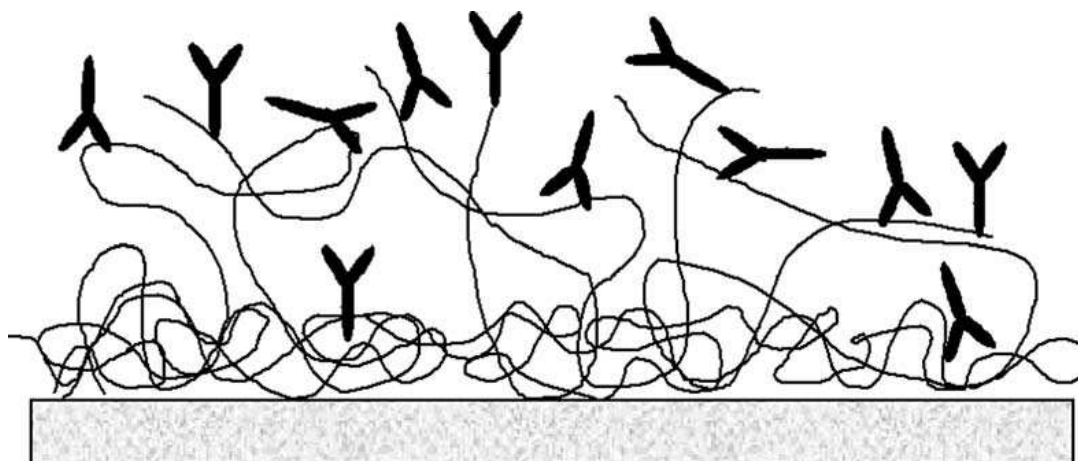
The copolymer of N,N-dimethylacrylamide (DMA), N,N-acryloyloxysuccinimide (NAS), and [3-(methacryloyl-oxy)propyl]trimethoxysilyl (MAPS) coated on glass slides had been shown to be able to bind amino-modified DNA by means of the NAS reactive group<sup>15</sup> The same reactive groups should be able to react with primary amines of lysines and arginines in proteins, as well. DMA, which forms the polymer backbone, could facilitates polymer adsorption on the glass surface, whereas MAPS covalently reacts with free silanols and stabilizes the coating with glass surfaces. Therefore, the performance of glass slides coated with the Copoly(DMA-NAS-MAPS) has been studied for application to protein microarrays.<sup>16</sup>

Two different experiments were carried out to illustrate the characteristics of this coating. In one experiment, the Fab portion of an antibody immobilized on the slide was efficiently recognized by the corresponding antibody. In the second experiment, the Fc domain of the capture antigen was specifically recognized.

The results of the two assays demonstrate that both the Fab and Fc domains of the antibody are freely accessible. This is a considerable advantage because it overcomes one of the major problems of microarray assays, that is, the insufficient accessibility of the ligand.

Copoly(DMA-NAS-MAPS)-coated slides immobilize antibodies in a random conformation, and no crosslinkers or spacers are required to separate the protein from the surface, as for most of the non-polymeric slides.<sup>17</sup> This is because the polymer chains act themselves as a spacer between the protein and the surface. In addition, the active esters, which bind the protein, are spaced along the chains. Therefore, only a few groups of the protein are covalently bonded with the surface functional groups.

In Fig. 3, a graphical representation of a copoly(DMA-NAS-MAPS) film with immobilized proteins is provided.



**Figure 3.** Graphical representation of Copoly(DMA-NAS-MAPS) polymer coating on glass support after hydration and antibody binding. The polymer chains close to the glass surface condense with themselves and with the silanols of the support. The chains at the surface-air interface are less dense due to the presence of loops and tails.

At the beginning of the second part of this PhD thesis, started with a few standard preparations of Copoly(DMA-NAS-MAPS) and coating of Copoly on glass surfaces, carried out as originally described by Pirri et al.

## Material and Methods

### Synthesis of Copoly (DMA-NAS-MAPS)

The monomers were dissolved in 6 mL of dried tetrahydrofuran (THF) in a 25-mL, round-bottomed flask, equipped with condenser, magnetic stirring, and nitrogen connection. The solution was degassed by alternating a nitrogen purge with a vacuum connection, over a 30-min period. R,R'-Azobisisobutyronitrile (AIBN) was added to the solution, which was then warmed to 50 °C and maintained at this temperature under a slightly positive pressure of nitrogen for 24 h. After the polymerization was completed, the solution was evaporated using a rotary evaporator and the white solid was dissolved in chloroform and precipitated by adding petroleum ether. The supernatant was eliminated, and the whole procedure was repeated two times. The polymer was dried under vacuum for 24 h at room temperature and stored at 4 °C.

<sup>13</sup>C NMR (DMSO),  $\delta$  (ppm): 174.6 (backbone carbonyl), 166 (succinimide carbonyl) 40-30 (methylene carbons). The degree of succinimide insertion was determined from the ratio of the integrals of backbone and succinimide carbons, and the NAS molar fraction was found to be 0.015. The average molecular masses ( $M_w$ ) and the polydispersity index of the different polymers were determined by gel permeation chromatography. Samples and standards were run in a 802.5, 804, 806 OH-PAK columns in series at 40 °C (Shodex) connected with UV Bruker detectors injecting 0.5% w/v sample solutions. The mobile phase was 100 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>. Five polyacrylamide standards with  $M_w$  of 21 900, 58 400, 79 900, 400 000, and 600 000 were used to calibrate the GPC (Polysciences).

### Glass Slide Coating.

The coating of glass slides requires surface pretreatment and adsorption of the polymer. In a first step, the slides were treated with 1 M NaOH for 30 min, washed with water and then treated again with 1 M HCl for 1 h, washed with water and dried. In the second step, pretreated glass slides were immersed for 30 min in a solution of the polymer, (0.2-1% w/v in a saturated water solution of ammonium sulfate (20% weight/volume). The slides were then washed extensively with water and dried under vacuum at 80 °C.

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## Chapter 6.

### **Copoly(DMA-NAS-MAPS) coating of silicon oxide surface and application to microarrays**

One of the targets of the second part of the PhD thesis has been to test a few additional aspects of the versatility of the copolymer Copoly(DMA-NAS-MAPS) in the functionalization of inorganic substrates typically used in bio analytical assays. This research activity has been carried out in the frame of an integrated project entitled Nanosystems for early Diagnosis of Neurodegenerative Diseases (NaDiNe) funded by the European Community that involved the Italian CNR (Istituto di Chimica del Riconoscimento Molecolare, Dr. Marcella Chiari) aimed at developing and integrating new technologies towards a fully automated lab-on-chip system for the simultaneous quantitative analysis of a multiplicity of proteins, peptides and their variants, down to concentrations in the picomolar range directly from plasma.

This part of the thesis would specifically address one of the many macro-objective of NaDiNe project and specifically the development of analytical microarrays with the aim to profile complex mixture of proteins in order to measure their binding affinities, specificities, and expression levels. Microarrays experiments involve immobilizing a set of well characterized ligands on a solid substrate surface and monitoring their interaction with an analyte in sample solutions. The work was expected to be carried out in close collaboration with NadiNe partners that would have provided protein probes and inputs for the design of the microarray experiments. To this purpose a statistically significant number of samples could be tested, by different partners and with different technologies, for known dementia biomarkers in CSF, such as Tau, p-Tau and A $\beta$  peptides, as well as for potential novel biomarkers including e.g. TDP43,  $\alpha$ -Synuclein fragments, MAP kinase Erk1/2 and GFAP. The data set would have served to complement the clinical and neuropsychological diagnosis with neurochemical CSF signatures (“neurochemical dementia diagnosis”). Depending on the set of biomarkers furnished by the partners of NadiNe consortium the target sensitivity should have ranged from 1 - 100 pg/ml.

The specific aim of this PhD thesis was related to the development of methods and procedures for the efficient analysis of the selected biomarker by microarray technology, exploiting the knowledge and experience of the group headed by Dr. Marcella Chiari on the use of Copoly(DMA-NAS-MAPS) as versatile coating agent of silicon or gold surfaces in protein microarrays development.

An important part of the activity would have been devoted to the characterization of the physical properties, the chemical composition, and the spatial distribution of the polymers on the surface.

State of the art techniques for surface characterization such as, for instance, X-ray photoelectron spectroscopy (XPS), atomic force microscopy, X-ray reflectivity could be used to this purpose in collaboration with external groups (University of Brescia, University of Boston). The results should provide a feed-back to the activity focused on polymer synthesis and coating allowing a rational optimization of array design, spotting and hybridization conditions, to improve the stability of the proteins immobilized on the surface and the overall assay performance.

Within the NaDiNe Project, this part of the PhD thesis was aimed at the immobilization of selected model antibodies onto Copoly(DMA-NAS-MAPS)-coated silicon surfaces.

The copolymer copoly (DMA-NAS-MAPS) was originally introduced as a new 3-D polymeric matrix for immobilizing onto glass slides amino-modified oligonucleotides<sup>1</sup> or proteins.<sup>2</sup> The copolymer has been coated successfully onto silicon dioxide layers by Cretich et al., who have reported that this system is able to enhance the sensitivity of fluorescence labeling and a confocal laser scanning apparatus, typically involved in microarray technology.<sup>3</sup>

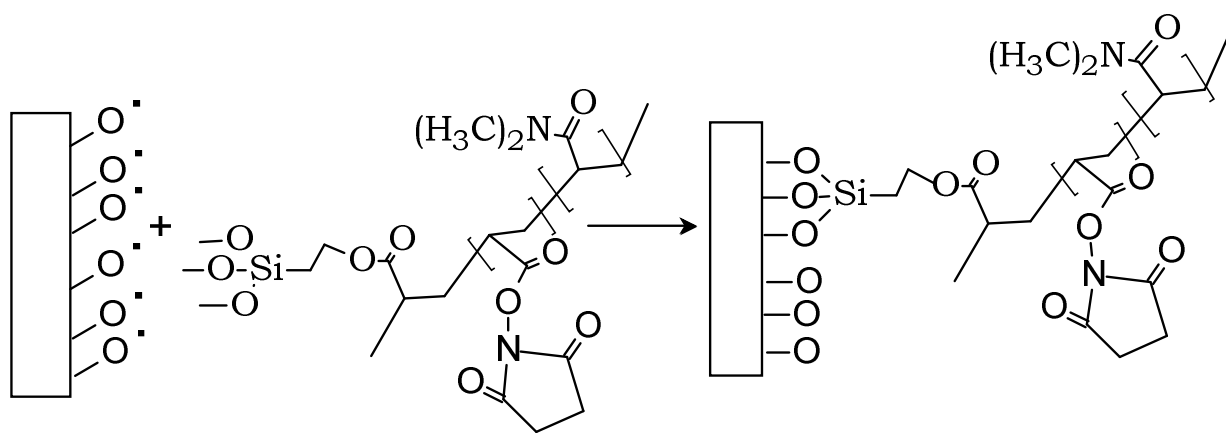
## **6.1 Preparation of the Copoly(DMA-NAS-MAPS)-coated silicon surface**

As a part of the PhD program, silicon dioxide layers were initially prepared according to the experimental procedure described by Cretich et al.<sup>3</sup> In this work, the improvement of microarray sensitivity was achieved by a crystalline silicon substrate coated with thermal silicon oxide functionalized by a polymeric coating with copoly (DMA-NAS-MAPS). The optimized layer of thermally grown silicon oxide (SiO<sub>2</sub>) of a highly reproducible thickness, low roughness, and fluorescence background provides fluorescence intensification due to the constructive interference between the incident and reflected waves of the fluorescence radiation. The oxide surface is then coated by copoly-(DMA-NAS-MAPS), which forms, by a simple and robust procedure, a functional nanometric film. The polymeric coating with a thickness that does not appreciably alter the optical properties of the silicon oxide confers to the slides optimal binding specificity leading to a high signal-to-noise ratio. The coated silicon slides, tested in protein and peptide microarrays for detection of specific antibodies, lead to a 5-10-fold enhancement of the fluorescence signals in comparison to glass slides.

In the second part of the PhD thesis, Copoly(DMA-NAS-MAPS) has been prepared according the original procedure and coated onto a silicon oxide (SiO<sub>2</sub>) surface for the determination of

biomarkers present in human serum for neurodegenerative diseases within the frame of an European project devoted to the identification of such biomarkers via microarray technique. The silicon oxide slides prepared by thermal grown, used for the assays have been purchased from SVM, Santa Clara, USA.

A self assembled monolayer (SAM) of Copoly on the surface of the SiO<sub>2</sub> slides has been efficiently achieved with plasma oxygen treatment of the silicon slides. Typically, monatomic (single atom) oxygen plasma is created by exposing oxygen gas at a low pressure (O<sub>2</sub>) to high-power radio waves, which ionise the gas. This process is realized under vacuum in order to create a plasma with generation of free radicals which interact with the silicon surface exposed to the treatment. This technique is commonly used to activate neutral surfaces thanks to the efficacy of the oxygen radicals generated. Specifically, the plasma oxygen treatment of SiO<sub>2</sub> slides activates the superficial silanol groups, that react with the silanizing groups of Copoly (MAPS) and realize the covalent attachment of Copoly onto the SiO<sub>2</sub> slides. In Figure 1 a schematic representation of the Copoly (DMA-NAS-MAPS) on the silicon oxide surface is represented.



**Figure 1.** Schematic representation of Copoly(DMA-NAS-MAPS) coating on silicon oxide surface.

## 6.2 Characterization of the Copoly(DMA-NAS-MAPS)-coated silicon oxide surface

A few analyses were carried out to characterize the Copoly(DMA-NAS-MAPS) coating specifically, atomic force microscopy (AFM) and AFM scratch test.

### Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) or scanning force microscopy (SFM) is a very high-resolution type of scanning probe microscopy. AFM provides a 3D profile of the surface on a nanoscale, by measuring forces between a sharp probe (<10 nm) and surface at very short distance (0.2-10 nm probe-sample separation). The probe is supported on a flexible cantilever. The AFM tip “gently” touches the surface and records the small force between the probe and the surface. The probe is placed on the end of a cantilever (which one can think of as a spring). The amount of force between the probe and surface is dependent on the spring constant (stiffness of the cantilever and the distance between the probe and the sample surface). This force can be described using Hooke’s Law:

$$F = -k \cdot x$$

F = Force

k = spring constant

x = cantilever deflection

If the spring constant of cantilever (typically ~ 0.1-1 N/m) is less than surface, the cantilever bends and the deflection is monitored.

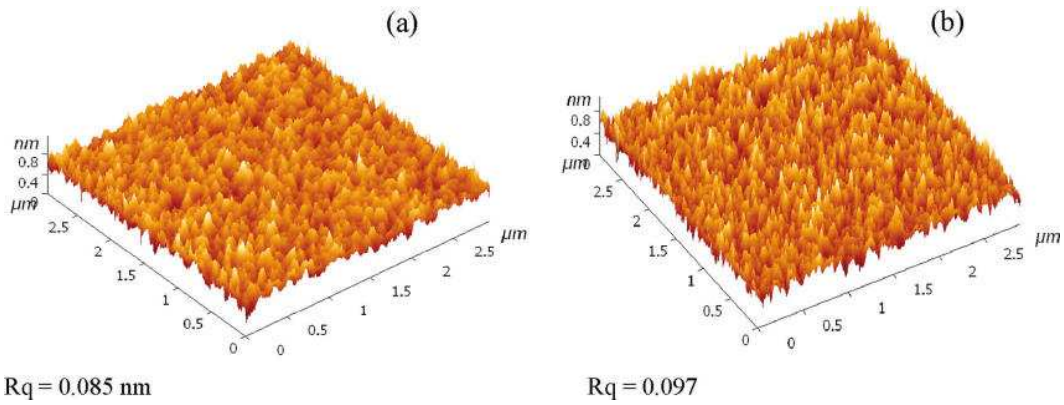
This typically results in forces ranging from nN (10<sup>-9</sup>) to μN (10<sup>-6</sup>) in the open air.

Probes are typically made from Si<sub>3</sub>N<sub>4</sub> or Si.

Different cantilever lengths, materials, and shapes allow for varied spring constants and resonant frequencies. Probes may be coated with other materials for applications such as chemical force microscopy (CFM) and magnetic force microscopy (MFM).

In the following picture (Figure 2) are reported the results of the AFM analysis of Silicon Oxide surface and Silicon Oxide Copoly coated surface.



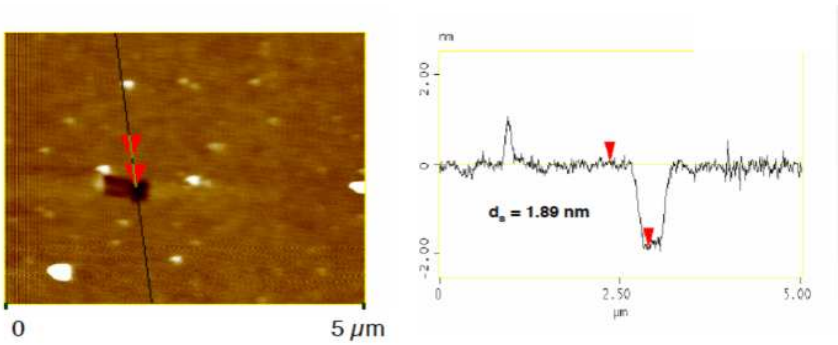


**Figure 2.** AFM image of SiO<sub>2</sub> surface (a) and SiO<sub>2</sub> copoly coated surface (b). The undulation is expressed in root mean square (rms) and correspond to a value of 0.085 and 0,097 respectively.

**Scratch Test**

The technique provides the measure of the thickness of the polymeric coating after a scratch on the surface made by the cantilever’s top. The depth of the channel so created represent the thickness of the polymer coating. In Figure 3 a picture resulting from a AFM scratch test of the SiO<sub>2</sub> Copoly coated surface is presented. The morphology of the polymer coating reflects the SiO<sub>2</sub> surface trend: the film is a very thin layer with undulation 0,2 nm height and 20-50 nm width. The undulation of the coating surface is expressed in root mean square(rms) and appears very similar to the SiO<sub>2</sub> surface undulation.

**Thickness of polymeric coating by AFM scratch test**



**Figure 3.** AFM scratch test of the SiO<sub>2</sub> Copoly coated surface.

### **6.3 Copoly(DMA-NAS-MAPS) coated on silicon surfaces and fluorescence enhancement**

One of the problems that still hinder the use of microarrays for analytical purposes, is the lack of the sensitivity of the adopted technique. Optical fluorescence detection of microarray spots is an established laboratory technique, but the demand for higher sensitivity has stimulated the research of new labeling strategies,<sup>4-10</sup> new substrates with increased loading capacity,<sup>11</sup> as well as new approaches to maximize target concentration on the surface.<sup>12</sup>

A simple way to achieve fluorescence enhancement may rely on optical interference (OI) coating technology using substrates with single or multiple films of well defined thickness that maximize photo-absorption of the dye molecules in the vicinity of the surface and reflect the emitted light, which would normally be lost through the substrate, toward the detector. This strategy does not require changing the experimental parameters provided that a conventional scanner can be used for the detection.

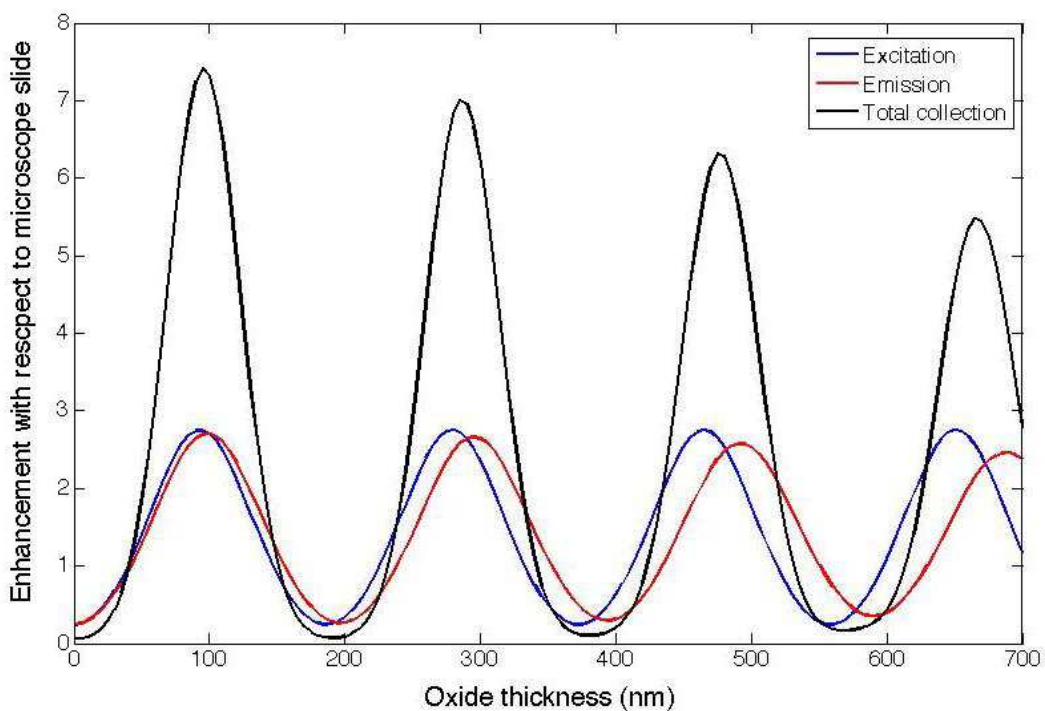
Recently, the enhancement of fluorescence on reflecting substrates has drawn considerable attention, especially for microarray applications.<sup>13,14</sup> Layered structures with carefully adjusted thicknesses have been designed and fabricated for increased fluorescence signal. Even though these substrates deliver improved performance over commonly used microscope slides, the complexity of their design and fabrication has been a drawback. Using a much simpler substrate consisting of a silicon reflector with a thin film of thermally grown oxide, similar enhancement levels to those of sophisticated multilayered structures can be achieved.<sup>15,16</sup>

The thermal growth of silicon dioxide is a well-established process, and controlled layer thickness with high reproducibility can be easily delivered. The pure oxide allows for adapting standard surface chemistries used on glass microscope slides for DNA or protein microarray applications. In general, organosilanization reactions are the chosen technique of surface derivatization. The silanization protocols however have poor reproducibility, suffer from high backgrounds, and do not provide high probe bioactivity.

Using a surface chemistry formed by the adsorption of Copoly(DMA-NAS-MAPS) on silicon oxide, a layer on the order of a few nano-meters is formed that does not affect the fluorescent enhancements predicted for the layered substrate. An additional benefit is the reduction of nonspecific background binding, which is critical in the practical application using substrates that enhance the fluorescence. Since the background fluorescence is also enhanced, a high background decreases the sensitivity.

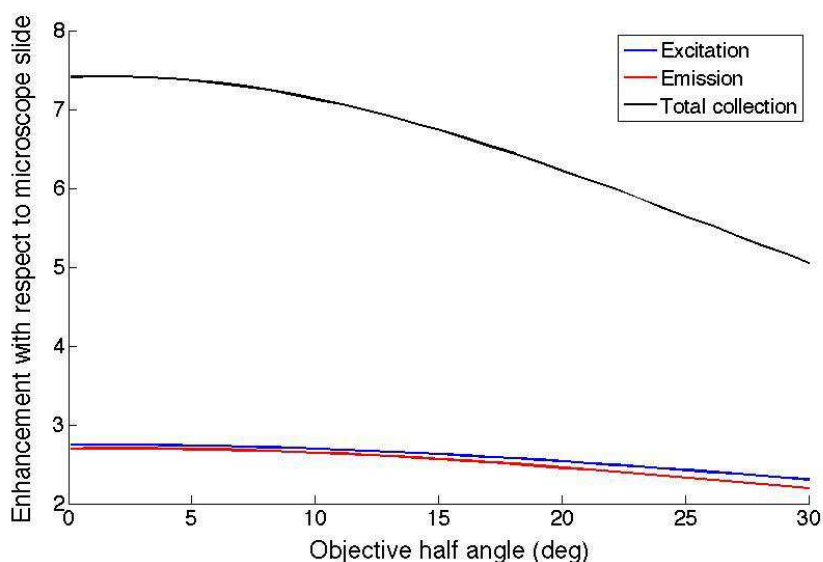
To demonstrate the enhancement of the excitation of and emission from the fluorescent molecules, electromagnetic models previously established<sup>17,18</sup> have been used. The normal incidence of a monochromatic excitation at 543 nm, and a collection in the 550-600 nm spectral range has been considered. Fluorescent molecules are considered to be located axially at the SiO<sub>2</sub>/Si surface, and the enhancement values are reported with respect to the detection on a glass microscope slide.

In Figure 4, the enhancements for the excitation and emission, as well as for the total collection are shown for a uniform distribution of dipole orientations. As the oxide thickness is varied, the excitation and emission intensities go through maxima and minima. The nodes of the standing wave for excitation are separated by  $\lambda_{ex}/2n_{oxide}$ , which approximately corresponds to an oxide thickness of 190nm. A 2.7 fold increase in both the excitation and emission intensities is apparent, which yields a maximum total enhancement of 7.5 fold.



**Figure 4.** Fluorescence enhancement on reflecting substrates at normal incidence of excitation and collection. The simulations for a) excitation, b) emission, and c) total collected intensity enhancement via utilization of the layered reflecting substrate for varying thickness of the top transparent oxide layer are shown. Monochromatic excitation at 543 nm, and collection in the 550-600 nm range are assumed.

For an oxide thickness of  $d_{ox}=95\text{nm}$ , which corresponds to the point of maximum enhancement, the numerical aperture of the objective is varied. The simulations in Figure 5 illustrate that the enhancement can be preserved over a range of objective cone angles that covers those used in commercial fluorescence scanning instruments.



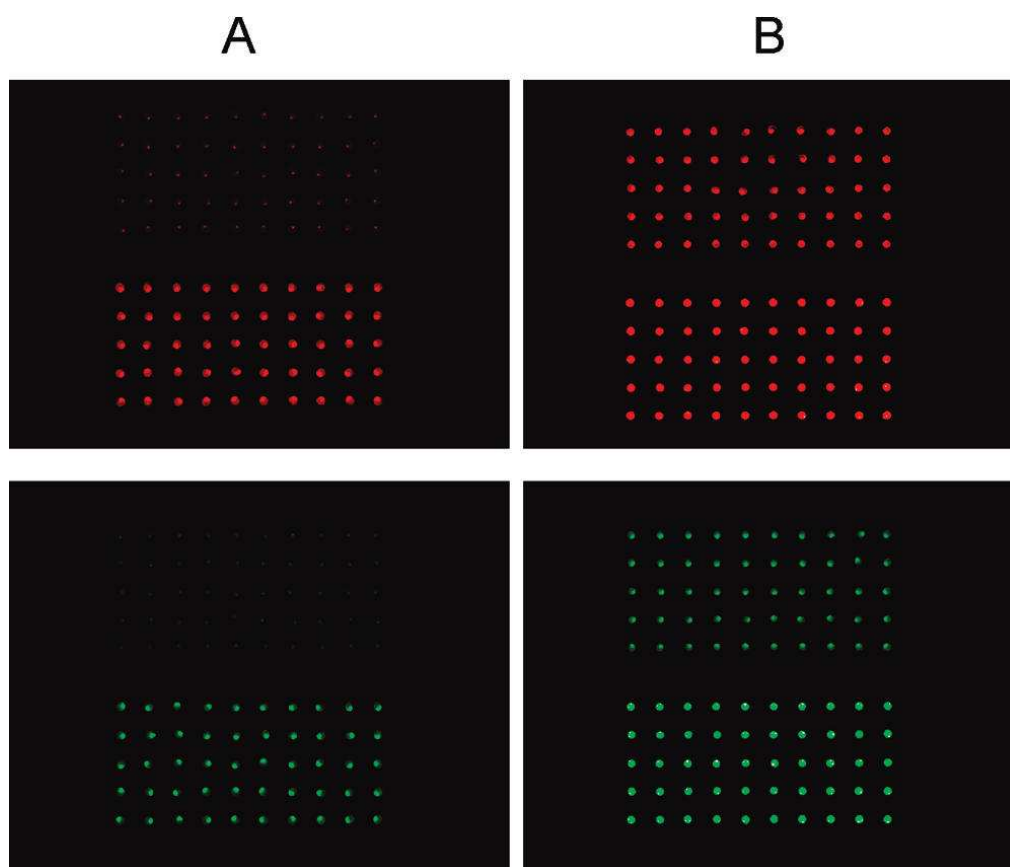
**Figure 5.** Fluorescence enhancement on reflecting substrates at varying angles of excitation and collection. The enhancement values shown correspond to excitation and collection through the full cone of an objective of indicated half angle. Monochromatic excitation at 543nm, collection in the 550-600nm range, and an oxide thickness of 95nm are assumed.

The fluorescence intensity and the signal-to-noise ratio of spots of Cy3 (excitation peak at 543 nm) labeled oligonucleotides (23mers) on a glass microscope slide and a  $\text{SiO}_2/\text{Si}$  substrate of 85 nm oxide were compared. Fluorescence measurements were carried out by scanning the surface immediately after the spotting of the oligonucleotides at 0.25 and 2.5  $\mu\text{M}$  concentrations.

Subarrays of 50 spots of Cy5 (upper part) and Cy3 (bottom part) labeled oligonucleotides were spotted at two concentrations (0.25 and 2.5  $\mu\text{M}$ ). The slides were scanned immediately after the spotting [laser power 22%, photomultiplier tube (PMT)]

As shown in Figure 6, the fluorescence was revealed only at 2.5  $\mu\text{M}$  in the glass slides and the fluorescence was more intense on the  $\text{SiO}_2/\text{Si}$  substrates (4 fold enhancement) with a signal to noise

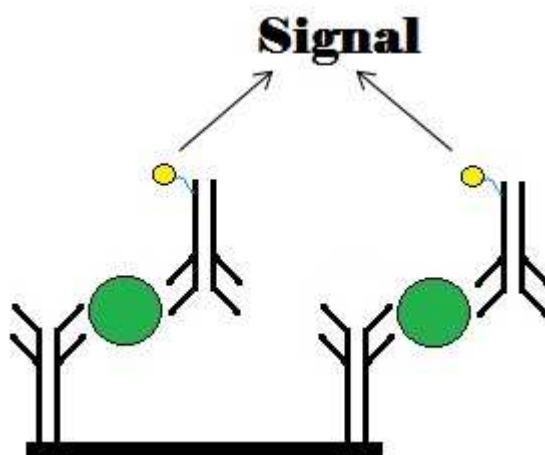
improvement of 7 fold. Furthermore, the low concentrated oligonucleotides were only detected on the SiO<sub>2</sub>/Si substrates.



**Figure 6.** Comparison of spot fluorescence intensity on glass (A) and silicon (B) slides. Subarrays of 50 spots of Cy5 (upper part) and Cy3 (bottom part) labeled oligonucleotides were spotted at two concentrations (0.25 and 2.5  $\mu\text{M}$ ).

## 6.4 Copoly (DMA-NAS-MAPS) coated silicon slides immunoassay of model antibodies.

The silicon oxide chips coated with Copoly(DMA-NAS-MAPS) have been tested with a protein/antibody couple by the sandwich approach previously described in Chapter 4 to verify the binding capacity of Copoly (DMA-NAS-MAPS) coated silicon slides.



**Figure 7.** Scheme of a sandwich assay

The "sandwich" assay is based on antibodies immobilized onto the chip that have to capture the protein of interest. A second labeled antibody, directed against a different epitope of the captured protein is used for the detection. This approach requires two different antibodies, each with affinity for different epitopes of the same protein of interest. Although the need of two antibodies often complicates the design of the array, the "sandwich" method is widely used as it increases the specificity of the antibodies array.

Commercial antibody couples usually available for ELISA or western blot techniques have been tested on copoly-coated SiO<sub>2</sub> slides to verify the specificity of the couples and the possible application in microarray technique.

In particular we have tested the following protein/antibodies couple:

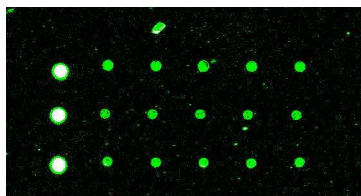
GFAP recombinant protein (Abnova), mouse mAb clone 273807 from R&D systems, mouse mAb clone SB61b-CY3 from Novus Biologicals

Glial fibrillary acidic protein (GFAP) is an established indicator of astrogliosis in neuropathology. As GFAP is expressed in different cell populations, variable cerebrospinal fluid (CSF) concentrations of these proteins might reflect disease-specific pathological profiles. In this respect the commercially available protein antibodies couple available is used in ELISA assays. The interest in developing a microarray assay for this protein comes from its potential role as biomarker in Alzheimer disease. Silicon/silicon oxide slides coated with Copoly(DMA-NAS-MAPS) have been chosen as microarray substrates owing to the high sensitivity provided by this material.

A solution of capture antibody in Phosphate Buffered Saline (PBS) (1mg/ml) was printed on coated slides to form a 15X15 array using a piezoelectric robotic system (spotter) at 20 °C in an atmosphere of 50% humidity. After spotting, all residual reactive groups of the coating polymer were blocked by dipping the printed slides in 50 mM ethanolamine/0.1 M Tris pH 9.0 at 50 °C for 15 min. After discarding the blocking solution, the slides were rinsed two times with water. The proteins (50 ng/mL) were coupled to the capture antibody at room temperature.

The detecting antibody (Cy3-labeled), dissolved in the hybridization buffer at a concentration of 0.04 mg/ml, was added and after a 1h incubation the slides were washed with PBS and water, then dried and scanned for fluorescence evaluation (in Material and Methods section a detailed description of the experiment is reported).

GFAP capture antibody is mouse mAb clone 273807 from R&D systems and the detecting antibody, labeled with Cy3, is mouse mAb clone SB61b-CY3 from Novus Biologicals. In Figure 8 the result of the sandwich assay of GFAP Copoly (DMA-NAS-MAPS) coated silicon slides is reported.



**Figure 8.** Fluorescence intensity of sandwich assay of GFAP on Copoly (DMA-NAS-MAPS) coated silicon slide.

The fluorescence of the corresponding labeled antibody (specifically mouse mAb clone SB61b-CY3 for GFAP in each spot of the array is the result of the binding capacity of the chip. The Copoly (DMA-NAS-MAPS) layer is able to bind the capture antibody that then binds the protein (GFAP). The bound protein is revealed by the incubation with the detection secondary fluorescent antibody.



## **6.5 Copoly (DMA-NAS-MAPS) coated silicon slides *versus* Quantum Dots enhanced fluorescence in a immunoassay of apolipoprotein E (ApoE).**

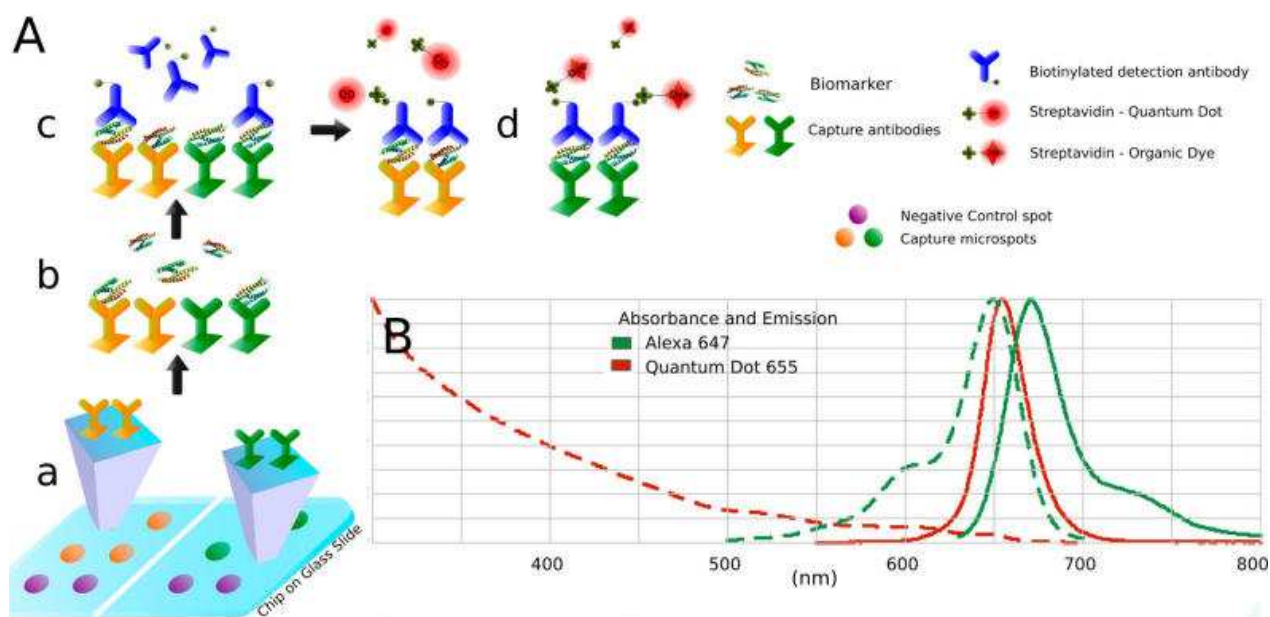
The chip constituted by silicon slides coated with Copoly (DMA-NAS-MAPS) has been used for the detection of apo-lipoprotein E (ApoE), a 34 kD protein implicated in lipid transport and lipoprotein metabolism, whose levels are ultimately controlled by the gene ApoE.

ApoE exists in three isoforms: ApoE2, ApoE3, and ApoE4 and is present in plasma lipoprotein particles such as chylomicrometers, very-lowdensity lipoprotein, and high-density lipoprotein. It is also found in large amounts at sites of neurological damage and appears to be involved in recycling of apoptotic remnants and of amyloidal aggregates. Diverse pieces of evidence suggest that abnormal ApoE levels may be implicated in dementia and central nervous system diseases.<sup>19,20</sup>

In a recent paper,<sup>21</sup> a group participating in the NaDiNe project has published a new assay to detect apolipoprotein E (ApoE) that relies on Quantum Dots (QDs) as highly effective reporters in microarrays.<sup>22-24</sup>

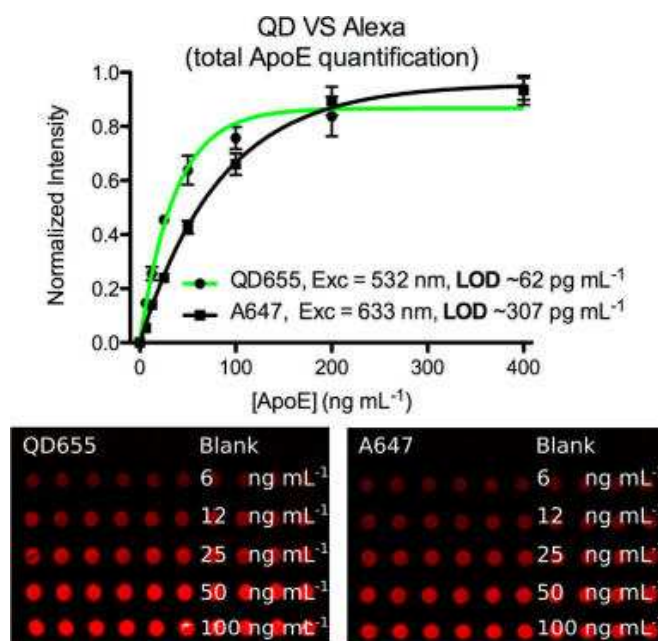
QDs are semiconductor nanocrystals that offer advantageous fluorescent properties that can be exploited for optical biosensing:<sup>25</sup> size tunable emission, narrow and symmetric photoluminescence, broad and strong excitation spectra, strong luminescence, and robust photostability. QDs exhibit better photonic performance in solid phase (i.e., directly exposed to air) than in liquid phase (e.g., as an aqueous dispersion).<sup>26</sup> The interaction of QDs in immunoassays has been widely studied by Zhu et al.<sup>27</sup>

In the cited work,<sup>21</sup> the performance of CdSe@ZnS quantum dots (QD655; maximum emission peak: ca. 655 nm) has been explored as reporter and compared with the fluorescent dye Alexa 647 (maximum emission peak: ca. 668 nm) in a sandwich immunoassay microarray designed to detect ApoE. The immunoassay in its two forms (QDs or Alexa 647) and the absorbance/emission spectra of the studied fluorophores are illustrated in Figure 9.



**Figure 9.** (A) Schematic of apolipoprotein-E (ApoE)-screening sandwich immunocomplex microarrays using either quantum dots (QDs) or the organic dye Alexa 647 as reporter. (a) Capture antibodies are spotted onto glass slides and free sites are blocked. (b) Samples containing the analyte are incubated. (c) Biotinylated detection antibodies are incubated. (d) Bound detection antibodies are reported by a streptavidin–fluorophore complex (streptavidin–QD or streptavidin–Alexa 647). (B) Absorbance (dashed line) and emission (solid line) spectra of the studied fluorophores; in red, QD 655; in green, Alexa 647.

The two versions of the microarray were compared for performance and then compared to an ApoE assay based on a conventional enzyme-linked immunosorbent assay (ELISA). The two versions of the microarray (QD or Alexa 647) were assessed under the same experimental conditions and then compared to a conventional enzyme-linked immunosorbent assay (ELISA) targeting ApoE. The QDs proved to be highly effective reporters in the microarrays, although their performance strongly varied in function of the excitation wavelength. At 633 nm, the QD microarray gave a limit of detection (LOD) of  $\sim 247 \text{ pg mL}^{-1}$ ; however, at an excitation wavelength of 532 nm, it provided a LOD of  $\sim 62 \text{ pg mL}^{-1}$ , five times more sensitive than that of the Alexa microarray ( $\sim 307 \text{ pg mL}^{-1}$ ) and seven times more than that of the ELISA ( $\sim 470 \text{ pg mL}^{-1}$ ). In Figure 10, results of the comparison of ApoE screening using the two microarrays (QD or Alexa 647 as reporter) under the same experimental conditions are reported.



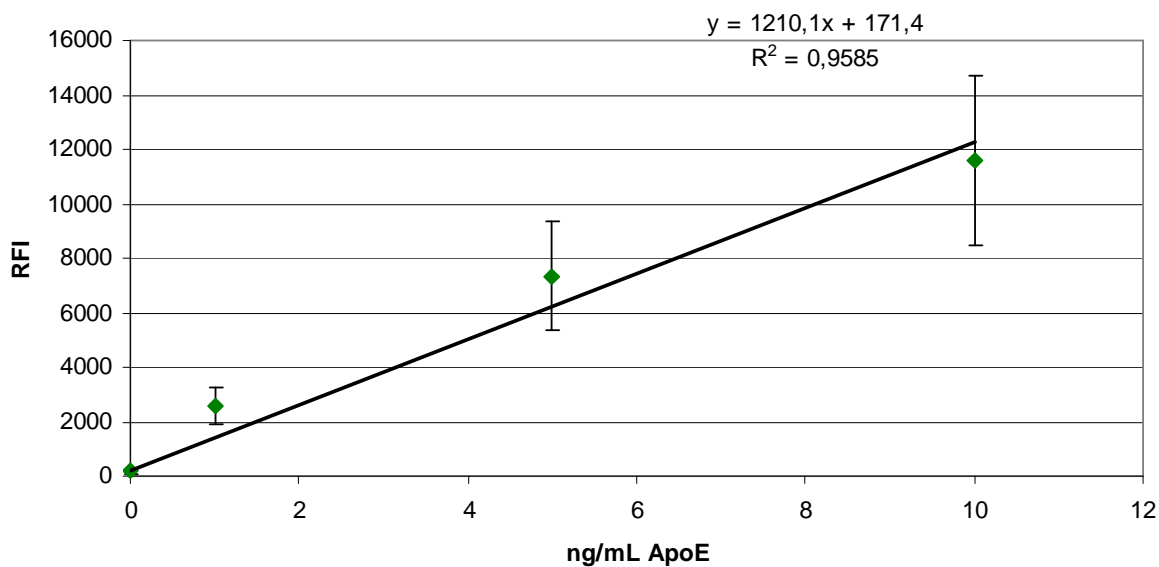
**Figure 10.** Comparison of ApoE screening using the two microarrays (QD or Alexa 647 as reporter) under the same experimental conditions. ApoE2, ApoE3, and ApoE4 were mixed (in equal proportions) so as to quantify total ApoE at different concentrations. The error bars were obtained from parallel assays of 10 spots. QD655, quantum dot 655; A647, Alexa 647; Exc, excitation wavelength; LOD, limit of detection.

Finally, serial dilutions from a human serum sample were assayed with high sensitivity and acceptable precision and accuracy.

Despite their advantages, QDs can exhibit some shortcomings, such as their heavy metal components (in the above case cadmium) are highly toxic and present intermittence in emission or “blinking”, the causes and mechanism of which are as yet not completely understood.<sup>28-31</sup> Furthermore, QDs are much larger than organic fluorophores in size and this must be carefully considered while designing a novel biosensing system, since biomolecules such as antibodies, enzymes, or aptamers are dependent on their structure to function and perturbation of the structure or behavior of biomolecules can constitute a problem.<sup>25</sup>

As a part of this Ph.D. thesis, ApoE has been assayed by static and dynamic incubations of silicon slides coated with Copoly (DMA-NAS-MAPS) spotted with mAb-capture for ApoE (antibody clone E276, from Mabtech kit for ApoE detection) with solutions containing increasing amounts of ApoE ranging from 0 to 100 ng/ml. After 2 hours of incubation at room temperature in static and dynamic conditions the surface was incubated with biotinylated revealing antibody (clone E887 from Mabtech kit for ApoE detection). Captured ApoE was then detected upon incubation with Cy3





**Figure 12.** Calibration curve (dose–response curve) produced by fitting the data of spot fluorescence intensity versus ApoE concentrations.

From the calibration curve of Figure 12 a limit of detection of 331 pg/ml was extrapolated. This value is significantly higher than that found in static conditions suggesting the importance of overcoming mass transport limitations in microarray experiments. It's important to note that the LOD obtained with Cy3 labeled streptavidin is similar to that provided by QDs proving that the use of silicon/SiO<sub>2</sub> substrates allow a significant sensitivity increase using conventional dyes.

# Material and Methods

## Chemicals

TRIS, ethanolamine, ammonium sulfate, Saline Sodium Citrate (SSC), Sodium Dodecyl Sulfate (SDS), Phosphate Buffered Saline (PBS) tablets, *N,N*-dimethylacrylamide (DMA) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) were purchased from Sigma (St. Louis, MO). *N,N*-acryloyloxysuccinimide (NAS) was from Polysciences (Warrington, PA).

GFAP Recombinant protein (Abnova), mouse mAb clone 273807 from R&D systems, mouse mAb clone SB61b-CY3 from Novus Biologicals. S-100 beta chain recombinant protein from GenWay, goat pAb and rabbit pAb from Santa Cruz Biotechnology. mAb-capture for ApoE (antibody clone E276, from Mabtech kit for ApoE detection; biotinylated revealing antibody (clone E887 from Mabtech kit for ApoE detection)

## Silicon Oxide surfaces

The silicon oxide chips with a dimension of 1.5x1.5 cm obtained by thermal grown have been purchased from SVM, Santa Clara, USA

## Surface treatment

The SiO<sub>2</sub> surface was treated for ten minutes within an Oxygen Plasma Generator (Harrick Plasma Cleaner), then immersed for 30 min in a Copoly(DMA-NAS-MAPS) solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level). The slides were then washed extensively with water and dried under vacuum at 80 °C for 15 minutes.

## Coating of silicon chips with Copoly(DMA-NAS-MAPS)

Copoly(DMA-NAS-MAPS) was synthesized as described in Chapter 5. Silicon substrates with 85 nm oxide were pretreated with 0.1 M NaOH for 15 min, washed with water, and dried. Microscope glass slides were pretreated with 1 M NaOH for 30 min and 1 M HCl for 1 h, washed with water, and dried. After the pretreatment, the substrates were immersed for 30 min in a copoly(DMA-NAS-

MAPS) solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level), rinsed with water and dried under vacuum at 80 °C.

### **GFAP protein functional test**

A 1mg/ml solution of mouse mAb clone 273807 from R&D systems, in PBS was printed on coated slides to form a 15 X 15 array using a piezoelectric spotter (SciFLEXARRAYER S5; Scienion). Spotting was done at 20 °C in an atmosphere of 50% humidity. After spotting, all residual reactive groups of the coating polymer were blocked by dipping the printed slides in 50 mM ethanolamine/0.1 M Tris pH 9.0 at 50 °C for 15 min. After discarding the blocking solution, the slides were rinsed two times with water. The slides were incubated for 2 h with GFAP protein 0.05 mgr/ml in the hybridization buffer (Tris-HCl, 0.1 M, pH 8; 0.1 M NaCl; 1% w/v BSA; 0.02% w/v Tween 20); washed with Tris-HCl (0.05 M, pH 9), 0.25 M NaCl, and 0.05% Tween 20; and rinsed with water before a 1-h incubation with the detecting antibody Cy3-labeled, mouse mAb clone SB61b-CY3 from Novus Biologicals, dissolved in the hybridization buffer at a concentration of 0.04 mg/ml. After extensive washing with PBS and water, the slides were dried and scanned for fluorescence evaluation. Scanning for fluorescence evaluation was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA); silicon oxide was analyzed using 90% of photomultiplier (PMT) gain and laser power.

### **ApoE protein preparation**

5 ug of ApoE protein have been dissolved in 1 ml of PBS + 0.5 mM DTT + 0.1% BSA, in static conditions for 20 min. Successive dilutions have been done in hybridization buffer (Tris-HCl, 0.1 M, pH 8; 0.1 M NaCl; 1% w/v BSA; 0.02% w/v Tween 20) + 1% BSA.

### **ApoE protein functional test**

A 0.5 mg/ml solution of mAb-capture to ApoE (E276) was printed on Copoly (DMA-NAS-MAPS) coated slides to form a 9X10 array using a piezoelectric spotter (SciFLEXARRAYER S5; Scienion). After spotting, all residual reactive groups of the coating polymer were blocked by dipping the printed slides in 50 mM ethanolamine/0.1 M Tris pH 9.0 at 50 °C for 15 min. After discarding the blocking solution, the slides were rinsed two times with water.

The slides were incubated for 2 h with ApoE protein in the hybridization buffer in dynamic conditions at the following concentrations:

- chip1 ApoE 0 ng/mL
- chip2 ApoE 1 ng/mL
- chip3 ApoE 5 ng/mL
- chip4 ApoE 10 ng/mL
- chip5 ApoE 100 ng/mL

Washed with Tris-HCl (0.05 M, pH 9), 0.25 M NaCl, and 0.05% Tween 20; and rinsed with water before a 1-h static incubation with mAb-biotin (E887) diluted 1:500 in hybridization buffer + 1% BSA to a final concentration of 1 ug/mL.

Final 1h incubation with Streptavidin-Cy3 diluted 1:500 in PBS.

After extensive washing with PBS and water, the slides were dried and scanned for fluorescence evaluation. Scanning for fluorescence evaluation was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA); silicon oxide was analyzed using 80% and 90%, of photomultiplier (PMT) gain and laser power.



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## **Chapter 7**

# **Copoly (DMA-NAS-MAPS) coating of gold for bio-sensing analysis**

The great development of microarray analysis technique that has taken place in the last ten years for the study of proteomics and genomics<sup>1</sup> involves the chemical functionalization of different substrates (glass, silicon, gold) for an effective and selective immobilization of bio-macromolecules like DNA, oligonucleotides and proteins.

In particular, there is strong interest in developing methods of functionalization of gold surface, as this metal is widely used in optical biosensors based on the phenomenon of surface plasmon resonance (SPR).

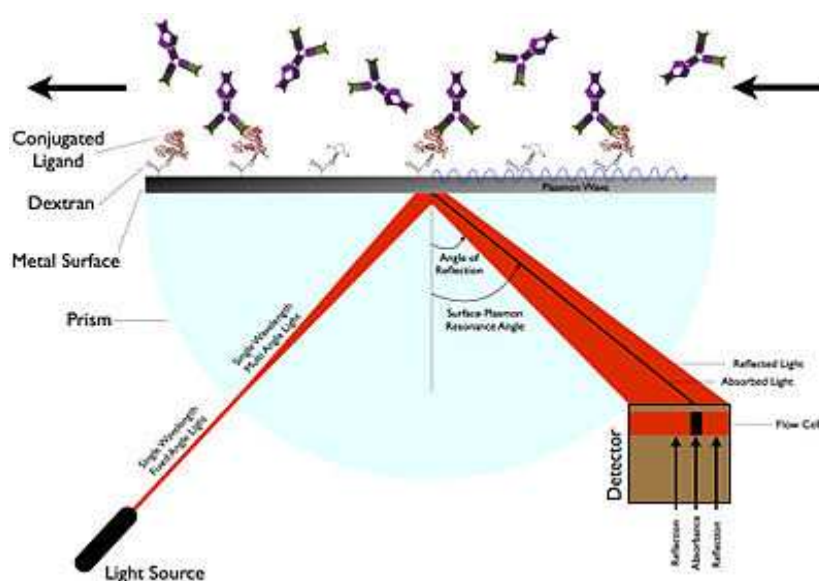
In the final part of this PhD thesis, we have realized a gold surface with a polymeric thin coating based on Copoly(DMA-NAS-MAPS). The polymeric coating of gold has been studied by means of atomic force microscopy (AFM) and X-Ray Photoemission Spectroscopy (XPS).

Finally, to prove the effectiveness of the polymer in immobilizing biomolecules, two different experiments have been carried out: a DNA hybridization experiment with chemiluminescence and a Surface Plasmon Resonance (SPR) experiment of fibroblast growth factor (FGF2)-heparin binding.

### **7.1 Principles and applications of Surface Plasmon Resonance (SPR).**

The underlying physical principles of SPR are complex. Fortunately, an adequate working knowledge of the technique does not require a detailed theoretical understanding. SPR-based instruments use an optical method to measure the refractive index near (within ~300 nm) a sensor surface that forms the floor of a small flow cell (20-60 nL in volume), through which an aqueous solution (henceforth called the running buffer) passes under continuous flow (1-100  $\mu\text{L}\cdot\text{min}^{-1}$ ). In order to detect an interaction, one molecule (the ligand) is immobilized onto the sensor surface. Its binding partner (the analyte) is injected in aqueous solution (sample buffer) through the flow cell, also under continuous flow. As the analyte binds to the ligand the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RUs) versus time (a sensor-gram).

Importantly, a response (background response) will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensor-gram to obtain the actual binding response. The background response is recorded by injecting the analyte through a control or reference flow cell, which has no ligand or an irrelevant ligand immobilized to the sensor surface. One RU represents the binding of approximately 1 pg protein/mm<sup>2</sup>. In practice, >50 pg/mm<sup>2</sup> of analyte binding is needed. Because it is very difficult to immobilise a sufficiently high density of ligand onto a surface to achieve this level of analyte binding, sensor surfaces with a 100-200 nm thick carboxymethylated dextran matrix attached can be adopted. In Figure 1 a schematic representation of the SPR technique is shown.



**Figure 1.** Schematic representation of the SPR technique

By effectively adding a third dimension to the surface, much higher levels of ligand immobilisation are possible. However, having very high levels of ligand has two important drawbacks. Firstly, with such a high ligand density the rate at which the surface binds the analyte may exceed the rate at which the analyte can be delivered to the surface (the latter is referred to as mass transport). In this situation, mass transport becomes the rate-limiting step. Consequently, the measured association rate constant ( $k_{on}$ ) is slower than the true  $k_{on}$ . A second, related problem is that, following dissociation of the analyte, it can rebind to the unoccupied ligand before diffusing out of the matrix and being washed from the flow cell. Consequently, the measured dissociation rate constant (apparent  $k_{off}$ ) is slower than the true  $k_{off}$ . Although the dextran matrix may exaggerate these kinetic artifacts (mass transport limitations and re-binding) they can affect all surface-binding techniques.

In summary, SPR exploits the ability of a thin layer of gold on a high refractive index glass surface to absorb laser light, producing electron waves (surface plasmons) on the gold surface. This occurs only at a specific angle and wavelength of incident light and is highly dependent on the surface of the gold, such that binding of a target analyte to a receptor on the gold surface produces a measurable signal.<sup>2</sup>

## 7.2 Gold surface coating

The most common approach to gold functionalization with a biomolecule involves formation of monolayers on the surface by spontaneous assembling of chemically reactive alkene thiols leading to formation of so called self-assembled monolayers (SAM).<sup>3</sup> By placing a gold substrate into a millimolar solution of an alkanethiol in ethanol a variety of chemically reactive groups can be introduced by a reaction between gold and thiols. This reaction is well-known and widely adopted due to the fact that it is straightforward and requires commercially available chemical reagents. In addition, the binding between gold and thiol is very stable owing to its covalent character.<sup>4,5</sup>

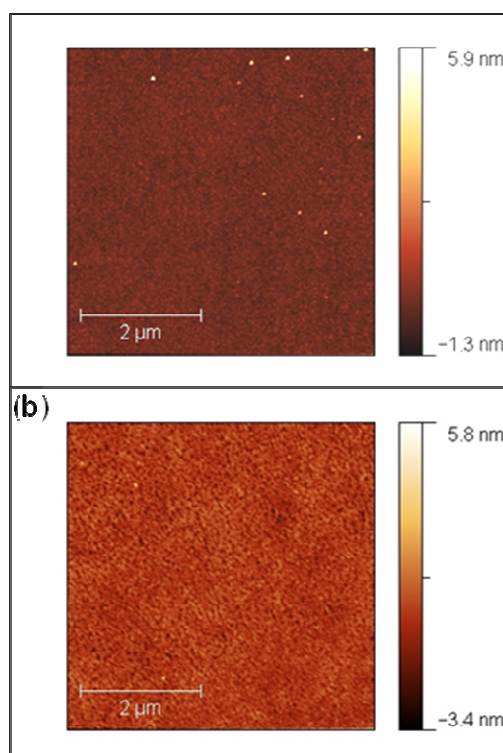
However, the formation of a well-assembled monolayer can depend on the purity of the alkanethiol being used. The presence of even low levels of contaminants can result in a disordered, non-ideal monolayer. Many typical impurities in thiol compounds are thiolated precursor molecules that have been not separated during the purification process.<sup>6</sup>

Tree-dimensional polymeric coating bearing functional domains for the immobilization of biological molecules are widely used in gold surface functionalization as they provide a surface that immobilizes proteins in their native conformation while resisting non-specific adsorption. For the functionalization of gold surfaces hetero-bifunctional poly(ethylene glycol) having a thiol group on one terminus and a reactive functional group on the other for use as a flexible spacer have been developed.<sup>7</sup> As previously stated, the coating of choice in SPR is based on dextran. A stable dextran layer is obtained by covalent attachment of the polymer to a functional sublayer formed on the surface by self assembling of a thiol bearing molecule. The characteristics of the dextran coated surface are optimal and the quality of this coating has greatly contributed to the success of SPR technology. However, the procedure for binding dextran is cumbersome and it requires multistep chemical reactions which may be difficult to control. An alternative approach to dextran surface modification could be constituted to polymer coating of the gold surface, that is robust and easy to perform even in laboratories that do not have a strong surface chemistry background.

### 7.3 Gold surface coating with Copoly(DMA-NAS-MAPS)

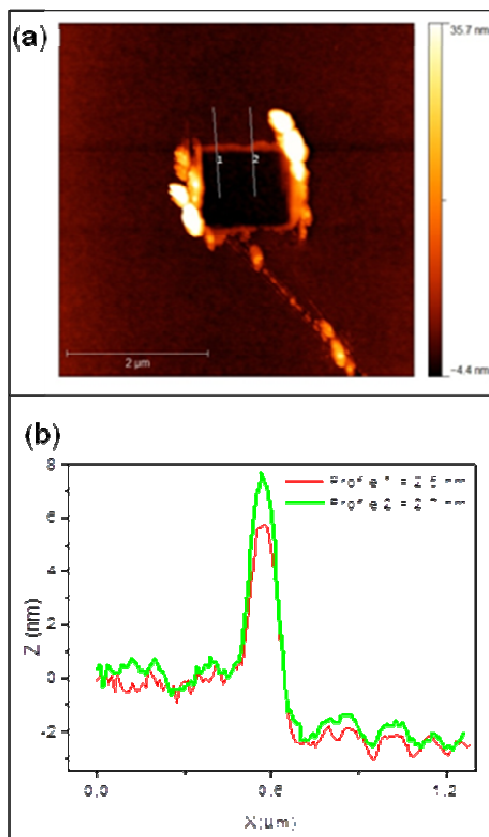
In the final part of this Ph.D. thesis, we have realized a gold surface with a polymeric thin coating based on Copoly(DMA-NAS-MAPS). Gold coated chips (SPR sensor chips from XanTec bioanalytics) were treated for ten minutes within an Oxygen Plasma Generator (Harrick Plasma Cleaner), then immersed in a Copoly(DMA-NAS-MAPS) solution, according to a well established coating procedure. Copoly(DMA-NAS-MAPS) forms a thin film on a gold surface, characterized by the polymer backbone that interact with the surface by weak, non covalent interactions such as hydrogen bonds, Van der Waals or hydrophobic forces. The pending silane hydrolysable monomers (MAPS) may stabilize the film chemically interacting with the gold surface whereas remaining monomers (NAS) are able to react with amines present in the protein later chains allowing the covalent binding of biomolecules to the surface.

The general appearance of the gold surface and polymer modified surface are presented in Figure 2 (panel a and b respectively). The roughness mean square (RMS) of the gold surface is about 0.36 nm on an area of  $5 \times 5 \mu\text{m}^2$ , while the polymer coated surface presents a RMS roughness of 0.60 nm suggesting a uniform polymeric coating following the gold morphology.



**Figure 2.** AFM image on a  $5 \times 5 \mu\text{m}^2$  area of the free gold (panel a) and polymer coated (panel b) surface. The roughness RMS is 0.5 and nm respectively

In Figure 3a a representative nano-scratch image of the layer and in Figure 3b some depth profiles (taken along the white lines) are shown. In the scratched area ( $1 \times 1 \mu\text{m}^2$ ) a roughness of 0.48 nm RMS is recovered. The AFM phase contrast (not shown) between the scratched area and the overlayer reveals also that in the two zone a different material with different stiffness is present, demonstrating that the scratch has removed only the polymeric coating. By evaluating the depth profiles, the layer thickness is about  $2.5 \pm 0.3 \text{ nm}$  (Figure 3), consistent with the thickness evaluated by scratch test taken on a  $\text{SiO}_2$  surface coated with the same polymer.<sup>8</sup>



**Figure 3.** Panel a:  $4.5 \times 4.5 \mu\text{m}^2$  AFM image of the sample after scratch; the scratched area is  $1 \mu\text{m}^2$ . Panel b: depth profiles from the lines of panel a. The estimated polymer thickness is  $2.5 \pm 0.3 \text{ nm}$

In the XPS spectra, four elements are presented: gold from the substrate and carbon, oxygen, nitrogen coming belonging to the polymer. In Table 1, the intensity ratios normalized to the cross section, transmission and escape depth between O1s and C1s, N1s and C1s, C1s and Au 4f are shown.

**Table 1:** Relative intensities of N1s, O1s and C1s peaks taken at 0°, 60° collection angle.

	N1s/C1s	O 1s/C1s	C1s/Au 4f
0°	0.17±0.02	0.17±0.02	1.47±0.01
60°	0.14±0.02	0.16±0.02	3.33±0.01

The surface was analysed at two collection angles: 0° and 60° between the analyzer entrance and the normal to the surface; in particular the latter configuration is more sensitive to the surface of the sample due to the finite escape depth of the emitted photoelectrons.

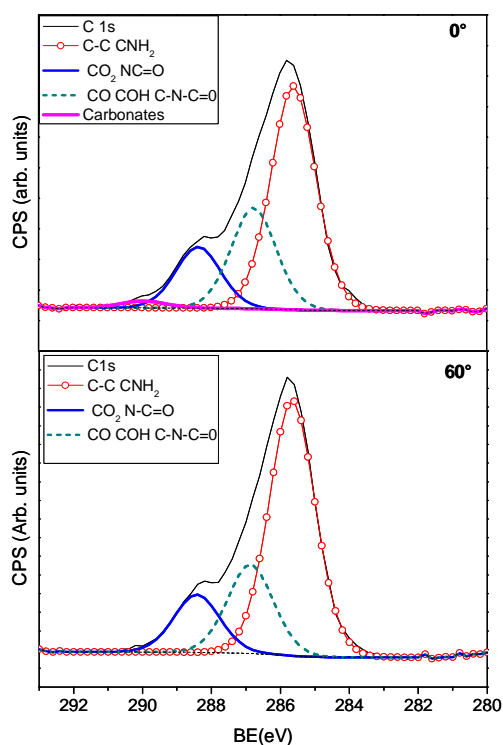
The nitrogen and oxygen content, evaluated from the intensity of the characteristic peak are the same: 17% of that of the carbon. At 60° for both the elements the signal seems to slightly decrease with respect to that from C1s (14% and 16% for N1s and O1s respectively); although this decrease is within the limit of the uncertainty of the measure, this trend is realistic, considering the presence of adventitious carbon at the surface or the intrinsic structure of the polymer

From C1s and Au 4f ratio at 0° and 60°, it is possible to calculate the thickness of the polymer; however, due to the uncertainty on the escape depth of C 1s and Au 4f photoelectrons in a polymeric matrix, the simple model of a continuous organic film on a bulky gold substrate seems to underestimate the thickness of the polymer. Also considering a partial wetting of the polymer on the surface (although AFM analysis reveal that Copoly(DMA-NAS-MAPS) completely covers the substrate) a value of  $1\pm 0.2$  nm is obtained. A more complex model considering the structure of the polymer and its influence on the attenuation on the photo-emitted electrons should be applied but this detailed investigation on the coating thickness is beyond the scope of this paper.

In figure 6 the fit of the C 1s peak shows the different contributions coming from the different coordination of the C atoms in the polymer. The fitting is performed with a Voigt lineshape, whose FWHM is consistent with that found in the C1s peak taken in the same condition from adventitious carbon arising from the exposure of other samples to residual gasses, even in UHV conditions.<sup>9</sup>

Panel a (0° collection angle) and panel b (60° collection angle) show common features; the main peak is located at 285.6 eV and corresponds to adventitious carbon (carbon in CNH<sub>2</sub> groups) and C-C bonds. The position of this peak gives moreover the energy calibration of all the spectra of the sample.

Two other contributions are present in both the panel of Figure 4: one at 286.7 eV is due to the presence of CO, COH CNC=O and the other at 288.4 eV comes from CO<sub>2</sub> and NC=O.



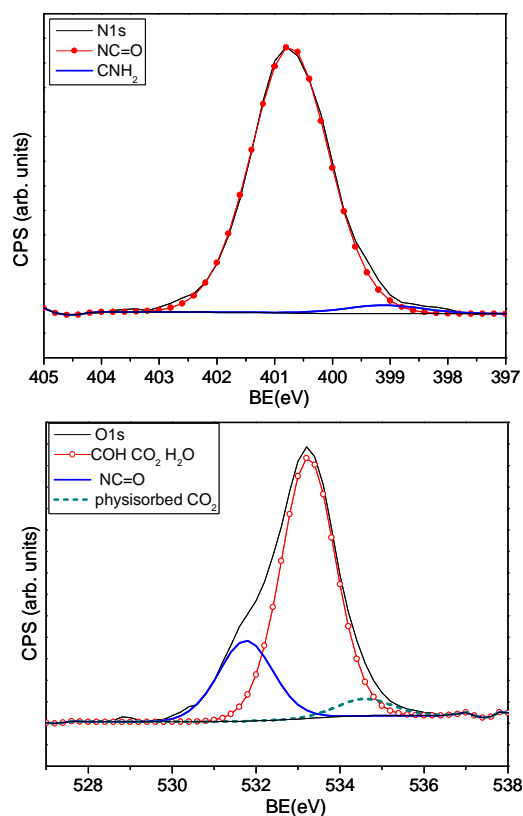
**Figure 4.** XPS spectrum of C1s taken at 0° (panel a) and 60° (panel b) collection angle; the deconvolution of the peaks reveals the presence of the specific group of the polymer: NC=O and CNC=O

This two last peaks are the fingerprint of the polymer coating of the gold surface (in particular the CNC=O and NC=O groups) and they account roughly for 22% and 15% of the whole C1s peak, consistently with the previous value found for O1s/C1s and N1s/C1s ratio.

At 0° collection angle another peak arising from carbon is present, but this signal can be attributed to some contaminants present on the bare gold surface.

Spectra at 60° collection angle of N1s and O 1s, are presented in Figure 5. Also in this case the peaks are decomposed by a sum of Voigt function with FWHM 1.62 eV and 1.53 eV respectively.





**Figure 5.** XPS spectrum of N1s and O1s at 60° collection angle; both the spectra show the feature coming from -NC=O groups

N1s presents two characteristic contributions: the main one at 400.7 eV, corresponding to N in NC=O compounds and the minor one at 399.1 eV corresponding to CNH<sub>2</sub>.<sup>10</sup>

This second contribution is only 5% of the entire peak and probably do not arise from the polymer but from contamination of the sample exposed to air; in the C1s peak this small feature contributes to the peak at 285.6 eV.

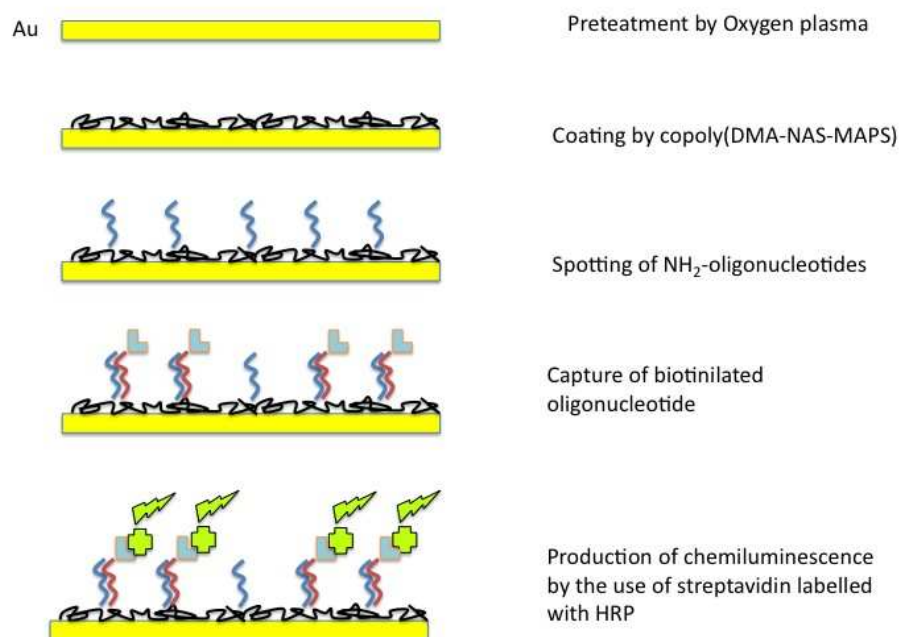
Regarding oxygen, three features give rise to the O1s peak: the main one at 531.7 eV is due to the NC=O compound, while the one at 533.3 eV is due to H<sub>2</sub>O, COH and CO<sub>2</sub>.<sup>10</sup> The minor at 534.9 eV might be due to physisorbed CO<sub>2</sub>.

All above features are consistent with those found for N1s and C1s peaks, but due to the superimposition of different species at the same energy in all the spectra, it is quite difficult to quantify the weight of a specific group.

However, both the AFM scratch test and the XPS analysis reveal that a polymer 2.5 nm thick is immobilized on the surface. In particular XPS reveals the presence of the specific N-C=O groups present in the Copoly(DMA-NAS-MAPS) and of the gold under-layer, suggesting that the coating procedure preserve both the substrate and the polymer coating.

## 7.4 DNA Hybridization experiments

A hybridization test was carried out to prove that the functional polymer bind amino modified oligonucleotides with great efficiency and low background noise. A simple DNA hybridization experiment was performed to prove that the coating has introduced chemical reactive groups on the gold surface. A scheme of a typical array experiment is shown in Figure 6.

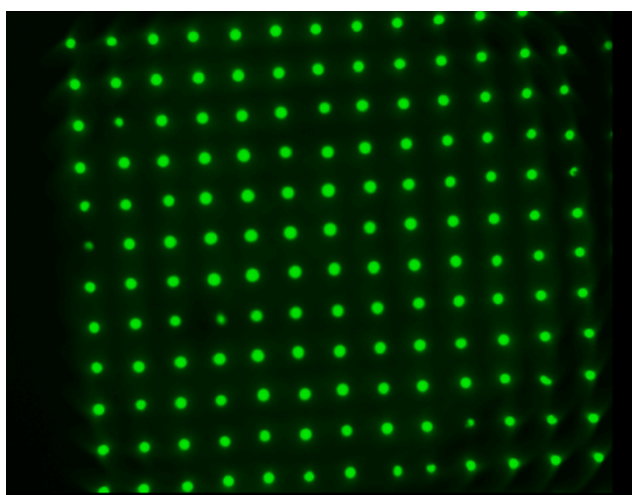


**Figure 6.** Scheme of a typical array experiment on gold surface

Replicates of an oligonucleotide ligand are spotted on the polymer coated gold using a piezoelectric spotter. After washing and blocking the surface unreacted sites, the array is probed with a sample containing a complementary target oligonucleotide labelled with biotin. A detection step with streptavidin labelled with horse radish peroxidase (HRP) is then performed. The hybridization

reaction was revealed by chemiluminescence using an enzyme, HRP, that catalyses the conversion of the enhanced chemiluminescent substrate into a sensitized reagent in the vicinity of the molecule of interest, which on further oxidation by hydrogen peroxide, produces a triplet (excited) carbonyl, which emits light when it decays to the singlet carbonyl. Enhanced chemiluminescence allows detection of minute quantities of a biomolecule. The strong signal to noise ratio of the oligonucleotide spots proves that the surface bind covalently amino-modified DNA molecules .

In Figure 7 the chemiluminescent HRP array experiment is shown.



**Figure 7.** Chemiluminescence signals of oligonucleotide spots after hybridization with complementary oligonucleotide labelled with biotin followed by incubation with streptavidin labelled with Horse Radish Peroxidas

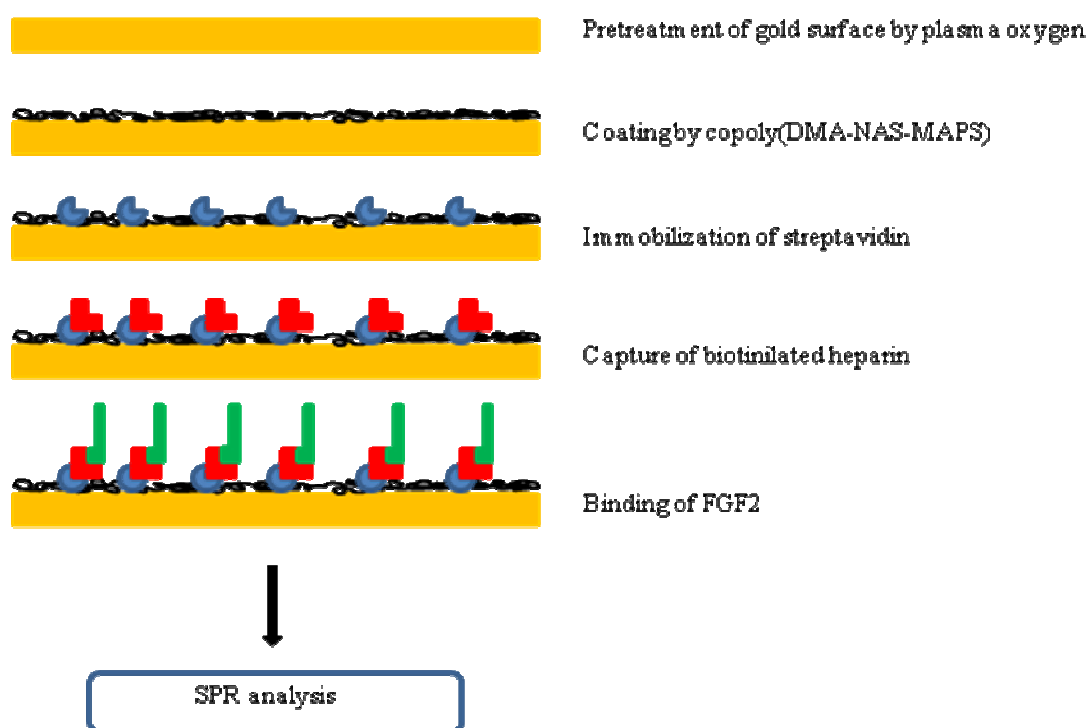
Chemiluminescence was chosen to prevent fluorescence quenching that would have occurred labelling the DNA with a fluorophore.<sup>12</sup>

In this approach, luminol ,one of the most widely used chemiluminescent reagents, is oxidized by peroxide and it results in creation of an excited state product called 3-aminophthalate. This product decays to a lower energy state by releasing photons of light. The distance from the surface is enough to prevent quenching.

## 7.5 SPR determination of Copoly(DMA-NAS-MAPS) functionalized gold sensorchip

Streptavidin was immobilized to a Copoly(DMA-NAS-MAPS)-functionalized gold sensorchip and used to capture biotinylated heparin. Then, heparin immobilized to the gold surface was evaluated for its capacity to bind FGF2, one of the most important angiogenic growth factors largely studied for its heparin-binding capacity.<sup>13,14</sup>

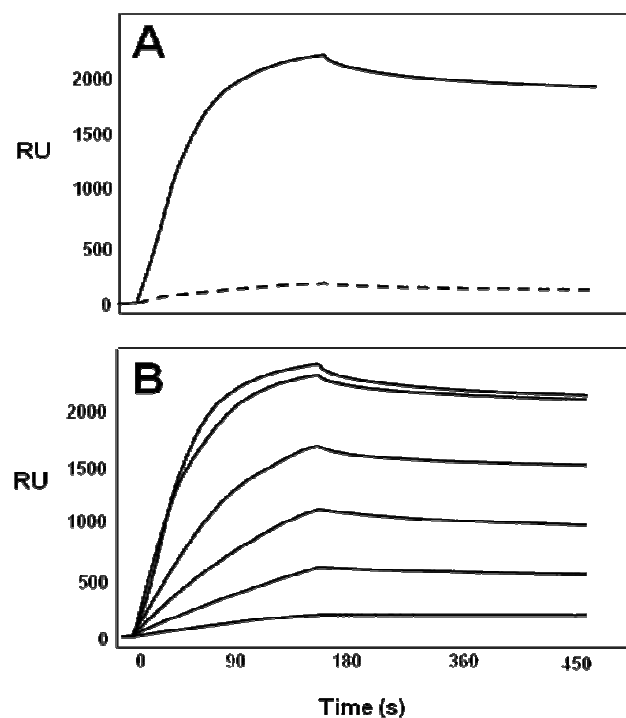
In Figure 8 a schematic representation of the projected experiment of the binding of FGF2 on gold surface is shown.



**Figure 8.** Scheme of the binding of FGF2 on gold surface

In parallel experiments, FGF2 binding assays were performed onto streptavidin and biotinylated-heparin immobilized to commercially available carboxymethyl dextran-functionalized gold layer<sup>15</sup> and the results obtained onto the two different gold surfaces compared. Injection of 100 ng/ml of heparin onto a Copoly(DMA-MASP-NAS)/streptavidin-functionalized sensorchip allows the immobilization of 233 resonance units (RU) equal to 17.1 fmol/mm<sup>2</sup> of heparin. In the same experimental conditions, the injection of heparin onto streptavidin immobilized to a carboxymethyl dextran-functionalized sensorchip leads to the immobilization of comparable amounts of the glycosaminoglycan (5.8-9.5 fmol/mm<sup>2</sup>).<sup>16,17</sup>

We then studied the capacity of Copoly(DMA-NAS-MAPS) /streptavidin-immobilized heparin to bind FGF2, a classical heparin-binding protein. As shown in Figure 9, when immobilized to a surface *via* Copoly(DMA-MAPS-NAS)/streptavidin, heparin retains its capacity to bind FGF2 in a specific way, as demonstrated by the absence of interaction with immobilized streptavidin. Increasing concentrations of FGF2 were injected over the heparin surface to evaluate the kinetic parameters of FGF2/heparin interaction. The binding data demonstrate that the interaction occurs with dissociation constant ( $K_d$ ) equal to 18 Nm(Figure 2b).



**Figure 9.** SPR analysis of FGF2/heparin interaction. **A)** Sensor-grams showing the binding of FGF2 (300 nM) to a functionalized gold layer coated with streptavidin alone (hatched line) or with streptavidin and biotinylated-heparin (straight line). **B)** Blank-subtracted sensor-gram overlay showing the binding of increasing concentrations of FGF2 (300, 150, 75, 36, 18 e 9 nM from top to bottom) to a Copoly(DMA-NAS-MAPS) functionalized gold layer coated with streptavidin and biotinylated-heparin. In both the panels the response (in RU) was recorded as a function of time.

This value together with the dissociation ( $k_{off}$ ) and association ( $k_{on}$ ) rate constant listed in Table 2 is close to those obtained from SPR assays performed with free FGF2 and heparin immobilized to commercially available carboxymethyl dextran-functionalized sensorchips.<sup>18</sup>

**Table 2.** Comparison between  $K_d$ ,  $k_{off}$  and  $k_{on}$  values obtained from Copoly- based and carboxymethyl dextran-based experiments.

	$k_{off}$ ( $s^{-1}$ )	$k_{on}$ ( $s^{-1}M^{-1}$ )	$K_d$ (nM)
Copoly(DMA-MAPS-NAS)	$1.54 \cdot 10^{-3}$	$8.22 \cdot 10^4$	18
Carboxymethyl dextran	$3.8 \cdot 10^{-4}$	$9.0 \cdot 10^3$	42.5

# Material and Methods

## Chemicals

TRIS, ethanolamine, SSC, ammonium sulfate, SDS, PBS, *N,N*-dimethylacrylamide (DMA) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) were purchased from Sigma (St. Louis, MO). *N,N*-acryloyloxysuccinimide (NAS) was from Polysciences (Warrington, PA). Oligonucleotides were synthesized by MWG-Biotech AG (Ebersberg, Germany). Streptavidin labelled by HRP (Horse Radish Peroxidase) from Jackson Immunoresearch. Heparin (13.6kDa) was obtained from LaboratoriDerivatiOrganici Spa (Milan, Italy). SuperSignal ELISA Femto maximum Sensitivity kit from ThermoScientific. Human recombinant fibroblast growth factor 2 (FGF2) was expressed in *Escherichia coli* and purified as described by Isacchi et al.<sup>19</sup>

## Gold surface preparation

Silicon wafers has been coated with 3 nm Cr (to promote gold adhesion) and 50 nm Au, by means of electron beam deposition. Copoly(DMA-NAS-MAPS) was synthesized and characterized as described in Chapter 5. The gold surface was treated for ten minutes within an Oxygen Plasma Generator (Harrick Plasma Cleaner), then immersed for 30 min in a copoly(DMA-NAS-MAPS) solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level). The slides were then washed extensively with water and dried under vacuum at 80 °C for 15 minutes.

## AFM measurements

AFM measurements were performed with a VEECO Innova AFM. The morphological characterization was carried out in tapping mode with 512 samples/line, 0.8 Hz of frequency, 4-10 μm scan range. The thickness of the polymeric layer was evaluated by AFM-tip scratch test . This technique consists in scratching the coating with the AFM tip while the normal force applied was set to a value high enough to penetrate the layer but low enough to avoid significant tip or substrate damaging. The scratch was made in Lift Mode (start height 0.3μm, lift height 0.3μm) with 30 scan cycles, 256 samples/line, 62.75 Hz, 1μm scan range. After scratching, the area was imaged in tapping mode with the same tip used for scratching. This was necessary in order to not “loose” the observed area after tip replacement.

## XPS experiments

XPS experiments have been performed in a multichamber ultrahigh-vacuum (UHV) system described in detail in reference 8.

In XPS measurements, photoelectrons were excited by Al  $K\alpha$  ( $h\nu = 1486.67$  eV) or Mg  $K\alpha$  ( $h\nu = 1253.6$  eV) radiation, and analyzed by a 150 mm hemispherical analyzer (SPECS Phoibos 150). The angular acceptance set for this work was about  $\pm 6^\circ$ , and the spatial resolution was 1.4 mm. Two modes of operation have been employed: 1) pass energy of 50 eV and Al  $K\alpha$  line (total resolution of 1.643 eV) to evaluate the stoichiometry of the polymer; 2) pass energy of 10 eV and Mg  $K\alpha$  line (total resolution of 0.841 eV) in order to study the lineshape of the characteristic peaks.

## Functional test

A 23-mer, 5'-amine-modified oligonucleotide synthesized by MWG-Biotech AG (Ebevsberg, Germany), 100  $\mu\text{M}$  stock solution, 5'-NH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-GCC CAC CTA TAA GGT AAA AGT GA was dissolved in 150 mM sodium phosphate buffer at pH 8.5. A 10  $\mu\text{M}$  solution of this oligonucleotide was printed on coated slides to form a 15 X 15 array using a piezoelectric spotter (SciFLEXARRAYER S5; Scienion). Spotting was done at 20 °C in an atmosphere of 50% humidity. The oligonucleotides were coupled to the surfaces by incubating overnight in an uncovered storage box, laid in a sealed chamber, saturated with sodium chloride (40 g/100 mL H<sub>2</sub>O), at room temperature. After incubation, all residual reactive groups of the coating polymer were blocked by dipping the printed slides in 50 mM ethanolamine/0.1 M Tris pH 9.0 at 50 °C for 15 min. After discarding the blocking solution, the slides were rinsed two times with water and shaken for 15 min in 4X SSC/0.1% SDS buffer, pre-warmed at 50 °C, and briefly rinsed with water. An oligonucleotide, complementary to the one spotted on the surface, at a 1.0  $\mu\text{M}$  concentration (2.5  $\mu\text{L}/\text{cm}^2$  of coverslip) was dissolved in the hybridization buffer (2X SSC, 0.1% SDS and 0.2mg/mL BSA) and immediately applied to microarrays. The complementary oligonucleotide has the sequence 5'-TCA CTT TTA CCT TAT AGG TGG GC-3' and is labeled with biotin in 5' position. The surfaces, placed in a hybridization chamber, were transferred to a humidified incubator at a temperature of 65 °C for 2 h. After hybridization, the slides were first washed with 4X SSC at room temperature to remove the coverslip and then with 2X SSC/0.1% SDS at hybridization temperature for 5 min. This operation was repeated two times and was followed by



two washing steps with 0.2X SSC and 0.1X SSC for 1 min at room temperature. Streptavidin labelled by HRP (Horse Radish Peroxidase) from Jackson ImmunoResearch at the concentration of 1  $\mu\text{g}/\text{mL}$  in PBS buffer was applied to the gold surface for 60 minutes. After a short rinse in PBS and water slides were dried and chemiluminescence was developed using the SuperSignal ELISA Femto maximum Sensitivity kit from ThermoScientific according to the manufacturer instructions. Chemiluminescence signal was registered at 0.2 sec exposure time using a homemade set up equipped with a Q Imaging CCD Camera.

### **Surface plasmon resonance (SPR) analysis**

For the Surface Plasmon Resonance experiment a naked gold sensorchip for SPR analysis (XantechBioanalytics, Dusseldorf, Germany) was functionalized with Copoly(DMA-NAS-MAPS) as follows: the gold chip was treated with oxygen plasma in a Plasma Cleaner, Harrick Plasma (Ithaca, NY, USA) to clean the surface. Immediately after the oxygen plasma treatment, the sensorchip was dipped in an aqueous solution of poly(DMA-NAS-MAPS) in ammonium sulphate at 20% of saturation level for 30 minutes, then it was rinsed with DI water, dried with nitrogen flow and cured under vacuum at 80°C for 15 minutes. The functionalized sensorchip was positioned in a BIAcore X instrument (GE-Healthcare, Milwaukee, WI) and the surface was conditioned with 3 consecutive 1-minute injections of 1 M NaCl in 50 mM NaOH before heparin immobilization. Biotinylated heparin [100 ng/ml in 10 mM HEPES buffer pH 7.4 containing 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 (HBS-EP)] was injected onto the sensorchip for 4 minutes at a flow rate of 5  $\mu\text{L}/\text{min}$ . Increasing concentrations of FGF2 in HBS-EP were injected over the heparin for 4 min at a flow rate of 30  $\mu\text{L}/\text{min}$  (to allow their association with immobilized heparin) and then washed until dissociation was observed. A sensorchip coated with streptavidin alone was used to evaluate the non-specific binding and for blank subtraction. After every run, the sensorchip was regenerated by injection of 2 M NaCl. Kinetic parameters were calculated by using the non linear fitting software package BIAevaluation using a single site model. In parallel experiments, streptavidin and biotinylated heparin were immobilized to a carboxymethyl dextran-functionalized F1 SPR sensorchip (GE-Healthcare, Milwaukee, WI) as originally described in ref. [23] and used as a standard.

## Conclusions

In the last part of the Ph.D. project, a new method for functionalizing a gold surface with Copoly(DMA-NAS-MAPS) is described. The polymer is deposited by dip coating after an oxygen plasma treatment; this simple procedure is followed by a washing in water and a AFM scratch test reveals the presence of a polymer completely coating the surface with a thickness of 2.5 nm. The XPS spectra from C1s N1s and O1s levels present the typical fingerprints of the polymeric overlayer, i.e. the features arising from C=O and N=O groups. Therefore with these surface sensitive techniques we have demonstrated that the coating procedure is effective in the immobilization of Copoly(DMA-NAS-MAPS) on gold. Immobilization of heparin to Copoly(DMA-NAS-MAPS) functionalized gold slides allows SPR binding assays whose kinetic results are comparable to those obtained with commercially available carboxymethyl dextran-functionalized sensorchips. These results enlighten the versatility of Copoly(DMA-NAS-MAPS) as a polymeric coating for the preparation of different protein microarrays and expand the initial successful binding of Copoly(DMA-NAS-MAPS) to glass and silicon surfaces through the MAPS monomer as already demonstrated in the experiments with GFAP and ApoE protein. The Copoly (DMA-NAS-MAPS) layer is able to bind the capture antibody that then binds the proteins. The bound protein is revealed by the incubation with the detection secondary fluorescent antibody moreover it is important to notice that the LOD obtained with Cy3 labeled streptavidin revelation of ApoE protein is similar to that provided by QDs proving that the use of silicon/SiO<sub>2</sub> substrates allow a significant sensitivity increase using conventional dyes.

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