SYNTHETIC NANOPORES AND NANOPARTICLES
FOR THE DETECTION AND THE MANIPULATION OF
BIOLOGICAL MOLECULES

Settore disciplinare: FIS/07

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ABSTRACT

In this work I present a novel approach to the analysis of biomolecules, and a study on two derived practical applications to evaluate its constraints, limits, and potential benefits, namely a biosensing device and a selective transport through membrane.

The new approach is based on a 100-800 nm pore etched in a silicon nitride membrane. A linear target molecule, such as DNA, is inserted in the pore and linked at both termini with anchors, one on each side of the pore. Since the complex is stable and the linked objects have a size that is much larger than the target molecule, manipulation, pore closure/opening, possible interactions, stretching and other forces, and in general several characteristics and behaviours of the molecules can be studied at the pore interface. The realization of such a device is preliminary to the development of novel pore-based analytical tools.

The principle was applied for the development of a biosensing device. Biosensing devices that perform electrical signal detection are facing the need of being both extremely small and highly sensitive, that is particularly challenging for conventional biosensors where the signal produced is proportional to the surface detecting area. Here, I report the production of a sensor device based on DNA specific displacement of a stable blockade in a synthetic pore section, due to objects associated with the interacting molecules. Thus the signal is proportional to the pore size and not to the surface containing the target/probe molecule.

First, I report the setting up of the single components of the device: a complex made of a DNA linker and two particles –the anchors-, the synthetic nanopored membrane and an electrophoretic cell together with an electromagnet –the sensing tools-. Then I show the results of transmembrane interactions between the objects both outside and inside the sensor device. The applications results related to the biosensor operation are then shown, reporting the detection of the hybridization or the strand-displacement between probes and targets DNA molecules. Finally, I show the operation of a trans-membrane transporter mediated by particles carriers, where the system is exploited to capture and import target molecules through the membrane.
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PART 1
INTRODUCTION
1 INTRODUCTION

In recent years, the introduction of cross-disciplinary approaches to the study of biology has brought countless benefits. The need to understand the complexity of living organisms involves genomics, proteomics, metabolomics and system biology: these fields of study are demanding tools deepening in sensitivity, precision, affordability, miniaturization, and inexpensiveness that can be attained only with technology of the state of the art in chemistry, physics, engineering, statistics, biology and the so-called nanotechnology.

Among the many approaches, nanotechnology gives a support reducing the complexity of the systems by supplying synthetic counterparts of biological aggregates involved in complex processes and re-inventing their original mean. An example of this biological aggregate is the cell membrane. The cell exploits the separation of two environments generated by its membranes to selective transport molecules through channels aided by specific carrier proteins. The ability to mimic the cell membrane using synthetic material and nanotechnology would open the fascinating perspective of applying in our instruments the same approach that has been selected as the most efficient by the natural evolution.

The goal of this project is to demonstrate the feasibility of molecular capture and carrier mediated transport through synthetic nanopores. As practical examples, it will focus on two applications that take advantage of the separation of two solutions by a synthetic nanopored membrane: a detection method for nucleic acids interactions and facilitated membrane-based separation.

In 1949 Mr. Wallace Coulter filed his patent application for the cell counter in which a punctured cellophane separated two electrodes connected to a source of electric current while cells suspended in ionic medium were passed through it simultaneously with the current. Coulter found that one cell crossing the aperture displaces a liquid equal to its own volume signalling a proportional voltage pulse between the electrodes passing the current through the aperture. So the counter detects change in electrical conductance of a small aperture as fluid containing particles is drawn through and alters the effective cross-section of the conductive channel. Recently, the Coulter counter has been revived at the molecular, rather than cellular, level. Indeed, a major exercise of integrating technology directed towards miniaturized laboratory and high sensitivity
has been, in the last decade, molecular characterization through artificial nanopores. In recent years the use of the alpha-haemolysin pore for the characterization of nucleic acids has addressed rewarding promises. The technique is usually applied to study properties of nucleic acids passing through the lumen or with sequencing purpose (Akeson et al., 1999; Chen et al., 2007; Deamer and Branton, 2002; Harrell et al., 2006; Kasianowicz et al., 1996; Keyser et al., 2005; Li et al., 2003a; Li et al., 2001; Meller et al., 2000; Storm et al., 2005a). However, the analysis of ionic current signatures as DNA translocates through, is a very difficult task given the translocation rate (1 to about 30 nt/μs at 100-120 mV depending on estimates) and the noise in the ionic current signal. Detection of DNA features requires an electronic system with a bandwidth which is currently not available when designing a bench-top diagnostic system.

In the sensor that will be described in this thesis, rather than as a counting engine, the change in electrical conductance of a synthetic nanopore is exploited to signal that a molecular interaction between biological features has occurred. This sensor differs from previous pore-based approaches in that the recorded ionic current variation events are not determined by the transient blockade caused by the analyte crossing through the synthetic pore, but by stable, yet analyte-dependent pore clogging caused by buoys associated with probe molecules. Thus the molecular events concerning a small number of molecules can trigger a physical and electric reversible switch that is easily recorded, paving the way to the development of portable and highly sensitive devices.

Not only molecular recognition governs all cellular activities, but also controls the relationships of the cell with the environment by precisely selecting the chemical compounds that are imported or exported. The cell accomplishes this goal through the use of specialized transporter (or facilitator) proteins. The sophisticated biomechanical system that is at the basis of biological membrane selectivity and transport remains unmatched by human manufacturing, despite the significant progresses that have been achieved in the construction of materials and objects at the nanoscale. Nevertheless, the idea of constructing nanoscaled machines that could mimic the through membrane cellular transport by fishing objects in an outer compartment and specifically pumping them in an inner compartment is fascinating and full of implications for future applications. Attempts to reproduce cellular transport have faced the difficulty of developing a suitable supporting system. To date, artificial membranes either use components purified from natural sources or are prepared by impregnating a porous
filter with a solution of lipid mixture (Funakoshi 2006; Galvez 1991). Lipid based membranes, however, have several shortcomings including lack of rigidity, reproducibility and stability, that severely limit their possible applications in nanoscale pumps.

In this second application, I attempt to define a fully artificial system: I explored the possibility of constructing a rigid, highly reproducible and configurable membrane transport system based on pored silicon nitride membranes and report the results of the characterization of the features of the molecular interaction through silicon nitride membrane pores.

Below I will give a brief introduction to the state of the art in techniques that have a major rule in the two mentioned applications that have been studied in this work.

1.1 DETECTION OF MOLECULAR INTERACTIONS

Molecular interaction between receptors and their ligands is important in life sciences. Such specific recognitions include those between complementary strands of DNA, enzyme and substrate, antigen and antibody, ligands and cell surface receptors as well as between cell adhesion proteins. These interactions are involved in many important biological processes, including genome replication and transcription, enzymatic activity, immune response, initiation of infection, and many other cellular functions. Their selectivity and specificity are exploited in nanobiotechnology for developing bioanalytical devices such as biosensors.

1.1.1 BIOSENSORS

A biosensor can be defined as any sensor that uses a biological component to bind specifically an analyte of interest and provide a physical signal that is in proportion to the amount of analyte. All molecular based biosensors rely on high specific recognition events to detect their target analytes. The elements of any biosensor include a molecular recognition layer and a signal transducer that can be coupled to an appropriate readout device. DNA is especially well suited for biosensing applications, because the base-pairing interactions between complementary sequences are both specific and robust. In a typical configuration, a single-stranded probe sequence is immobilized within the recognition layer, where base pairing interactions recruit the target DNA to the surface. The hybridisation is then detected by many different principles like radioactive,
fluorescence, electrochemical, microgravimetric, enzymatic and electroluminescence methods (e.g. Kricka, 2002). They provide different sensitivity to the molecules detected. Optical biosensors based on fluorescence have detection limits approaching 10^7 molecules/cm² (Epstein et al. 2002). Surface Plasmon Resonance (SPM) reports changes in the refractive index of a thin metal film substrate upon absorption of the analyte: to achieve sufficient detection limit the amount of the material deposited has to be increased to amplify the hybridization signal. Gold nanoparticles solution linked to probe nucleic acids changes its color upon hybridization of target molecule. This color-change was used for a colorimetric differentiation of polynucleotides with single base imperfections; the detection could be improved by subsequent transfer of the complexes onto reverse-phase silica plates (Elghanian et al. 1997, Reynolds et al. 2000, Storhoff et al. 1998). About 10 fmoles of an oligonucleotide could be detected with this unoptimized system. An alternative readout strategy is to monitor mass changes in the immobilized recognition layer that occur upon target binding using a quartz microbalance (Wang et al. 1999) or microfabricated cantilevers (Fritz et al. 2000). However, electrochemical methods better suite DNA diagnostics because electrochemical reactions give an electronic signal directly and because immobilized probe sequences can be readily confined to a variety of electrodes substrates. Direct electrochemistry readout, based on adsorption stripping voltammetry has been able to detect 40 femtomoles of substrate (~ 2x10^10 molecules) (Jelen et al. 2002). Indirectly electrochemical detection by Ruthenium mediated oxidation of guanine has been coupled to a reverse transcription PCR assay to monitor the over-expression of genes in tumour samples (Armistead and Thorp 2002). Sensitivity of the systems extends down to 550 attomoles of target DNA (~3x10^8 molecules). Strategies in which target DNA is labelled with redox-active reporter molecules are reported to detect ~10^10 molecules. In an alternative approach to chemical labeling scheme, redox active reporter molecules that associate non-covalently with the double helix have been used for DNA analysis with high reported sensitivity (Millan and Mikkelsen 1993). Although all of these biosensors get very high levels of sensitivity they do not match yet the single molecule sensitivity. Compared with bulk studies, there are several advantages of single-molecule approaches. Properties measured in bulk studies represent ensemble averages of a population of molecules, while some studies or application need to detect the single molecule.
1.2 SINGLE MOLECULE ANALYSIS

All single-molecule methods rely on the attachment of labels to the molecule of interest, which either scatter light (such as micron-sized beads) or emit it (such as fluorophores or quantum dots), to allow visualization of molecules. For force-based methods (motion, torque, extension etc.), these labels must be attached to the single molecule sufficiently well to withstand the forces being applied. Mechanical properties-reporter labels can be attached by using specific covalent interactions, specific noncovalent interactions, or nonspecific interactions. Magnetic tweezers provide a stable platform for measuring slow molecular processes involving both force and torque, avoid completely the use of potentially damaging photon fluxes and they involve relatively simple, straightforward instrumentation. AFM uses a compliant cantilever to exert force upon a single molecule bound by one end to the cantilever probe tip and by its other end to a cover glass surface. AFM has been applied with success to study unfolding in a number of proteins, but most extensive investigations have been carried out for the protein titin, a filamentous molecule from muscle sarcomeres that carries multiple-tandem repeat domains of several distinct types and is believed to furnish passive elasticity to muscle tissues. Several groups characterized the nanomechanical properties of single titin molecules (Rief et al. 1997, Williams et al. 2003). Optical tweezers have been used, among the others, to investigate the elastic properties of nucleic acids (Seol et al 2004, Smith et al. 1996), to measure protein-DNA interactions (Kotch et al. 2002), and to characterize the folding of nucleic acids (Liphardt et al. 2001, Onoa et al. 2003, Woodside et al. 2006).

Single molecule detection methods include also the spectroscopic methods. The single-molecule-based technique fluorescence correlation spectroscopy (FCS) allows inherent averaging over a large number of single-molecule passages through the measurement volume, thus it is ideally suited to assess molecular movements (Rigler et al. 1993). Fluorescence resonance energy transfer (FRET) has elevated fluorescence colocalization experiments to a new level of specificity, providing a direct measure of proximity on molecular length scales. In a typical FRET experiment, a biological macromolecule is labelled with a donor and an acceptor fluorophore at two different positions. Upon excitation of the donor, energy is transferred non-radiatively via induced dipole–dipole interaction (Forster, 1959) to the acceptor. The main
fluorescence fluctuations arise from conformation-dependent FRET: in particular, the structural dynamics of all kinds of oligonucleotides, such as Holliday and fourway RNA junctions (McKinney et al. 2004) and ssDNA flexibility (Murphy et al. 2004) have been studied extensively.

1.3 NANOPORE DETECTION

In the 1990s, it was proposed that it might be possible to use nanopores as resistive-pulse sensors for DNA. The first experimental results were reported in 1996 by Kasianowicz and co-workers on the α-haemolysin pore. Bacteria *Staphylococcus aureus* secretes the α-haemolysin protein which spontaneously inserts itself in a lipidic membrane generating a pore of 1.4 nm diameter (Song et al. 1996). The small constriction of this pore allows the passage of single-strand DNA (ssDNA), suggesting the possibility of sequencing the nucleic acids by simply measuring differences in ionic conductance while different bases crossed the pore (Kasianowicz et al. 1996). Detection of target molecules have been accomplished with nanopore-based sensors developed by incorporation of probe molecules permanently tethered to the interior of the pore (Howorka et al., 2001b; Movileanu et al., 2000). However, these schemes require that the events of molecular recognition occur within the channel, and the probe-analyte dimer must be small enough to enter the pore lumen. Other approaches require a specific configuration of the molecule being analyzed (Vercoutere et al., 2001; Vercoutere et al., 2003).

Although biological pores have proved to be very useful for a range of interesting translocation experiments, they do exhibit a number of disadvantages such as fixed size and limited stability. Typically the pores, and also, in particular, their embedding lipid bilayer, can become unstable if changes occur in external parameters such as pH, salt concentration, temperature, mechanical stress, and so on. Fabrication of nanopores from solid-state materials presents advantages over their biological counterpart such as very high stability, control of diameter and channel length, adjustable surface properties and the potential for integration into devices and arrays.

1.3.1 SYNTHETIC NANOPORES FOR DETECTION

Four basic approaches have been used to fabricate nanometre-scale channels within synthetic membranes. The most common of these is based on localized etching of the
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monolith using, for example, a focused ion beam (FIB). The second relies on various forms of lithography, the third involves the use of a template, and the fourth relies on the channels present within nanotubes.

Etching a hole into an insulating layer has been one way to create pores. Researchers have also etched holes in glass slides for electrophysiological measurements on cells (Schmidt et al. 2000, Fertig et al. 2001). A different technique has been to guide the etching by shooting a metal ion through a polymer layer (Siwy and Fulinsky 2002). Importantly, one can monitor the ion current across the layer during the etching, thus controlling the etching. In this way, pores with nanometre dimensions, down to 2 nm, have been made. Golovchenko and co-workers reported a novel technique — ion beam sculpting — by which they fabricated single nanopores in Si₃N₄ membranes with nanometre control (Li et al. 2001). The group used a focused ion beam to mill a hole in the membrane: depending on the ion rate and temperature, pores could enlarge and shrink, allowing the fine-tuning of pores in the nanometre range. Pores with nanometre dimensions were made in this way and provided a starting point for DNA translocation measurements.

Using the techniques from silicon microfabrication, free-standing membranes of Si, SiN, or SiO₂ can be made. Using electron-beam lithography and subsequent etching, pores of 20 nm diameter were produced (Storm et al. 2003). A way to fine-tune the nanopores to a small size with sub-nanometre resolution was developed after the finding that, while imaging with TEM, large holes, with a diameter greater than the membrane thickness, grew in size, whereas small holes shrunk. Other groups pursued nanopores in multilayer membrane materials that may provide additional electronic probes for detection of DNA (Gracheva et al. 2006). Solid-state nanopores have mainly been used to measure the translocation of individual DNA molecules. Individual DNA molecules within a mixture containing different size molecules could be distinguished (Storm et al. 2005b). However, a narrow pore only allows the linear passage of DNA in a head to-tail fashion. Instead, in wider pores, it has been observed that DNA can traverse in a folded manner (Storm et al. 2005b, Li et al. 2003b). An attempt has been made to measure changes in the mutual capacitance as DNA molecules pass through a nanopore in a semiconducting multilayer (Gracheva et al. 2006) rather than a nanopore in a single insulating membrane. It is also possible to translocate non-polynucleotide polymers, e. g. to observe the passage of single protein molecules (Siwy et al. 2005, Han
et al. 2006). Nanopores have also been used as a template for nanoelectrodes with diameters down to 2 nm (Heng et al. 2004), and have recently been used to explore new regimes for electrochemistry (Krapf et al. 2006).

1.4  MANIPULATION OF PARTICLES

The control of particles has been attempted by several methods. Often the manipulability of a single particle is not feasible, although some methods are prone to develop in this direction.

Controlled manipulation of large quantities of microparticles has been accomplished on a surface using inertial forces. Motion can be induced by applying a periodic parabolic wave form to a shear-polarized piezoelectric plate coupled to a substrate on which the particles reside. The technique attempt is to control manipulation and separation of large collections of particles without the need for a fluid medium (Eglin et al. 2006).

The acoustic standing wave technology combined with microtechnology has been developed for advanced particle and cell separating microfluidic systems (Laurell et al. 2007). The acoustic technology offers attractive features, such as reasonable throughput and ability to separate particles in a size domain of about tenths of micrometers to tens of micrometers.

Electric fields can be used for the on-chip manipulation and assembly of colloidal particles (Velev 2006). Charged particles suspended in water respond to alternating (AC) or direct current (DC) electric fields: the derived dielectrophoresis and AC electrophoresis could be used in droplet-based microfluidic chips, biosensors, and devices for collection of particles from diluted suspensions. An attempt to control single particles by this method was made taking advantage of the qualitative and quantitative differences of the cooperative motion of particles in dc vs. ac fields, re-positioning particles by alternating between the two modes (Kim 2002).

Optical trapping is an alternative method to manipulate single or multiple molecules in a very controlled way.

Optical traps exploit the radiation forces of laser light to manipulate microscopic particles. The object of manipulation can be of different natures: colloid particle, molecule, cell, virus, micromechanism component etc. (Soifer et al. 2004). Optical tweezers were used to trap and position a single superparamagnetic particle of 2 µm
over a spin valve sensor, with the particle then detected with a corresponding drop in the voltage across the sensor (Lui et al. 2008). A single-beam optical trap was set-up within an associated fluorescence microscope system which can be useful to measure forces and collect fluorescence signals upon biological systems simultaneously (Lee et al. 2007).

Magnetic particles are currently used in routinely laboratory protocols as separators for molecules purification. But the ability of manipulate them in single or multiple sets and the possibility to link several kind of molecules has lead to the development of different applications. Several studies on manipulation were based on large-scale permanent magnets (Strick et al. 1996, Fulconis et al. 2004) or electromagnets (Gosse and Croquette 2002, Bausch et al. 1998, Hosu et al. 2003), showing that the forces in the piconewton range could be used on super-paramagnetic beads attached to biomolecules. Movable permanent magnets have successfully moved tethered-bead DNA molecules (Strick et al. 1996, Fulconis et al. 2004). Permanent magnets have the advantages of simplicity, portability, and no power requirement. Alternatively, electromagnets are preferred due to their excellent controllability during operation. For example, one can turn on or off, or even change the magnitude of the applied current to control the movement of a magnetic bead bonded with a DNA molecule (Haber and Wirtz 2000, Gosse and Croquette 2002). Magnetic tweezers can manipulate magnetic probes also inside living cells (De Vries et al. 2005). A microelectromagnet matrix and a ring trap was used by Lee et al. (2007) to position and control magnetic nanoparticles. Specifically, they were able to manipulate superparamagnetic particles in two-dimension using a matrix and to trap them using a single circular trap. An application for lab-on-a-chip 3D-manipulation of a droplet containing magnetic particles has been reported (Lehmann et al. 2007). The scope of the work was to move, merge, mix, hold and separate droplets on the chip surface to perform sequential bioanalytical processes on a chip.
PART 2

MATERIALS AND METHODS
2. MATERIALS AND METHODS

In this section materials and methods for the production of the single components of the device and methods to assemble them are presented.

If not specified materials are purchased from Sigma-Aldrich, Germany. All the solutions were sterilized by autoclaving at 121 °C for 21 minutes.

2.1 WORKING WITH NUCLEIC ACIDS

Oligonucleotides for work with particles or for PCR amplification were purchased from MWG biotech, Germany (Tab.2.1).

<table>
<thead>
<tr>
<th>NAME</th>
<th>SEQUENCE 5’-3’</th>
<th>MODIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13F</td>
<td>TTG TAA AAC GAC GGC CAG</td>
<td>-</td>
</tr>
<tr>
<td>M13R</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>-</td>
</tr>
<tr>
<td>M13F NH$_2$</td>
<td>TTG TAA AAC GAC GGC CAG</td>
<td>5’-NH$_2$</td>
</tr>
<tr>
<td>M13RP</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>5’-P</td>
</tr>
<tr>
<td>M13R NH$_2$</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>5’-NH$_2$</td>
</tr>
<tr>
<td>LIG5P</td>
<td>CCGTGAAGTtttcatgagtaggaaacacagctatg acacat</td>
<td>5’-Phosphate</td>
</tr>
<tr>
<td>5PP0LIA3NH$_2$</td>
<td>TAC TCA TGA AAC TCA CGG (A)$_9$</td>
<td>5’-P; 3’-NH$_2$</td>
</tr>
<tr>
<td>M13RFINT</td>
<td>GGA AAC AGC T(fluoro)AT GAC CAT</td>
<td>T int-fluorescein</td>
</tr>
<tr>
<td>M13F bio</td>
<td>TTG TAA AAC GAC GGC CAG</td>
<td>5’-Biotin</td>
</tr>
<tr>
<td>SHM13RC</td>
<td>ATG GTC ATA GCT GTT TCC</td>
<td>5’-SH-C6</td>
</tr>
<tr>
<td>M13RFLUO</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>5’-Fluorescein</td>
</tr>
<tr>
<td>M13RCH bio</td>
<td>ATG GTC ATA GCT GTT TCC</td>
<td>5’-Biotin</td>
</tr>
<tr>
<td>NH$_2$ M13RC</td>
<td>“</td>
<td>5’-NH$_2$-C6</td>
</tr>
<tr>
<td>M13R bio</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>5’-Biotin</td>
</tr>
<tr>
<td>M13F FLUO</td>
<td>TTG TAA AAC GAC GGC CAG</td>
<td>5’-Fluorescein</td>
</tr>
<tr>
<td>BIOM13FRC</td>
<td>CTG GCC GTC GTT TTA CAA</td>
<td>5’-Biotin</td>
</tr>
<tr>
<td>FLUOM13FRC</td>
<td>“</td>
<td>5’-Fluorescein</td>
</tr>
<tr>
<td>M13RA</td>
<td>GGA AAC AGC TAT GAC CAT</td>
<td>-</td>
</tr>
<tr>
<td>SDL4BIO1</td>
<td>CCAAGGCCAATCTGAACCTGCCTAGG</td>
<td>5’-Biotin</td>
</tr>
<tr>
<td>M13R Abio</td>
<td>GGA AAC AGC ATT TAA ATA</td>
<td>5’-Biotin</td>
</tr>
</tbody>
</table>

Table 2.1 List of oligonucleotides used as primers for PCR and other manipulations
2. MATERIALS AND METHODS

2.1.1 PRODUCTION OF DOUBLE STRANDED OLIGONUCLEOTIDIES

Oligonucleotides were reacted with their complements in equimolar proportion in a water solution containing 2xSSC (0.3 M NaCl, 0.03 M NaCitrate, pH 7.0). The mix solution was incubated in a water bath at 60 °C and allowed to cool at room temperature for two hours. The samples were run on an acrylamide gel electrophoresis in 1x TBE buffer (from 5x TBE: 21.6 g Trizma-base, 11 g Boric acid, 8ml EDTA 0.5 M, pH 8.00 for 400 ml).

2.1.2 PCR

PCR amplification was made with primer pairs M13F/M13R (with and without SFB modification), M13Fbio/M13RP, M13Fbio/M13Rbio, M13Fbio/M13RFluo in a TProfessional (Biometra GmbH, Goettingen, Germany) thermocycler. PCR mix was made as follows:

<table>
<thead>
<tr>
<th>PCR mix for 25 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.50 µl 10 x PCR buffer</td>
</tr>
<tr>
<td>2.50 µl dNTP (200 µM)</td>
</tr>
<tr>
<td>16.65 µl H2O</td>
</tr>
<tr>
<td>1.00 µl Primer 1 (10 mM)</td>
</tr>
<tr>
<td>1.00 µl Primer 2 (10 mM)</td>
</tr>
<tr>
<td>0.35 µl Taq polymerase (4 U/µl)</td>
</tr>
<tr>
<td>1.00 µl DNA</td>
</tr>
</tbody>
</table>

10x PCR buffer was: 15 mM MgCl₂, 500 mM KCl, Tris-HCl 100 mM.

Primers were re-suspended from lyophilised stock into H₂O and brought to 100 pmol/µl stock solution. Dilutions were made with H₂O.

For SFB modified primers final concentration in PCR reaction was maintained at 0.4 mM, loading a variable volume of solution depending on concentration after desalting.

Deoxiribonucleotide (dNTP) stock solution (200 µM), was made using dNTPs
purchased from Roche Diagnostics GmbH, Penzberg, Germany.

DNA was amplified following this program:

**PCR program**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>0.5</td>
<td>35</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

PCR product was purified with PCR purification kit (Roche) eluted in 50 µl H₂O and stored at -20 °C. Product concentration was estimated measuring absorbance at λ=256 nm by spectrophotometer. After amplification, products were stored at -20 °C.

### 2.1.3 STANDARD ELECTROPHORESIS

#### 2.1.3.1 Agarose gel electrophoresis

PCR product was checked by agarose gel electrophoresis. Electrophoresis in agarose gel was accomplished using 1xTAE buffer (TAE 50x solution is 48.4 g Trizma base, 11.42 ml Acetic acid, glacial, 0.5 M EDTA, pH 8.0) as running buffer. Slab gels were prepared by pouring a 1% to 4% solution (MP Agarose, Roche in 1x TAE) into a suitable gel case and let polymerize for 30 min. Running voltage varied from 50 to 100 V depending on dimension of gel and nucleic acids length. Running time varied from 30 min to 120 min depending on dimension of DNA fragments and on voltage used. DNA ladders used were marker VIII (Roche) when working with oligonucleotides or marker VI (Roche) when working with PCR product from 600 to 2500 bp (base pairs) length. Loading buffer 6x (0.25% xylene cyanil, 0.23% bromophenoblue; 15% fycoll 400 in H2O) was loaded in the gel in proportion of 1:5 with nucleic acid solution.

Gels were stained in a solution containing 10 µM ethidium bromide in 1x TAE for
10 min and washed out in 1x TAE for 10 min. Visualization was taken under UV lamp system (UVP) and image collected with Canon Powershot S2 IS camera and processed with Visionworks LS (UVP) software.

2.1.3.2 Denaturing acrylamide gel electrophoresis

Single stranded PCR products were analyzed using denaturing acrylamide gel electrophoresis.

Purification of DNA from proteins was carried out by phenol/chlorophorm extraction.

Phenol/chlorophorm (1:1) in equal volume to the enzymatic reaction was added and the 1.5 ml tube was centrifuged at 6000 rpm for 6 min (Eppendorf centrifuge 5415 D). The supernatant was discarded and 1 ml of 95% EtOH was added to precipitate DNA by centrifuging at 13200 rpm for 3 min. After discarding of EtOH the precipitate was washed in 1ml 70% EtOH and centrifuged at 13200 rpm for 3 min. Discarded the supernatant, the DNA was re-suspended in 50 µl H₂O.

H₂O was added to volume and the solution was autoclaved at 121 °C for 21 min.

Acrylamide solution was prepared in water to a final volume of 100 ml as follows:

- 42 g Urea
- 20 ml TBE 5x
- 16.6 ml Acrylamide/bis-acrylamide (19:1)
- 63.4 ml H₂O

The solution was filtered (0.45 µm) and degassed. To 90 ml of the solution, 120 µl TEMED and 120 µl APS (10% solution in water, fresh prepared) were added. The solution was poured into a pre-cast glass box and allowed to polymerize for 1.5 hours. DNA digestion product was denatured for 10 minutes at 100 °C and put in an ice-bath. Eight µl of denaturing loading buffer (20 mM EDTA, 0.05% Xylene cyanole, prepared in 95% formamide) were added to 12 µl of DNA solution. Electrophoresis was run in 0.5 x TBE buffer at 150 V for 3.5 hours. Single stranded nucleic acids were visualized
by silver staining.

For silver staining the following solutions were prepared in water.

Fixer solution was 7.5% acetic acid. Silver solution, freshly prepared was 1.5g/L AgNO₃ and 0.056% formaldehyde. Developer solution, freshly prepared was 30g/l Na₂CO₃, 0.056% formaldehyde 400μg/l sodium thiosulfate.

The gel was immersed in the fixer solution for 30 minutes then washed 3 times in deionised water. 2-5 minutes each silver impregnation was made putting the gel in silver solution for 25 minutes and then rinsed with deionised water for 20 seconds. The gel was then put into developer solution for 5 minutes. The reaction was stopped in fixer solution for 5 minutes at 4 °C. Visualization was under visible light and photograph was taken with Canon Powershot S2 IS camera.

2.1.4 HYBRIDIZATION DETECTION OF PCR PRODUCT BY DOT-BLOT.

2.1.4.1 Solutions for Dot-blot

In Tab.2.II the compositions of the solution used for Dot blot hybridization are listed. D indicates denaturing buffer, N neutralizing buffer, W washing buffer, B buffer, H is hybridization solution, BSS is Blocking Stock Solution. SSC is Sodium Salt Citrate and SDS is Sodium Dodecyl Sulfate.

<table>
<thead>
<tr>
<th>D1</th>
<th>D2</th>
<th>N</th>
<th>Pre-H and H</th>
<th>WA</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M NaCl</td>
<td>0.5 M Tris-HCl, pH 7.5</td>
<td>0.5 M Tris-HCl, pH 7.5</td>
<td>5x SSC</td>
<td>2x SSC</td>
<td>0.1x SSC</td>
</tr>
<tr>
<td>0.5 M NaOH</td>
<td>1.5 M NaCl</td>
<td>0.1% Sarkosyl</td>
<td>0.1% SDS</td>
<td>0.1% SDS</td>
<td></td>
</tr>
<tr>
<td>B1, pH 7.5</td>
<td>B1 + Tween 20</td>
<td>B2</td>
<td>B3</td>
<td>Color solution</td>
<td></td>
</tr>
<tr>
<td>100 mM Maleic acid</td>
<td>0.3% Tween 20 in B1</td>
<td>10% Blocking stock solution in B1</td>
<td>100 mM Tris-HCl, pH 9.5</td>
<td>45 μl NBT solution</td>
<td></td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>100 mM NaCl</td>
<td>100 mM NaCl</td>
<td>35 μl X-phosphate solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM MgCl₂</td>
<td>10 ml B3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.II List of solutions used for Dot-blotting of PCR product.*
2. MATERIALS AND METHODS

2.1.4.2 Probe sequence

The probe sequence SDL4bio was drawn from the forward sequence of the PCR product of DNA extracted from the ascomycete fungus *Diaporthe helianthi*. The probe sequence was built basing on the rules of 50% GC, no self annealing and no loop in the sequence, no generation of dimers. Probe sequence was 5’-CCAAGGCAATCTGAACTGCGGTAC-3’ reverse complement of the portion underlined.

DL4 M13forward sequence (5’-3’)

AATTCGATTACTATAGGGCACGCGTGACGACGGCCCCGGCTGGTCTCTTCTCTTCACA
AGGGGGTTGTTATCTTTGACACACATTAGGAGGTGATCGATCGATTTTTGCAGAAAAGACTGCCCT
GACCCCTCGAGGTGGACGGGGAAAGATAACGGCGATGGGAGAAGATCAAGGCGGATGGGGGCTACG
TGAGCACCGGTGCTTTATGCGCGCCACTCTCTACCCGCACGGTACCTGCTCTCTGACTGGCG
GAAGCTCTTTCGCGGGCTCAGCGGCTTTATCAATGCGGGAAGCCATTATGATCGATCGCTATTATCGG
GGCGATTTCAGTATCAGCTCGAGAAGCGAGAAGCGAGAGAGGCGACGCTATTATCGG
CATCGGTGTCGGAGATTTCATGACGCGGACACGGCATACCACTCATAATCACTA

2.1.4.3 Protocol for blotting

A nylon membrane (Boehringer, Mannheim) was cut into pieces of suitable dimension for the pre-cast case. A Whatman foil was soaked in 2x SSC and leaned in a Petri glass dish. The nylon membrane was leaned on the Whatman foil. 10 µl of sample PCR product in 2x SSC were spotted on the membrane and let dry in open air. Three Whatman foils were put in three different Petri glass dishes after soaked the first in D1 solution, the second in D2 solution and the third in N solution. The membrane was leaned on the first foil for 5 min, on the second for 1.5 min and on the third for 5 min. The membrane was then dried in open air and in oven at 120 °C for 30 min inside two incubators. The membrane was put in 20 ml pre-hybridization solution for 2 hours at hybridization temperature (45 °C). Before hybridization the probe SDL4bio was denatured at 100 °C for 10 min and then put in an ice bath, after spin down of the solution. The membrane was put in 5 ml
hybridization solution, 140 pmoles of biotin-modified probe were added and the solution was incubated overnight with shaking at 45 °C. Washing of the membrane was done 2 times for 5 min in WA and 2 times 5 min in washing B at room temperature. The membrane was after equilibrated in 20 ml buffer 1. Before streptavidin-AP reaction the membrane was incubated in Buffer 2 for 30 min. After that the membrane was put for 30 min in 50 ml buffer 2 containing 50 mU of streptavidin-AP conjugate. Washing was carried out in B1 containing Tween 20 two times for 20 min. The membrane was pre-equilibrated for 1 min in B3 and then was incubated in 10 ml colour solution. Positive results developed a dark spot. Positive control was a biotinilated ds oligonucleotide, while negative control was water.

2.1.5 OTHER ENZYMATIC MANIPULATIONS

2.1.5.1 Single stranded DNA production

Lambda Exonuclease (USB) catalyzes the stepwise and processive hydrolysis of duplex DNA from 5’-phosphoryl termini to 5’ mononucleotides. Lambda exonuclease digestion was made using 10x buffer supplied with the enzyme using 6 units (U) of enzyme each 2 µg of dsDNA product with one end modified with a phosphate (M13R5P). Reaction was performed at 37 °C for 30 min.

2.1.5.2 Ligation

T4 DNA ligase (Roche) catalyzes the formation of phosphodiester bonds between neighbouring 3´-hydroxyl- and 5´-phosphate ends in double-stranded DNA. One unit of T4 DNA ligase joins more than 80% of 1µg Rsal digested Lambda DNA in 30 µl 1× ligation buffer after incubation at 15 to 25°C for 16 h. Single stranded DNA from enzymatic digestion was purified using Qiaquick PCR purification kit and re-suspended in 30 µl H₂O. SsDNA, LIG5P and 5PPOLIA3NH2 were first separately denatured in a water bath at 100 °C for 10 min, and then put directly in a water bath at 16 °C. Then they were put in equimolar proportion in 1.5 ml tube adding 10 x incubation buffer from the supplier to 1x concentration and T4 DNA ligase in suitable amount. Reaction was let occur at 16 °C overnight.
2.1.5.3 Enzymatic digestion

The double stranded PCR product was digested with restriction enzymes, following supplier protocols. *Sal* (Roche Diagnostics GmbH) cuts once, recognising the site G↓TCGAC and generating two fragments with blunt ends. One unit of enzyme catalyzes the complete digestion of 1 µg Lambda DNA in 1 hour at 37 °C in a total volume of 25 µl using 1x L buffer supplied with the enzyme. All reactions were performed in these conditions calculating the exact amount of enzyme needed in respect of DNA or bead-DNA to be digested.

2.2 WORKING WITH PARTICLES AND NUCLEIC ACIDS

In working with particles purity of reagents is essential. Buffers were produced using only HPLC water from Aldrich and then filtered with 0.22 µm filters (Amersham Biosciences). Washing steps with paramagnetic particles were accomplished with the help of a neodymium magnet proper rack, while other kind of particles were separated by centrifugation. Particles were stored at 4 °C.

2.2.1 PARTICLES

<table>
<thead>
<tr>
<th>Particle name</th>
<th>Diameter</th>
<th>Core</th>
<th>Surface</th>
<th>Binding capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latex-m Micromod</td>
<td>150 nm</td>
<td>superparamagnetic</td>
<td>NH2</td>
<td></td>
</tr>
<tr>
<td>Nanomag-D streptavidin</td>
<td>130 nm and 250 nm</td>
<td>superparamagnetic</td>
<td>streptavidin</td>
<td>1.5 µg streptavidin/mg particle (60-70 molecules streptavidin/particle)</td>
</tr>
<tr>
<td>Micromer</td>
<td>250 nm</td>
<td>Latex</td>
<td>biotin</td>
<td></td>
</tr>
<tr>
<td>Nanomag-D 250 nm</td>
<td>250 nm</td>
<td>superparamagnetic</td>
<td>NH2</td>
<td>230 nmol/mg</td>
</tr>
<tr>
<td>Kisker polystyrene particles</td>
<td>820 nm</td>
<td>Latex</td>
<td>streptavidin</td>
<td>-</td>
</tr>
<tr>
<td>Roche 0.2-2 µm</td>
<td>0.2-2 µm</td>
<td>superparamagnetic</td>
<td>streptavidin</td>
<td>350 pmol free biotin, 150 pmol biotin labelled oligonucleotide, 10 pmol biotin-labelled dsDNA fragment (1.5 kb)</td>
</tr>
</tbody>
</table>
2. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Dynal</th>
<th>1.00 µm</th>
<th>superparamagnetic streptavidin</th>
<th>2500 pmoles free biotin, 250 pmol 200 bp biotinylated dsDNA, 100 pmol 500 bp biotinylated dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluosphere</td>
<td>1.00 µm</td>
<td>streptavidin</td>
<td>2500 pmoles free biotin, 250 pmol 200 bp biotinylated dsDNA, 100 pmol 500 bp biotinylated dsDNA</td>
</tr>
<tr>
<td>Qdots</td>
<td>24 nm</td>
<td>streptavidin</td>
<td>1-5 biotin molecules/particle</td>
</tr>
</tbody>
</table>

*Table 2. List of particles used.*

2.3 MODIFICATION AND CONJUGATION OF NUCLEOTIDES AND PARTICLES

Chemical modification of particles and nucleic acids was made using materials and protocols from Solulink (Solulink Inc., San Diego, California). The products include SANH (6-hydrazinonicotinamide 290.2 MW), SFB (succinimidyl 4-formylbenzoate 247.1 MW) and 2-HP (2-Hydrazonepyridine 182.1 MW). Modification buffer 10x for Solulink reagents reacting with NH₃ modified particles was phosphate buffer 1 M pH 7.2, 1.5 M NaCl, 1x conjugation buffer was MES buffer, pH 4.7 or pH 6.3 containing 0.1 M MES, 0.9% NaCl. Modifying reagents SANH and SFB were dissolved in organic solvent (DMF) at 50 µg/µl concentration.

2.3.1 MODIFICATION OF PARTICLES

Magnetic particles Nanomag and latex particles Micromer were reacted with SANH in 1x modification buffer following this recipe.

1 ml Nanomag/Micromer
0.1 ml 10x modification buffer
5.8 µl SANH/DMF solution

Samples were incubated in a 1.5 ml Eppendorf tube for 2 hours with gentle shaking. Washing of unreacted chemicals was done with 1x modification buffer. Particles were re-suspended in 500 µl 1x modification buffer. An aliquot of 10 µl was re-suspended in MES buffer for quantification of modified particles.

Quantification of modified particles was accomplished with visualization of nucleic acids linked after conjugation reaction.
2. MATERIALS AND METHODS

2.3.2 MODIFICATION OF OLIGONUCLEOTIDES
Oligonucleotides were modified as follows

30 µl M13F5NH₂ (1 µg =178 pmol)

3 µl 10x modification buffer

1 ul SFB/DMF (50 ng/ul starting from 50 µg/ul and diluted 1:1000 in H₂O)

Samples were incubated for 2 hours at room temperature. Microspin column Amersham biosciences was used to desalt nucleic acid after pre-equilibration in 1x modification buffer obtaining 50 µl product. An aliquot of 10 ul was re-suspended in MES buffer for quantification.

2.3.2.1 SFB quantification
A 1.0 mM working solution of 2-hydrizinopyridine-2HCl solution in 0.1 M MES buffer, pH 5.0 was prepared as follows. Five mg 2-hydrizinopyridine-2HCl were dissolved in 100 µl DMF. 91 µl of this solution were added to a 50 ml conical tube containing 50 ml 100 mM MES Buffer (pH 5.0). Ten µl of SFB-modified nucleic acids solution (~100 pmol/µl in 1x conjugation buffer) were transferred to a new 1.5 ml microfuge tube containing 490 µl 2-HP reagent. Another reaction tube (negative control) containing 490 ul 2-HP reagent and 10 µl of 1x conjugation buffer was prepared. All reaction tubes were incubated at 37 °C for 30 minutes. Absorbance at 350 nm of both reactions was measured in a spectrophotometer using a quartz cuvette.

2.3.3 CONJUGATION REACTION WITH MODIFIED OLIGONUCLEOTIDES AND PARTICLES
Solulink modified particles (1 mg/ml) were reacted with the modified oligonucleotides or modified PCR product in 1x conjugation buffer overnight at room temperature. The proportion particles/nucleic acids in the reaction was 1 streptavidin to 20 biotin.

Streptavidin particles were reacted with biotinilated oligonucleotides or PCR products in 1xWBB for 30 minutes at room temperature. The proportion
particles/nucleic acids in the reaction was 1 streptavidin to 10 biotin.

Storage buffer was used to conjugate Qdots to nucleic acids with a molar proportion of 1:10.

Incubation buffer for biotin/streptavidin binding was 1xWBB starting from 2x Binding & Washing (2xWBB) Buffer which was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.0 M NaCl.

Storage buffer is 1xWBB with 0.1 M NaCl.

2.4 ELECTROPHORETIC MINI-CELL AND DETECTOR SET-UP

2.4.1 MINI-CELL

The mini-cell and its lid were turned in a PMMA piece following the model in fig.2.1. Depending on the experiment, buffer solution used was Tris-HCl, pH 7.00 with 1 M or 0.1 M NaCl, 1x WBB, 0.1x WBB.

![Figure 2.1 Electrophoretic mini-cell project.](image)

2.4.2 DETECTOR SETUP

For experiment involving current measures, the dedicated cell was built as depicted in Fig. 2c. The silicon membrane frame as described above was placed between two sealing silicone rubber o-rings generating two chambers separated by the pored membrane. The two compartments were maintained vertical. An
electromagnet was positioned nearby the pore inside the top chamber. Electrophoretic solution was loaded into the two compartments. Platinum wire mobile electrodes were dipped in each chamber solution and connected to a current/voltage converter and a power supply set at 5 V. Data acquisition and processing was made with LabJack (LabJack Co., Lakewood, CO) and DAQFactory (Azeotech Inc., Boulder, CO).

Figure 2.2 Detector set-up.

2.5 THE ELECTROMAGNET

An electromagnetic device was home made in order to have a localised tip magnetic force acting on the superparamagnetic particle positioned on the pore. A 0.5 mm copper wire was coiled two folds on a paramagnetic needle, then the magnetic tip was enveloped into a paraffin film (Parafilm M, Sigma-Aldrich). Connection to a power supply typically working at 1.5 V allowed a 0.25 A current through the wire. Magnetic tip positioning nearby the membrane pore was achieved with the aid of a mechanical micromanipulator (Filippi, Padova, Italy).

2.6 PORE FABRICATION

2.6.1 MEMBRANE PRODUCTION

Silicon (100) wafers (Jocam srl, Milano, Italy) 100 nm Si₃N₄ coated on both sides were used to fabricate silicon nitride membranes 56 µm wide into frames 15 mm sided. An optical mask was made starting from a squared glass sheet (75x3 mm) coated with 300 nm chrome. Polymethylmetacrylate (PMMA 950 KDa) positive resist (Shipley) was spinned on the sheet followed by electron beam exposure of the pattern shown in fig.2.a. PMMA was developed in metylisobutylketone and 2-isopropanol (MIBK:
IPA=1:1) solution (Shipley) and chrome layer was removed by chemical etching. Resist was completely removed in hot acetone solution. Fig. 2.5b depicts the lithographic steps to obtain a Si₃N₄ membrane. The optical mask was used to expose silicon nitride wafer coated with S1828 optical resist (Shipley) under UV light into optical stepper for MUV lithography (I). The wafer was further developed in MF322 solution (Shipley) (II). Silicon nitride exposed after developing was etched with O₂/CF₄ = 28.5/1.5 (sccm), at 150 W power and 250 V Bias for 2 minutes in Systec RIE 600 System (III). After total removing of resist with acetone (5 min at 50 °C), exposed silicon was wet etched in 5 M KOH solution at 80 °C for 10 hours (IV).

![Figure 2.3 2D-project of the mask for UV stepper (a) and lithographic steps for Si₃N₄ membrane](image)

### 2.6.2 LITHOGRAPHY PRODUCTION OF PORES

Pore production was made starting from the frame depicted in fig. 2.3 IV. The entire process is depicted in fig. 2.4.

Chrome and gold were growth in electrolytic cell on Si₃N₄ membrane (a). On the metal layer, the negative resist SAL601 (Shipley) was spun at 2000 rpm for 1 minute (b). The wafer was baked on hot plate at 105 °C for 1 minute. Electron beam exposition of the pattern was made (c) and then pre-baking of the sample at 105 °C for 1 minute. Parameters for exposition were set at 50 pA current, 0.1 ms dot dwell time and 500x500 µm² exposition field. After exposition post-baking of the sample was made at 105 °C for 1'15”. Pattern was developed with MF312 for 1 minute and washed in H₂O (d). Before electrolytic growth of Nickel, a RIE etching was made for 20 seconds to remove
residual resist following these parameters: 50 Watt power, 50 V Bias, 40 mTorr pressure, O$_2$/CF$_4$= 28.5/1.5 (sccm). Nickel growth was made at 150 mA current for 15 seconds obtaining a layer of 50 nm (e). Removal of SAL601 forming the dot was made by RIE-O$_2$ following these parameters: 100 Watt power, 200 V bias, 100 mTorr pressure, for 7 minutes (f). The exposed area was removed by physical etching in RIE instrument (g).

First, the gold layer was removed with Argon ions following these parameters: 100 Watt power, 270 V bias, 10 mTorr pressure, Ar/CF$_4$=28.5/1.5 (sccm) for 5 minutes. Second, the chrome layer was removed 120 Watt power, 305 V bias, 10 mTorr pressure, Ar/CF$_4$=28.5/1.5 (sccm) for 10 minutes. The last step was the removing of Si$_3$N$_4$ layer by etching with O$_2$/CF$_4$ = 28.5/1.5 (sccm), at 150 W power, 250 V Bias and 35 mTorr pressure for 2 minutes (h).

Figure 2.4 Panel showing lithographic steps for pore production in Si$_3$N$_4$ membrane.

2.6.3 FIB PRODUCTION OF PORES

Single pores were milled without further processing of the wafer obtained after lithographic process described in paragraph 2.6.1 in NOVA 600i system (FEI Company, Hillsboro, USA) employing focused ion beam (gallium ions) at 10 KeV energy high tension and 50 pA current. Pores wide from 450 to 800 nm were obtained with time exposures from 10 to 60 seconds respectively (Fig. 1b-c). Pores array 700 nm diameter were milled employing 10 KeV FIB EHT and 500 pA current, dose matrix was 16000 uA/cm2. Upside down turning of wafer into the FIB stage was done to ratify pore opening on both sides. To avoid unspecific binding of nucleic acids and particles to the
wafer surface and unspecific amperometric signalling during electrophoresis due to possible residual metal on the silicon nitride surface, no gold sputtering before milling of pores in the FIB instrument was carried out. Imaging of the entire process was simultaneously taken with integrated SEM instrument. The wafer was observed after turning upside down into the FIB stage to ratify pore opening on both sides.
PART III
RESULTS
This chapter is divided in three sections.

First, I present a new approach to the characterization of biomolecules through small pores, the project of a general device concept and how the technical problem could be dealt with.

Secondly, I present the result of the experimental work that has been carried out to develop the tools and methods for the implementation of the new device concept.

Finally, the results of the assembly of the above elements for the production of two practical applications are presented.

### 3.1 THE SYSTEM PROJECT: A NEW APPROACH

In recent years, the technique of Coulter-counter has been improved to detect single molecules flowing through a small pore, driven by electrophoresis. The transient crossing method limits the investigation potential of the tool. In fact, (i) the molecules cross the pore at a speed that is very challenging at the present state of the art of detector electronic devices, (ii) the molecules can be sensed only by a pore of comparable dimension and (iii) no further analyses can be accomplished on the same molecule once it has crossed the pore. Confining and controlling biomolecules inside the pore while detection is carried out could cut down most technical constraints and significantly broaden the possibilities of characterization. The aim of this project is to develop strategies and methods to stably locate and manipulate them in the sensing pore.

Fig. 3.1 shows the basic principle of molecular trapping within a pore that I planned. A linear target molecule, such as DNA (shown as a red line in the figure) is stably linked on both sides of the pores with objects of a size larger than the pore. Since the complex is stable and the linked objects have a size that is much larger than the target molecule, manipulation, pore closure/opening, possible interactions, stretching and other forces, and in general several characteristics and behaviours of the molecules can be studied at
the pore interface. The realization of such a device would pave the way to the development of novel pore-based analytical tools.

3.1.1 SENSOR CONCEPT AND ITS APPLICATIONS

In order to translate the principle of the new approach into practice, I have focused on two applications.

In fig. 3.2, the basic feature of the major application of this project is presented. A silicon nitride ($\text{Si}_3\text{N}_4$) membrane produced by lithography technique in a silicon frame is used to separate a solution generating two chambers. Single or multiples nanopores are milled by focus ion beam (FIB) inside the $\text{Si}_3\text{N}_4$ membrane (a). The aperture generated permits the contact of the solution from the top chamber to the bottom chamber. If a potential is applied by electrodes dipped in each chamber an ionic current is generated. Superparamagnetic particles larger than the pores are loaded in the top chamber (b) and reach the apertures exposing a portion of their surface in the bottom chamber (c). The magnetic particles are functionalized with nucleic acids that will interact through the nanopore/s with particles or molecules loaded in the bottom chamber while they are settled in the pore (d).

The sensing strategy used in this work is based on the ionic current passing through the pore, whose value defines either an “ON” or an “OFF” state triggered by molecular interaction. The general concept is that the reciprocal recognition of two molecular species, reacting through a membrane pore, may produce a distinct signal by obstruction of the pore lumen (e, f). Pore closure (OFF) is recorded by measuring current passing
through the pore. In order to produce a stable pore blockade, the reacting molecular species are linked to relatively large buoys that cannot cross the pore. The recognition event elicits a distinctive response by the amperometre, opening the field to the detection of several biomolecular events.

In this project the sensor is interrogated by applying an electromagnet that cannot remove the paramagnetic bead settled in the pore if DNA hybridization has occurred (OFF) and therefore the paramagnetic bead is constrained in the pore by molecular buoys on the other side (f). The alternative sensor operation is obtained introducing a third molecular species (target) (g) having high affinity to one of the reacted molecular species that removes the block in the pore by displacing the less specific molecule (h). The sensor is interrogated by applying an electromagnet that can remove the paramagnetic bead settled in the pore (ON) only if DNA strand displacement has occurred and therefore the paramagnetic bead is not constrained in the pore by molecular buoys on the other side (i).

Fig. 3.3 shows how the separate components of the device are assembled to obtain the amperometric sensor. The silicon frame containing the nanopore/s is sealed between two o-rings generating the chambers of the electrophoretic cell and the electromagnet is fixed upon the upper chamber by a micromanipulator.
Figure 3.2 Sensing strategy of the project

Figure 3.3 Representation of the sensor components (electromagnet, pore membrane and electrophoretic cell).

The sensor technique is exploited in a second application, the selective transport of molecules by the magnetic particle from the bottom chamber to the top chamber crossing single or multiple pores. A small hydrostatic pressure is maintained in the upper compartment to ensure a small flow along the pores and limit passive diffusion of target analytes from the lower compartment to the upper compartment. A drawing at the nanoscale of the device used in this work is depicted in fig.3.4. The core elements of the device are paramagnetic beads (named “transporters” below) that have been functionalized to specifically recognize molecules (targets) through pores of the membrane (a). The transporters are delivered to the pores of the silicon nitride membrane (that are smaller in diameter and therefore cannot be crossed). They are intended to stably locate at the pores and transport objects from the lower to the upper compartment, against the flux. The through membrane transport is composed of three basic steps. First, the transporters are delivered to the pores to expose their specificity determinants in the lower compartments generating a OFF signal (b). Second, the specificity determinants react with their target in the lower compartment, forming stable transmembrane complexes (c). Third, the loaded transporters are removed by the
electromagnet from the pore generating an ON signal and other, unloaded, transporters (d) take their place.

![Diagram](image)

**Figure 3.4** Schematic representation showing (a) the selective transporter recognizing a molecule through the pore; (b) delivery of transporters to the pore by current flux generating an “OFF” signal; (c) formation of stable transmembrane interaction between transporter and target molecule; (d) removal of transporters against the flux generating an “ON” signal and opportunity for an unloaded particle to settle in the pore.

3.1.2 TECHNICAL CONSIDERATIONS

In the following paragraphs technical considerations for the implementation of the single elements of the device are given.

3.1.2.1 Biomolecular complex

In this project the biomolecular complex is formed by a target and a probe nucleic acid complementary in sequence. The types of construct considered were in order (Fig. 3.5): (a) two single stranded DNA one of those is a linearised PCR product linked by a probe molecule; (b) a linearised PCR product linked to the second particle by a probe sequence; (c) two complementary oligonucleotides; (d) three oligonucleotides involved
3. RESULTS

in a strand-displacement. As it will be discussed later the strategies employing oligonucleotides were preferred for the first implementation of the developed technology.

![Figure 3.5 Types of DNA construct connecting the particles](image)

Several protocols to link particles to nucleic acids were considered and tested to find the most appropriate. The principle adopted was based on these considerations: the complex formed has to be very stable in different conditions, the linkage has to be highly specific and with little steric encumbrance. Methods to link nucleic acids to particles include adsorption of molecules on particle surface, reaction between two different chemicals attached on the two different species considered and affinity reaction based on hydrogen bonding between multiple units molecules. The first method was discarded because it does guarantee neither stability nor specificity. It is noteworthy that the streptavidin/biotin complex is very reliable for its stability and its efficiency. It is one of the most chosen methods to tether particles to single molecule nucleic acids for force measurements acting on the helix (Smith et al. 1996). In comparison, the yield of other chemical attachment methods is lower. The general concept of a nucleic acid molecule as linker between two particles has to be adapted to the linking approach chosen and to the intrinsic properties of the linking molecule itself. One of the objects has to attach to the DNA in a later instance, because in the final implementation the second object has to react from a trans-chamber with the first particle. If a PCR product is considered as linker the chain could be stretched.
throughout a narrow pore, while if oligonucleotides are considered it is compulsory to have at least one of the two interacting objects protruding over the membrane in a trans mode.

The amount of PCR product is a critical parameter. The maximum yield of a PCR reaction is around 350 ng/µl, which implies for a 600 bp (base pairs) PCR product a number of moles equal to 0.884 pmol/µl. Binding capacity of 1 mg of particles range from 150 pmoles of biotin labelled oligonucleotide or 10 pmoles of 1.5 kilobase (Kb) of dsDNA for Roche streptavidin particles, to 2500 pmoles of free biotin or 250 pmoles of 200 bp dsDNA for Dynal streptavidin particles. Efficiency decreases with dimension of particles so that, e.g. a 150 nm particle has 60-70 molecules of streptavidin on its surface. This implies that to produce 100 ng of particles completely covered by nucleic acid, PCR product employed should exceed 100 pmol to be effectively linked to the particle chosen, an amount not easily yield in massive production. On the contrary, working with oligonucleotides gives no problem in administration of complex final quantity, because they are produced in large quantity (e.g. 200 µl containing 100 pmoles/µl).

Hybridisation of single stranded nucleic acids is ruled by ionic strength of the solution, temperature and affinity of the two strands. The two strands are kept together by hydrogen bonds between complementary sequences. The higher the sequence affinity the stronger is the stability of the double strand. When temperature gives energy higher than the sum of the energy of the hydrogen bonds, the double strand is broken (denaturation). Usually sodium salt concentration (SSC) defines the stringency of the reaction that is the increased specificity of hybridization: the more the concentration, the less the specificity. Melting temperature changes depending on salt concentration.

Strand displacement indicates a substitution of a single chain in the double helix with a more specific one. In detection systems, the more specific sequence is the target. It can be a short sequence oligonucleotide or a portion of a long chain nucleic acid, usually a PCR amplification product. The longer the probe sequence, the more specific the interaction, but the higher the temperature reaction required. Melting temperature of the perfectly matching sequence is 46 °C and melting temperature of imperfectly matching sequence is 28 °C: strand displacement reaction has to take place at less than
28 °C. Otherwise, using an incubation temperature comprised between 28 °C and 46°C, less complementary oligonucleotide displaces automatically.

### 3.1.2.2 Particles

Particle choice depends on the application required. The particle controlled by the electromagnet has to be superparamagnetic because no residual magnetization has to last in it, mainly to prevent particle aggregation. Superparamagnetic particles are widely used mainly as rapid separators agents of biomolecular species. Magnetic particles, due to their high manipulability, were used for really diverse purposes such as gene delivery (Planck et al. 2003), micromanipulation of biological molecules by cantilevers or optical tweezers (Gosse and Croquette 2002) or as ordered array separators for nucleic acids (Doyle et al. 2002). Here the magnetic property of the particle is exploited to manipulate it with an electromagnet inside an aqueous solution.

Particle dimension depends on the complex construct. Particles less than 130 nm in diameter need a supermagnet to be manipulated. To reduce complications in project set-up we decided to use particles bigger than 130 nm. Dimension of particle is not important for generating ON/OFF signal as pore diameter can be chosen accordingly. But the less is the diameter of the particle the more is the weight of the nucleic acid driving force. If the driving force is the electric charge of the DNA there is no need to use gravity or pumps and there is the possibility to built an horizontal cell. Deciding to use electrophoresis as carrying agent implies considering the driving capacity of charged DNA. To have a single molecule detection device it should be mandatory to link one DNA to each bead but in this case the DNA has to be very long and the particle very small. Otherwise, electrophoresis could be used only to extend DNA and not to drive particle in the pore, so that there is no necessity to modulate particle dimension and weight in dependence of DNA charge. In case of complex formed with oligonucleotides there is no need of extension at all. Another important factor is the chemical composition of the particle. First, the specific weight of the particle has to be equal or higher than buffers employed. The surface charge has not to contrast the moving of the particle in the solution this means that no net ionic charge should be on the surface. Then the surface has to be hydrophilic because the project is developed in aqueous medium. Particles have to tolerate different chemicals and pH conditions, first in the
modification reaction environment, second in the hybridization buffer and third in the electrophoretic buffer if different. Particles used in this project have an hydrophilic surface and specific weight close to the water.

3.1.2.3 Current measures

Electrophoresis is used to separate molecular species that differs in charge: when associated to a matrix (agarose gel, acrylamide gel) discrimination depends on mass and shape too. Negative ions in buffer solution confer negative solvatation spheres to nucleic acids which migrate towards positive electrode. The idea of detecting molecules by measure current changes while they are forced to pass an obstacle is quite old. The change in conductance of a small electrolyte channel is the principle for particle detection in Coulter-counter. Today evolution of technology permits production of 1 nm synthetic pores and incorporation in teflon membranes of 2 nm proteic pores, but the exploited principle is the same, while the sensitivity of the instrument changes. The diminished dimension of the lumen of the pore crossed permits to receive information on molecules, which are similar in dimension, namely single and double stranded nucleic acids. In this work, I chose to tether DNA to a large buoy to overcome the need of powerful detection systems and of slowing down the velocity of crossing molecule. Because the bead governs the movement, the studied species is no more pulled by electrophoresis voltage inside the pore but only by gravity. Bead in addiction is able to generate an obstruction on very large pores, which permits to be relatively independent on the background signal because voltage applied and consequently current measure can be chose at will. When measuring a current in a solution it is mandatory to have ions carrying the charge and moving from one electrode to the other. For very small apertures like 2 nm pore, the charge transported has to be very high in consideration that low ions concentration can cross it. On the contrary, in this project, ionic species concentration can be modulated at will.

3.1.2.4 Trans-membrane interaction

One important characteristic of this system is that super-paramagnetic beads in the upper chamber has to expose a considerable part of their surface on the trans side of the Si₃N₄ membrane to interact with particles of similar diameter loaded in the other side.
This is particularly true if the nucleic acids linked to the surface are shorter than the thickness of the membrane (100 nm in this project). Even choosing a nucleic acid much longer than 100 nm it could be difficult to have it in an extended configuration throughout the pore. In this project I preferred to balance dimension of the pore towards dimension of the magnetic particle to let biomolecules available on the other side of the membrane rather than try to extend a long DNA molecule throughout the pore. This has been possible thanks to the properties of the detection device in which the size of the pore does not limit the sensor applicability. As depicted in fig.3.6, the diameter of the pore should be modulated on the shape and diameter of particles. Relative measures are proportional to real measure. The dark section represents a pore into silicon nitride membrane respectively of 500, 600 and 700 nm diameter. Brown circle is paramagnetic particle of 1 µm and grey circle is latex particle of 0.8 µm. The orange dotted line indicates theoretical point after which particle is exposed in trans side. In all cases, particles encounter each other but the most favourable setup is the 700 nm pore (c), as it will be clarified in result section.

![Figure 3.6](image)

**Figure 3.6** Trans-membrane interaction of particles depending on diameter of pore: (a) 500 nm, (b) 600 nm, (c) 700 nm.

### 3.2 THE SYSTEM SETUP: SINGLE COMPONENTS

#### 3.2.1 MANIPULATION OF NUCLEIC ACIDS

The following paragraphs present results on the production of long and short nucleic acids both in single and in double stranded format, and their use to obtain isothermal switching between hybrid conformations.
3. RESULTS

3.2.1.1 Oligonucleotide hybrids and displacement

The hybridization between the complementary oligonucleotides M13R and M13RCfluo was verified by acrylamide gel electrophoresis with ethidium bromide staining and strand displacement of mismatching oligonucleotides by agarose gel electrophoresis. Hybridization was accomplished by cooling of incubation solution to room temperature. In Fig. 3.7a, the major bands in lane 2 and 3 demonstrated the formation of an hybrid of the two single stranded oligonucleotides (shown in lanes 1 and 4 before hybridization). The extra band in lane 3 was presumably due to residual ss-oligonucleotide that did not hybridized due to the absence of a preliminary high temperature pre-treatment needed to denaturate secondary structures. The same gel was visualized under UV light before and after ethidium bromide staining. Before ethidium bromide only oligonucleotide incorporating a fluorophore can be clearly seen (lane 2, 3 and 4), while non-fluorescent oligonucleotide is only visible in lane 1 of the Ethidium bromide stained gel (the stain is faint because EthBr stains dsDNA inefficiently). To assess effectiveness of strand displacement a similar experiment was set up. As shown in fig. 3.3b, the fluorescent oligonucleotide M13RCfluo when mixed with the non-perfect hybrid M13R/M13RCA (lane 2) displaces M13RCA forming the green fluorescent, perfect hybrid M13R/M13RCfluo (lane 3) which migrates slower than M13RCA flue alone (lane 4). Because particles cannot tolerate high temperatures and fig3.4a, lane 4 showed that hybridization is not well accomplished without pre-heating treatment, it was decided to separately produce the ds oligonucleotide and then link it to one of the particles (namely, the magnetic one). In the case of strand displacement, the method was the same.
3.2.1.2 Production of long DNA

Long double stranded DNA was produced by PCR amplification with Taq polymerase. The product was visualized by gel electrophoresis. In fig.3.1a, lane 2 and lane 3, the fluorescent band is the dsDNA product, 600 base pairs (bp) in length, compared with the DNA ladder marker VI (lane 1). Amplification of the negative control (no template; lane 4) gave no amplification, as expected.
PCR amplification with modified oligonucleotides as primers gave similar results, without influence on the migration pattern. 5'-P modifications were introduced in one primer to allow strand selective nuclease digestion.

Lambda exonuclease enzyme digested a single strand (ss) of the PCR product with a M13R primer phosphorilated at 5’. Silver staining of the digestion products was used to visualize single strand DNA in denaturing acrylamide gel. In fig.3.8b, lane 2, 3, 4 and 5 the arrow shows a slow migrating band that corresponds to the single stranded PCR product. It migrates slower than dsDNA due to secondary structures formation. Sample loaded after subsequent digestion incubation times of 5, 15 and 25 minutes, showed increased activity of exonuclease. The dsDNA band was fully digested after 25 minutes, as is visible in the gel electrophoresis (lane 5). The product was used after purification in ligation reaction with probe LIG5P. LIG5P is an oligonucleotide whose sequence is complementary on one-half to the ssPCR product and on the other half to the oligonucleotide POLIA (fig.3.9). It is the connector between two nucleic acids which in the will be linked to two different particles. In fig.3.10 lanes 4 and 5, the fragment evidenced by the upper arrow, migrated slower than PCR product (lane 2) and lambda exo digested (lane 3), indicating that ligation was effective.

![Ligation of two single stranded nucleic acids](image)

*Figure 3.9 Illustration of the ligation of two single stranded nucleic acids (ssPCR product and POLIA) mediated by a complementary oligonucleotide (SDL4).*
As further control, Dot-blot technique proved the hybridisation of probe SDL4BIO1 to the single stranded PCR product. In fig.3.11, spot 1 is the standard non containing nucleic acids. Spot 2 and 3 are positive controls with 10 pmol biotinilated and 1 pmol oligonucleotides respectively. The dark spots (4 and 6) indicates that the probe hybridized to the PCR product in comparison with the negative control (5), which does not contain the biotinilated oligonucleotide.

3.2.2 MODIFICATION AND MANIPULATION OF NUCLEIC ACIDS AND PARTICLES

3.2.2.1 Modification of nucleic acids and particles

In this paragraph methods to modify nucleic acids and particles and methods to
3. RESULTS

attach them each other are reported.

First, the chemical method on hydrazone bond forming between SANH and SFB modified objects was assayed, using protocols provided by Solulink. Solulink attachment protocols were applied to modification with SFB of PCR product after and before lambda exonuclease digestion and on oligonucleotides used as primers for PCR. An oligonucleotide without NH₂ terminus was used as negative control. Standard positive sample was the product SFB/2-HP (1 mM), which forms a stable hydrazone bond absorbing at 360 nm length.

Tab. 3.I shows absorbance values of positive control, negative control, sample modified with SFB to which 2-HP was added, and a control for 2-HP absorbance. Fifty nmoles of SFB modified nucleic acids were reacted for quantification with 500 nmoles of 2-HP. Formation of the complex SFB/2-HP was used as positive control for hydrazone formation. The absorbance value of the sample is in between the absorbance values of negative control and positive control. The absorbance of 2-HP was set as zero to quantify oligonucleotide modification. The difference between absorbance value of negative control and standard 2-HP, namely 0.0144, was interpreted as a spurious value for oligonucleotide. Subtracting this value to absorbance value of the sample, and comparing with positive control value, a percentage of oligonucleotide modification equal to 50.3% was obtained.

<table>
<thead>
<tr>
<th></th>
<th>2-HP (1mM)</th>
<th>Sample + 2-HP (1mM)</th>
<th>Ctrl- + 2-HP (1mM)</th>
<th>SFB+2-HP (1mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs₅₆₀</td>
<td>0.0500±0.0000</td>
<td>0.1100±0.0050</td>
<td>0.0644±0.0050</td>
<td>0.1900±0.0050</td>
</tr>
</tbody>
</table>

Table 3.I Absorbance values at 360 nm of SFB modified oligonucleotides after reaction with 2-HP to form the stable hydrazone molecule.

To assess attachment of ds PCR product to particles after formation of hydrazone bond between SFB and SANH, respectively attached to nucleic acids and to particles, an enzymatic assay was used. On this assumption, if any molecule of PCR product has ever been attached to particles, with restriction enzyme action should has been released from the particle surface. The fragment released after recovered in the first washing step, was run in gel electrophoresis. The concept is explained in the panel of fig.3.12.
Before this test, DNA free in solution was digested. Fig.3.13 shows a 600 bp PCR product before (lane 5) and after (lane 4) digestion with the restriction enzyme SalI. The formation of two distinct bands visible in lane 4 proved that enzymatic digestion worked. The enzyme SalI was chosen because generates two fragments the shorter of those is the one, which remains linked to the particle after digestion. This approach facilitated the visualization of the digested fragment recovered after first washing of particles. In lane 2 is shown the first recovered wash of magnetic particles added to a PCR product without NH₂ terminus and digested by SalI: the two visible bands are perfectly matching the pattern of PCR digested product in lane 4, meaning that the magnet is suitable to separate digested fragments from a solution containing particles. In lane 3 is shown the first recovered wash of magnetic particles attached by Solulink chemistry to the PCR product after digestion by SalI: no fluorescent bands were detected. This result was interpreted as unsuccessful attachment of particles to dsDNA by Solulink chemistry or as inability of the enzyme to cut in PCR products attached on particles. Repetition of the same experiment showed never a positive result.
The experiments were repeated changing incubation buffer pH from 4.7 to 6.3. In fact, use of acidic pH generated the formation of turbidity in separation washing solution. The behaviour was explained by the Micromod particles manufacturer as a destruction of the particle shield by pH equal to or lower than 4.7. Modification of incubation buffer did not change the result shown in fig.3.13 as fig.3.14 shows. In lane 4 is visible a slower migrating band after digestion of PCR product (lane 3): the same band is not visible in lane 2, indicating that no PCR product was linked to particles or that enzyme was not able to cut.

An oligonucleotide with on one end NH₂ molecule and on the other end a fluorescent molecule (FPOLINH₂) was used to visualize at optical microscope the
formation of the hydrazone bond both with solution at pH 4.7 and at pH 6.3. Different quantities of Fluorescein functionalized oligonucleotide were used as standards of fluorescence (fig. 3.15). Using a numerical conversion of standards and samples, a quantification of the fluorescent nucleic acids linked per particle was obtained. No fluorescence was detected after mercury lamp excitation and comparison with standards, stating that Solulink chemistry could not be used to link DNA to particles.

![Image](image_url)

**Figure 3.15 Fluorescent standards photographs taken under epifluorescent microscope after 1 second exposition at the camera objective.**

(a) 50 pmoles; (b) 15.6 pmoles; (c) 3 pmoles; (d) 1.8 pmoles.

### 3.2.2.2 Attachment of nucleic acids to particles

Streptavidin/biotin linking method was tested in several ways and on several particles showing the expected efficient rate. Quantification of fluorescent nucleic acids linked was made by comparison to the standards of fluorescence reported in fig.3.15. Both Roche and Dynal particles linked the oligonucleotide quantity reported by the manufacturer that is 150 pmoles/mg for Roche particles and 1000 pmoles/mg for Dynal particles. PCR fluorescent product was linked only to Roche particles. In fig. 3.16 is shown an epifluorescence photograph of Roche magnetic particles after linking of a PCR product with on one end a biotin and on the other end a fluorescein. Even after several washes, the green fluorescence is persistent on the particles (a). In negative control with fluorescent FPOLINH$_2$, no fluorescence was detected (b). Estimated moles of PCR product linked were 20 pmoles/mg of Roche particles.
3. RESULTS

![Image of Roche streptavidin particles functionalized with green fluorescent biotinilated PCR product (a) and with FPOLINH as negative control (b).]

3.2.2.3 Attachment of particles each other using nucleic acids as linker molecules

Different attachment protocols were tested to verify if two different particles could be linked each other with a nucleic acid in-between as linker molecule. Fig.3.17a shows Roche magnetic particle surrounded by gold nanoparticles linked by thiol chemistry. The intermediate is a ds oligonucleotide modified on one end with a biotin and on the other end with SH (M13Rbio/SHM13RC). For negative control a biotinilated nucleic acid without SH terminus was used: no gold particles were detected on Roche particles (fig.3.17b). Fig.3.17c shows the attachment of polystyrene Micromod particles to Roche magnetic particles mediated by ds oligonucleotide with biotin at both ends. The binding capacity was from 1 to 5 Micromod particles per Roche one. In the negative control, a non-biotinilated double stranded oligonucleotide was used: as shown, Micromod particles were not able to attach to Roche ones (fig.3.17d). As the pictures show, thiol attachment chemistry guarantees a complete covering of the magnetic particle by the gold particles, while only a maximum of 5 latex beads linked to one Roche particle.

![Image of Roche particle surrounded by gold particles (a) and its negative control (b). Roche particles linked to Kisker 250 nm latex particles (c) and its negative control (d).]

For the easy visualization by optical microscopy of interaction occurred between
different particles, fluorescent particles Fluosphere and Qdots were introduced in the protocol. In this way, samples were observed by epifluorescence microscopy without need of the preparation and mounting needed for TEM or SEM imaging. Only Dynal MyOne streptavidin C1 as paramagnetic particles were used for these experiments due to the above reported characteristics. Fluosphere and Qdots were linked to Dynal using the dsoligonucleotide M13Rbio/M13RCbio as linker bridge. To avoid cross-linking between Dynal particles, Fluosphere and Qdots were introduce one hundred times more concentrated.

Fig 3.18 shows Dynal particles completely enfolded by Qdots while only one or two Fluosphere were detected per Dynal particle. While Qdots circumvent the entire surface of the particles, only about 70% of Dynal MyOne linked to Fluosphere. All considering, linking two comparable diameter beads was 2/3 less efficient than linking particles with diameter difference of 50 times.

![Figure 3.18 Dynal particles interacting with Qdots (a) and Fluosphere (b).](image)

Strand displacement of mismatching oligonucleotides was tested on particles. The biotinilated dsoligonucleotide M13Rbio/M13Afluo was linked to Dynal streptavidin: the fluorescent product is shown in fig.3.19a. After incubation with Qdots/M13RCbio, green fluorescent Dynal particles became red fluorescent (fig.3.19b), with 100% yield. The experiment assess that the substitution of the fluorescent mismatched oligonucleotide with the red fluorescent Qdot carrier of the matching oligonucleotide was complete.
3. RESULTS

Figure 3.19 Strand displacement on particles. Particles fluorescence due to attached dsoligonucleotide (a) become red fluorescent after strand displacement with Qdots modified oligonucleotides (b).

3.2.3 PARTICLES

TEM and SEM assessed size uniformity of particles. As shown by fig.3.20a Roche are not uniform particles measuring 1 µm (as declared by the manufacturer) because the size range goes from 0.2 µm to 2.0 µm. Dynal instead are uniform particles measuring 1 µm as shown in fig.3.13b.

Figure 3.20 SEM and TEM photographs for comparison between Roche particles (a) and Dynal particles (b) size uniformity.

When the pore in the membrane is blocked by a particle exposing out of it in the trans-side, different kind of particles, which can be even smaller than the pore diameter, can be introduced in the bottom side. Fig.3.21a shows 0.8 µm Latex Micromod particles. To facilitate visualization some of the particles were chosen fluorescent like
Dynal Fluosphere (fig. 3.21b) and Qdots emitting at 610 nm length (fig. 3.21c).

Figure 3.21 Particle types for trans-membrane interaction. (a) Micromod latex particles of 800 nm diameter; (b) Dynal Fluosphere fluorescent particles of 1 µm diameter; (c) Qdots particles of different colours depending on diameter.

3.2.4 ELECTROMAGNET

A magnetic force can be exerted by a rare earth magnet or an electromagnet. The most powerful natural magnet is made of Neodymium. Electromagnet can be easily built and a localized field can be generated in a very confined space. Many tests were done with flat neodymium magnet, but due to the low accessibility of the particles nearby the pore, it was never able to act on them. The electromagnet was built coiling two folds a copper wire of 0.3 mm along a 3 cm paramagnetic needle (Fig. 3.22).

Figure 3.22 Hand-made electromagnet: copper wire is coiled around a ferromagnetic tip. Each end of the wire is connected to a power generator.

3.2.4.1 Electromagnet activity on particles in free solution

Electromagnet activity was evaluated under optical microscope at 160x
3. RESULTS

magnification. One microlitre solution containing one hundred Dynal streptavidin particles was put on a microscopy slide while the magnet was positioned at different distances from the surface of the drop. Current passing in coils was 0.22 mA. The electromagnetic force acted in a range of action of maximum 1 mm distance from the 1 µm particle. Superparamagnetic Dynal particles presented a different behaviour depending on buffer solution in which they were immersed. They tended to aggregate in chain or in globular features of several numbers of particles during electromagnetic influx. The introduction of a surfactant like Tween 80 induced globular structures formation of 10 to 20 particles (fig3.23), which were maintained even after the electromagnet was switched off. Changing NaCl concentration from 0.1 to 1.0 M increased the aggregation of particles when the electromagnet was on.

Figure 3.23 Globular agglomerates of particles after electromagnet activation in 0.1 M NaCl and 0.1% Tween 80 buffer.

3.2.4.2 Electromagnet activity on particles in pores

In tests conducted after settling of the particles on the array of pores different behaviours were observed. The bigger the diameter of the pore the less the ability of the magnet to remove the particles. In particular, chain-like structures between particles formed when electromagnet was activated and particles were never removed from pores wider than 650 nm. They were almost totally removed from pores from 400 to 600 nm diameter dimension. A correlation between volume of solution weighting on the particles and the ability of the magnet to remove particles was observed: when one µl of solution was on the membrane the particles were easily removed from the thinner pores, while with amount of solution from 5 µl on, the electromagnet was scarcely efficient. When working with the magnet on particles settled in single pores, removing of particles
was accomplished, even if with chain formation, from pores of all diameters considered.

3.2.5 ELECTROPHORETIC CELL

The sequence of the experiment in the electrophoretic cell is reported in fig. 3.24. On the bottom piece of the electrophoretic cell an o-ring punctured with a tip and with the platinum electrode diametrically opposed was placed. On the o-ring was put the membrane with backside up and on it the second o-ring with the second platinum electrode punctured. The entire complex was sealed with a cap fixed by four plastic screws. One hundred µl of electrophoretic buffer were loaded in the bottom chamber. Two tweezers connected to the amplifier crimped the electrodes using the screws as stable support. One hundred µl of electrophoretic solution were loaded on the upper chamber. Power was turned on and current measured. First magnetic particles were loaded from the top open space by a tip, then waiting for pore closure stated by current measure diminishing. Introduction of the electromagnet tip in the top chamber was the next step, while verifying that no changes in current measure happened. Power was turned off and second kind of particle was loaded in the lower chamber. After incubation of 15 min, power was turned on to verify that no changes in current measured have occurred. For strand displacement experiment, voltage was turned off again and the strand displacer oligonucleotide was loaded in the bottom chamber. After an incubation of 3 hours in wet conditions, current was measured again. Electromagnet was turned on with power off and then current measured again.
3.2.6 SYNTHETIC MEMBRANE

A Si$_3$N$_4$ membrane was produced starting from a silicon wafer covered on both sides with 100 nm layer silicon nitride. Using lithography methods, a window containing a membrane of 56 µm side was produced in the silicon frame.

Starting from the Si$_3$N$_4$ membrane two methods were contemplated to made a pore: standard lithographic technique and direct ion milling. To produce a circular pore into a window with lithographic techniques is necessary to expose a negative resist as SAL601 to electron beam to obtain a pillar and proceed with electrolytic growth (fig. 3.25a, b, c, d). The critical passage was the removing of the exposed resist after electrolytic growth.
of Nickel. Several methods were tested starting from hot acetone solution, to nanoremover developer, to oxygen RIE. No good results were obtained, as it is visible from the photographs (fig. 3.25e). This was due to the chemical properties of the resist, which after exposition to electrons became a polymer very hard to remove.

![Photograph](image)

Figure 3.25 Some of the lithographic steps to produce a nanopore. (a-b) Removal of negative resist SAL601 after exposition to SEM reveals the pillars. (b-c) Electrolytic growth of Nickel around the pores. (e) Unsuccessful removal of SAL601 after electrolytic growth.

Therefore, the approach of membrane ion milling was chosen. Several tests were conducted to produce pores of standardized dimensions. In fig. 3.26 is shown the result of different time expositions to the ion beam.

![Graph](image)

Figure 3.26 Pores of increasing diameters after increasing milling times.

Arrays of pores were produced to find the best distance from pore to pore and the
best diameter for trans-membrane molecular interactions. In fig. 3.27 are reported two examples. In the first SEM photograph, arrays of pores with distance from centre to centre of 1, 3, 5 and 7 μm with a pore diameter of 800 nm are shown. In the second photograph, with a distance from centre to centre of 3 μm were milled pores of 400, 650, 750 and 780 nm diameter are shown.

![Array of pores. (a) Distance from centre to centre of the pore changes. (b) Diameter of pores changes at fixed distance of 3 μm from centre to centre.](image)

Progressive positioning of plain magnetic particles in the pore is shown in fig. 3.28. The experiment was conducted out of electrophoretic cell: a clean membrane containing 4 arrays of pores of 400-500-600-700 nm (fig. 3.28a) was put on an o-ring on a microscopy glass with front side-up and one thousand particles in 0.1XWBB were loaded on it. Particles reached the pores and obstruct them completely after 3 minutes. The particles accumulated quicker on larger pores and slower on smaller ones (fig. 3.28b-c-d).

![Progressive positioning of plain magnetic particles in pores.](image)
3.2.7 TRANS-MEMBRANE EXPERIMENTS

In this section experiments conducted on Si₃N₄ membranes containing multiple pores are reported. The scope was to monitor the interactions occurring between functionalized particles depending on diameter of pore, distance from pore to pore centre, dimension of particles and linking method.

Trans-membrane array experiments were conducted using Dynal particle in the back side of the membrane and Kisker fluorescent, Fluosphere or Qdots on the front side. The experiments were done out of the electrophoretic cell. The sequence of the experiment was the same as described in electrophoretic cell set-up chapter. The aim was the capture throughout the pore of the non-magnetic particle in the trans-side by the magnetic particle as depicted in fig. 3.6.

In the first case Dynal particles were functionalized with the biotinilated anti-fluorescein antibody, while Kisker particles were linked to green fluorescent biotinilated oligonucleotides. The interaction between the antibody and the fluorescent terminus permits the linkage between the two particles. Fig. 3.29a shows the SEM image of the arrays of 700 nm pores at different distances set in the membrane (top left is 1 µm, bottom left is 5 µm, bottom right is 3 µm and top right is 7 µm). In Fig 3.29b-c, the result under optical microscope is shown: the membrane is positioned under the optical microscope with front side-up in the photographs. The membrane is transparent to light, which illuminates from bottom to top. After loading from back side-up, the magnetic particles settled in the pores in agglomerates (fig. 3.29b). Fig.3.29c shows the epifluorescence photograph after incubation and washing of Kisker particles from the front side-up: the fluorescent particles settled in the pores where a section of the magnetic particle was exposed. Kisker particles clogged in the area of pores milled at 1 µm distance, while for 3, 5 and 7 µm distance they were singularly distributed. No interaction occurred where no magnetic particles were exposed to the trans-side, as can be seen comparing the two corners up right of the pictures b and c: the pores are empty. Repeating the experiment with the same membrane, Kisker particles did not link with the same efficiency to their counterpart in the trans side: In fact, only 5% of the experiments showed this efficiency rate of 92/100 pore closure.
With Qdots and Fluosphere, only the streptavidin/biotin method to link double stranded oligonucleotides to both particles was used. SEM images (fig.3.30) show the permanent interaction of Dynal particles with Fluosphere through array of 700 nm pores. Fig.3.30a shows pores with a distance of 4 µm from pore to pore centre. Fig.3.30b shows the back side of the membrane where Dynal are settled after loading. Fig.3.30c shows the front side with reacted Fluosphere particles. Only 21/100 pores were stably occupied by interacting particles. Fig.3.30d is an enlargement of the fig.3.30c showing Fluospheres perfectly settled in the pores.
Fig. 3.31 shows Qdots-streptavidin progressively linking to magnetic particles functionalized with biotinilated oligonucleotides exposed through the pores, while diffusion on the membrane proceeds. Fig 3.31a shows the membrane with array of pores of different diameters (top right is 500 nm, bottom right is 600 nm, bottom left is 700 nm, top left is 800 nm). Photographs (b-c) were taken respectively after 30 and 60 minutes after loading of Qdots in the front side. Particles settled in the pores starting from the external lines (b) and after 1 hour arrays were completely saturated (c). After washing Qdots remained only in the pores (d) demonstrating that a specific interaction occurred with trans membrane particles. The same experiment was repeated 30 times with Qdots showing in each case 100% efficiency. Fig 3.23e shows the same transmembrane reaction using Fluosphere streptavidin in place of Qdots: 32 of 144 pores were stably occupied by interacting particles. The same experiment was repeated 30 times and the efficiency of the reaction was comprised in the range of 3-32 interactions on 144 pores. To verify if nucleic acids as linking means could bring instability to the interaction, Dynal streptavidin were directly reacted with Fluosphere biotin in trans membrane experiments. Again, the returning result after 30 experiments was an efficiency of 2 to 30 interactions per 144 pores membrane. Either using nucleic acids or
not, Dynal and Fluosphere reacted with different efficiency depending on the pore diameter. In fig.3.31e interactions between particles through 500 nm pore (up-right) occurred in 3/36 while through 700 nm pores in 15/36.

![Images of particle interactions](image)

Figure 3.31 Trans-membrane interaction between Dynal particles and Qdots: progressive occlusion of the pores before by Qdots (a, b, c) and permanent Qdots after washing (d); (e) Fluosphere trans-membrane interaction with Dynal particles after washing.

The efficiency observed was different for Fluosphere and Qdots: in particular, while Qdots reacted even through pores of 400 nm diameter, Fluosphere could not interact stably through pores smaller than 700 nm. Trans-membrane experiments evidenced that streptavidin/biotin linking method for trans-membrane interaction is affordable because the yield in Dynal/Qdots experiment is 100%.

### 3.3 THE SYSTEM AT WORK: APPLICATIONS

#### 3.3.1 ELECTROPHORETIC MEASURES

##### 3.3.1.1 Preliminary tests

Electrophoretic measures were taken as described in methods.

In this study, the narrowest pore used for electrophoretic measures had a diameter of 150 nm. Voltage was set from 0.5 to 10 V depending on the experiment. Thanks to the large pore diameter, the ionic strength of the buffer solution can be modulated...
from 0.01 to 1 M. Preliminary tests were done with 500 nm single pore membrane using 10 mM Tris-HCl buffer with NaCl as the ionic species.

The graph in fig.3.32 shows current measures taken using different types of septum between the upper and the lower chamber of the electrophoretic cell. An insulating material such as PDMS, PMMA or Si$_3$N$_4$ (samples 2, 4 and 6) did not let ions cross the septum so that the current measured was around zero. Without septum or with a septum containing one large pore (samples 1, 5) ions can move from one electrode to the other generating the 100000 nA current measure. The same value was also measured for silicon window with Si$_3$N$_4$ membrane sputtered with gold, even without pore aperture (sample 10) and for a sputtered membrane with 100 nm pore, both after HF etching (sample 12). The high values were generated by the conductive gold layer. A residual layer due to imperfect etching on a nanopored membrane generated altered current values compared to non-metal membrane (data not shown), thus invalidating the real measures of current crossing the pore. Baking of sample 10 lowered the current measure to 300 nA. Intermediate values were obtained from microscopy glass (10000 nA), Si$_3$N$_4$ with a single pore of 100 nm diameter (3000 nA) and nitrocellulose membrane (4000 nA). The current measure on microscopy glass was probably due to oxidized silicon on the surface, thus generating an invalidated current measure. The 30 nA measure of sample 8 was imputed to the oxidized silicon surface exposed after KOH etching of the membrane.
Table 3.II shows current measures through single and multiple pores of different diameters and after changing voltage applied. NaCl concentration was set at 0.1M. Increasing the diameter of the pores or the number of pores, the current increased accordingly. Current measure increased from 1000 nA to 5000 nA through pores from 150 nm to 500 nm diameter. The measure of the current passing through 2 pores (150 nm + 400 nm) was equal to the measure of the current passing through a single pore whose diameter is the sum of the separated pores. For applied potential of 1V and one 800 nm pore membrane, the measure (75 nA) was not discernible from background measure (30 nA). When working with array of pores (144 pores of 700 nm diameter in this case), current measures was higher than with single pores. For example, at 5 V, through an 800 nm pore passed an ionic current whose value is 5000 nA while in the same conditions, but with array of 700 nm pores the value is 30000 nA.

Figure 3.32 Graph reporting current measures for different septum used between upper and lower chamber of the electrophoretic cell. 1: No septum; 2: PDMS; 3: microscopy glass; 4: PMMA; 5: PMMA with punctured pore; 6: Si₃N₄ silicon wafer; 7: Si₃N₄ membrane in silicon window with gold sputtering; 8: Si₃N₄ membrane in silicon window without gold sputtering; 9: Si₃N₄ membrane with 100 nm pore; 10: Si₃N₄ membrane in silicon window with gold sputtering and HF treatment; 11: Si₃N₄ membrane in silicon window with gold sputtering, HF treatment and baking at 100 °C for 20 min; 12: Si₃N₄ membrane with 100 nm pore with gold sputtering, after HF treatment and baking; 13: Nitrocellulose septum with 450 nm pores.
3. RESULTS

Table 3.11 Current measures in the electrophoretic cell with different sets of pores and voltage applied.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Pore diamet.</th>
<th>Array 144 pores of 700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 V</td>
<td>150 nm</td>
<td>5000 nA</td>
</tr>
<tr>
<td></td>
<td>500 nm</td>
<td>6500 nA</td>
</tr>
<tr>
<td></td>
<td>150 nm + 400 nm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>800 nm</td>
<td>Out of scale</td>
</tr>
<tr>
<td>5 V</td>
<td>-</td>
<td>5000 nA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30000 nA</td>
</tr>
<tr>
<td>2 V</td>
<td>-</td>
<td>1500 nA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5000 nA</td>
</tr>
<tr>
<td>1 V</td>
<td>-</td>
<td>75 nA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 nA</td>
</tr>
</tbody>
</table>

Below, some graphs are reported, with variation of current (y axis) versus time (x axis).

Fig. 3.33 shows the current measure when a septum of silicon nitride covered wafer was inserted between the upper and the lower chamber of the electrophoretic cell. As silicon nitride is not conductive, the measure was interpreted as background rather than a real measure of the current generated by the wafer.

![Figure 3.33 Current measure with 10V applied potential on silicon nitride membrane without pores.](image)
3.3.1.2 Electrophoretic measures with particles

Magnetic particles were loaded in the upper chamber at a concentration of 1 mg/ml and with one thousand particles per experiment. Loading of particles into upper compartment of electrophoretic cell generated a current flux interruption in the 99% of experiments both with single or multiple pore membrane: the event happened some second to 1 minute after particle introduction. Fig. 3.34 shows an example of current change after introduction of magnetic particles on single 500 nm pore membrane at 10 V power and 0.1 M NaCl solution. Upon particle encountering the pore a decrease in current was measured from 4000 nA to 1000 nA. In the example reported the decrease in current corresponded to the 75% of the initial current measured. On a total of 52 experiments with single pored membrane as septum inside the electrophoretic cell, ionic flux decrease through the pore varied from 25% to 95% upon particles reaching the pore, with a mean percentage value of 55±21%.

![Figure 3.34 Current measure with 10V applied potential after particle introduction on membrane containing a 700 nm pore.](image)

Fig.3.35 shows the decrease of current measure after loading of particles upon a membrane containing 144 pores of 700 nm mean diameter. With an applied voltage of 5 V, the current decreased from 27000 nA to 1000 nA. Meanly, on a total of 43 experiments, the percentage of current decrease was 55% from starting point to stabilization point when particles were clogged on the pores. Before reaching the lowest
value, the current measure varied between the higher and the lower values in a not uniform way. This was partially explained observing the particles reaching the pores in optical microscope: they settled in pores in different times, and some of them lifted up after settled.

![Magnetic particles introduction](image)

*Figure 3.35 Effect of particle introduction upon membrane containing array of pores on current measure with 5V applied potential.*

### 3.3.1.3 Electromagnetic control in electrophoretic cell

Electromagnetic control of pore opening by current measure was tested on single pore and array of pores.

A single pore holding membrane was settled inside a vertical electrophoretic cell, with a needle shaped electromagnet dipped in the upper chamber. With an 800 nm diameter pore, an applied potential of 2 V, a buffer ionic strength determined by 0.1 M NaCl, current measured passing through the pore was typically around 1500 nA (ON state). A Dynal/dsDNA complex was loaded on the back (upper) side on an electrophoretic cell powered with 2 V. When a current drop from 1500 nA to 500 nA (OFF state) was detected upon occurrence of particle encountering the pore, the electromagnet was turned on briefly, and an increase in ionic current passing through the pore was measured, a behaviour that was interpreted as a return to the ON state by pore opening. When the electromagnet was turned off, current measure dropped again
3. RESULTS

to a value corresponding to the OFF state (Fig. 3.36).

![Graph showing current measurements over time with annotations for different states: (i) Magnetic particles loading, (ii) Electromagnet on, (iii) Electromagnet off.]

*Figure 3.36 Electromagnet effect on current measure when acting on particle settled on single pore with 5V applied potential.*

This behaviour was interpreted as a pore closing. When the same experiment was repeated without loading magnetic particles, no current changes were detected.

Experiments to control particles with the electromagnet upon an array of pores were conducted using a membrane containing 144 pores with a mean diameter of 700 (fig. 3.37). With 5V applied potential and buffer ionic strength determined by 0.1M NaCl a current of 36900 nA was measured. After loading of magnetic particles current measure reached 35600 nA. First activation of the electromagnet after current drop (a) generated a current increase until 36260 nA (b). Repeating the sequence of activation (c, e) and deactivation (d, f) of the electromagnet the same trend of current measures was found. In point (c) current was 36000 nA, in point (d) 36500 nA, in point (e) 35700 nA and in point (f) 36350 nA. Repeating ON/OFF states determined a loss of current measure, which from initial state ON at 36900 nA dropped to 36300 nA.
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3.3.2 TRANS MEMBRANE EXPERIMENTS INSIDE ELECTROPHORETIC CELL

Particles functionalized with M13Rbio/M13RCbio were loaded in the upper chamber and the system was let stay until current measure reached the OFF state (fig.3.38a). With the electromagnet turned off and the pore closed, Qdots-streptavidin were injected in the lower chamber. Electrophoresis was stopped and the streptavidin-Qdots allowed reacting with the exposed biotin of the trans-membrane magnetic particle/M13Rbio/M13RCbio complex for at least 15 minutes. When electrophoresis was reactivated, it was verified that the ionic current was on the OFF state (fig.3.38b). At this point electromagnet was turned on and no increase in the ionic current measured was detected (fig.3.38c), while in control experiment, carried out with non-complementary oligonucleotides, the current could be returned to the higher values corresponding to the ON state (fig.3.39b-c). It was argued that Qdots reacting with Dynaldsoligo blocked the particles in the pore and so prevented the pore to be opened by the electromagnet and the ionic current to be re-established. Observation of the sample in the epifluorescence microscope (fig.3.40) confirms that Dynaldsoligo were clustered on the pore and that Qdots react with them through the aperture, clustering on the opposite side of the membrane.
Figure 3.38 Current measure of interaction of Dynal particles with Qdots with the matching intermediate DNA, M13Rbio/M13RCbio.

Figure 3.39 Current measure of interaction occurring between Dynal particles and Qdots with non-complementary DNA as intermediate.
For trans-membrane strand displacement assay in the electrophoretic cell, the construct Dynal/M13RCbio/M13RAbio/Qdots was used to reach the OFF state of the device. The cell was powered off, and M13R was loaded on the lower chamber and incubated at 25 °C for 6 h. When the cell and the electromagnet were powered on again the ON state could be re-established. Fig.3.41 reports the actual current readings obtained in a typical experiment: the initial ON state (a), the OFF state signal due to Dynal encountering the pore opening (b), the stable pore occlusion following the introduction of Qdots (c), characterized by an OFF state that cannot be reverted by electromagnet operation (d), and finally restoration of ionic current signal (ON state) after oligonucleotide displacement and electromagnet activation (e). After strand displacement, the switch to the ON position following electromagnet operation did not always fully restore the current to the exact initial value.
3.4 SELECTIVE TRANSPORTER

In this application, magnetic particles captured specific molecules in the lower chamber of the electrophoretic cell and transported them in the upper side.

3.4.1 DELIVERY OF TRANSPORTERS

The transporters have been tethered with a double stranded DNA stretch of different lengths (from 20 to 2000 nucleotides, corresponding to 6.8 to 680 nm if fully extended). As shown in introduction, the DNA stretch has a terminal modification that determines the specificity of the transporter. In the experiments reported here, a terminal biotin is used as specificity determinant for the capture of streptavidins or streptavidin-modified objects.

Delivery of the transporters to the pores was obtained by launching the particles in the upper compartment. Fig. 3.28 showed a launch of transporters over a membrane including 4 arrays of pores of different diameters. As shown, the transporters accumulate at the pores, preferably on the arrays with larger pores, where the fluid flow is higher. Although the transporters settled on the membrane in isotonic solutions, the use of buffers with different salt concentration for each compartment can provide a partial control of particle movement velocity and stability in the pore: in particular a faster delivery of particles was observed when moving from NaCl 0.1 M in the upper camber to NaCl 1.0 M in the lower chamber. On the contrary if the upper solution has...
more concentrated ionic species (NaCl 1.0 M) than lower solution (NaCl 0.001 M) transporters never settled on pores but were conveyed to the sides of the membrane by the solution flux coming upwards (fig.3.42).

![Image](image.png)

**Figure 3.42** Upwards solution flux from upper to lower chamber due to different NaCl concentrations, impedes magnetic transporters to settle in the pores.

As reported, different pore sizes have been tested on membranes containing arrays of different pores. Aspect ratio has resulted to be particularly critical for the efficiency of the second device.

In pores with a diameter of 900 nm the transporters remain permanently blocked into the pore and cannot be removed (fig.3.43a). Pores with diameter ≤ 700 nm showed limited efficiency in transmembrane capture as compared with pores of 700-800 nm. Pores of 800 nm diameter were used in the subsequent experiments.
Figure 3.43 Behaviour of transporter in large pore (a) and trans-membrane interaction efficiency (b). (a): single large (900 nm) pore permanently clogged by the bead (SEM); (b): trans-membrane recognition in arrays of different diameter pores (top right 500 nm, bottom right 600 nm, bottom left 700 nm, top left 800 nm) with magnetic particles settled and interacting Fluosphere green fluorescent particles.

The size of the objects to be captured in the lower compartment greatly affects the efficiency of the system. Fig.3.44 shows the capture of the protein streptavidin (less than 5 nm according to Hendrickson and co-workers 1989), streptavidin coated quantum dots (about 20-30 nm) and latex sphere (700 nm) through pores array. Pores were milled at distance of 3 µm from centre to centre and with a diameter of 500 nm (up-left), 600 nm (up-right), 700 nm (bottom right) and 800 nm (bottom left). The efficiency of capture was high (> 99%) in the case of streptavidin (a) and quantum dots (b), but rather low (< 30%) for the latex spheres (c). In fact, every pore was covered by fluorescent streptavidin or Qdots, while only 28 Fluosphere were linked over 144 pores. This evidence suggests that Fluosphere are less suitable as transported molecules.
3. RESULTS

![Image](image1.png)

Figure 3.44 Optical microscopy with membrane lower side up of trans-membrane link to transporters of fluorescent streptavidin (a), Qdots (b) and Fluosphere (c).

3.4.2 TRANSPORTER IN ELECTROPHORETIC CELL

Once the transporters have captured their target, the next step was the removal of the reacted transporters from their position and the substitution with new transporters. The removal was obtained using the hand made electromagnet positioned at 1 mm distance from the pores. Removal of particles was most conveniently studied using a single pore membrane inside the electrophoretic cell. With a 2V applied potential, the intensity of a typical current passing through a single 800 nm pore was in the order of microamperes (fig.3.45). After launching in the upper compartment, the settlement of a transporter at the pore caused a nearly complete blockade of the ionic current. The action of an electromagnet placed nearby the pore was used to remove the transporter from the pore, thus restoring the ionic current due to the opening of the channel. Fig.3.37 shows a typical experiment of closing and opening of the pore by electromagnet operation. With the return to previous conditions a new, yet unloaded transporter is driven to the pore.
To monitor their import from the lower compartments, target objects were labelled with fluorescent labels and observed under a fluorescence microscope. Objects such as Fluospheres, which were larger than the pores, could not cross the membrane, thus their recognition by the transporters resulted in permanent blockade of the pore. Conversely, fluorescein labelled streptavidins reacted with the transporters without affecting their mobility. In the case of quantum dots the fate of the transporters depended on the density of the attached DNA threads, as multiple available moieties resulted in the formation of clusters of the quantum dots at the pores and their permanent blockade (fig.3.46). The density of the specific moieties could be tuned during preparation of transporters by balancing the ratio of functionalized and not functionalized DNA that is linked to the paramagnetic bead. A method to precisely quantify the imported molecules and the efficiency of the system is presently under study.
PART 4
DISCUSSION
4. DISCUSSION

The interest in biosensing methods that perform electrical signal detection is high, as they cut down consistently equipment costs and are amenable to integration in portable instruments: however, approaches that conjugate low cost and high sensitivity are, to date, still technically challenging (Boon et al. 2000; Gooding et al. 1998; Haham and Lieber 2004; Kim et al. 2004; Li et al. 2003). Size reduction is another major task in biosensor development, as it is the pre-requisite for portability and suitability to probing at cellular scale. The miniaturization of biosensors, however, faces two conflicting design requirements: they need to be both extremely small and highly sensitive. As sensors become smaller, so does their surface area. The signal produced by a biosensor is proportional to its surface area and will diminish as the size of the sensor shrinks. The background current of the sensor will also lessen with size, but the challenge in miniaturizing biosensors is to maintain a signal to noise ratio that enables effective and sensitive detection of the analyte. This means coating the tiny sensing surface with a high density of biomolecules. The reliable production of thin, robust and highly active biosensing layers on small electrodes has posed a significant barrier for the development of useful microelectrode biosensors.

Here, I advance the idea of a novel biosensing strategy, where the molecular detection reaction is not directly responsible of a current/potential shift, but it is rather the trigger of a nanomechanical event that is more easily monitored. Such an event was conveniently identified as a nanopore opening/closure.

Since Bezrukov et al. (1994) have reported that the alpha haemolysin pore protein in a lipid bilayer could mimic the action of the Coulter-counter at the nano scale, similar approaches for the characterization of molecules were directed toward analytical purposes, pursuing it during their transit in a nanochannel (Muthukumar, 2003; Nakane et al., 2003). Although this technology has found noteworthy applications in biosensing of specific molecules according to their current-voltage signatures or kinetics (Halverson et al., 2005; Kim et al., 2007), the exploitation in the field of biosensing and diagnostics at the nano scale has been hampered by the sensitivity and speed needed for the detection, as well as by the critical experimental setups (Kim et al., 2007). Promising modifications of the initial approach for the diagnostic field were obtained by
4. DISCUSSION

engineering the alpha-haemolysin pore (Capone et al., 2007; Cheley et al., 2002; Gu and Bayley, 2000; Movileanu et al., 2001), while significant progress in improving the stability of the system has been achieved with the use of synthetic rather than biological pores. Despite the progress in the manufacturing technology, that permits the production of 1 nm synthetic pores and the incorporation of 2 nm proteic pores in teflon (Akeson et al., 1999; Howorka et al., 2001a), glass (Sandison et al., 2007) and silicon supports (Peterman et al., 2002) the use of these bio-inorganic hybrid devices in diagnostics is still in its early infancy. With the few exceptions (discussed below), the methods proposed to date rely on the transient capture or detection of molecules (Deamer and Branton, 2002; Fologea et al., 2007; Lee et al., 2004; Meller et al., 2000). Here, I exploited the recent progress in pore manufacturing to demonstrate the principle of operation of a novel biosensing method, based on a DNA driven valve in the submicrometric scale.

The development of a new biosensing concept implied the need to face several technical problems for its implementation. On one end, the development of each single component of the device in accordance with the final project setup was carried out. This included the production of a DNA/particle complex suitable for trans-membrane interaction; the project of the electrophoretic cell as detector mean together with the electromagnet as manipulator; the development of the nanopore detector in accordance with DNA/particle complex and with electrophoretic setup. On the other end the implementation of the aforementioned elements into the multicomponent sensor device for practical application was performed. This included the trans-membrane experiments between particles outside the electrophoretic cell and the current measures experiments.

The highly selective recognition mechanism of DNA hybrids is at the basis of this sensor operation. The oligonucleotides hybridization and strand displacement were used in the project setup as signal activators. In fact, because of their manipulability, not only they were used as detection means but also as carrier means, being able to selectively transport biotin, streptavidin and particles. Production of double stranded 1200 bp (base pairs) nucleic acid was accomplished by PCR amplification of a DNA sample extracted from Diaporthe helianthi fungal pathogen. Further manipulations such as exonuclease lambda-exo digestion and hybridization both of biotinilated probe SDL4BIO1 and bridge connector SDL4-POLIA were carried out to link beads to each others. Although not yet used in this thesis work for biosensing, the ability to produce a
1200 bp (or even longer) DNA hybrid with the method here developed is important because it will allow the use of small pores in future applications. The long DNA molecule could extend through the membrane, overcoming the need for transmembrane exposition of the beads.

Chemical attachment chemistry by Solulink manufacturer was used to generate an hydrazone bond between a DNA-SFB and a particle-SANH. Despite the positive results obtained in the modification of nucleic acids with SFB linker molecule, the final hydrazone bond between it and SANH modified particles was never detected. As particle manufacturer suggested the best explanation is that the solution pH value lower than 4.7 and the presence of organic solvent (DMF) irreversibly destroyed the shell of the particles. Attempts to attach SANH to particles using mild incubation conditions were unsuccessful. Formation of S-S bridge between particles and DNA was an alternative chemical attachment method used. Gold particles were successfully attached by a linker DNA molecule formed by a biotinilated double stranded oligonucleotide with an SH-terminus to Roche streptavidin. Although the positive result and the possibility to use this method in trans-membrane transport, gold particles were not used in this electro-sensor device because of their metallic properties.

The method that provide most reliable and efficient results was streptavidin/biotin linking one. On linking particles each other the yield of the reaction was inversely proportional to the dimension of the particle linked. It was found that biotinilated Fluosphere (1 µm diameter) linked to Dynal streptavidin in 70% of cases and with a maximum of two particles linked per Dynal particle; in comparison biotinilated Qdots (20 nm diameter) had 100% of success both in covering all the Dynal particles and their entire surface. The fact that the efficiency of interaction between particles diminishes proportionally with the dimension of the particle linked was conceivably due to several factors. Diffusion capability is inversely proportional to particle size. Large particles tend to precipitate and washing steps can bring away a major part of heavier particles (Fluosphere) in comparison with lighter ones (Qdots). The same reasons preclude the trans-membrane interaction between particles of similar dimensions, added the sterical disadvantage given by the channel-pore obstacle. Chemical attachment approaches should be taken into account when reducing size of the entire set-up: in that case dimension of contour molecules could hold more importance. In this work set-up, due to the quasi-micrometer range of action of the biosensor, the streptavidin/biotin
complex was regarded as sterically small and was chosen as the preferred linker for all the molecular interactions studied for its affordability.

For this project applications the stability and uniformity of the size particle hold a primary importance: too large particles could not expose their surface across the pore and those to small would cross it going on the other side of the membrane. Due to size difformity. Roche particles caused failure in trans-pore experiments, conversely Dynal magnetic particles were found to be much more uniform in size. Moreover they were stable in solution, while Roche particles turned darker after use. Several different diameters particles were tested before assessing that Dynal were the most affordable with the only inconvenient that serial production is only for 1 µm particles coated with streptavidin. Fluorescent particles were also used in this work and their performances evaluated. Fluorescent particle should be able of maintain the fluorescence for prolonged time. The only disadvantage of Qdots in comparison with Fluosphere was their light sensitivity, which forces the operator to work applying dark conditions. Fluosphere on the contrary showed a high durability in fluorescent signal (in the order of ten days) due to the particular structure of the fluorescent core.

In the final implementations of the project an electromagnet was used to remove particle/s from pore/s. It was necessary to test the hand made electromagnet on particles in free solution and on particles on self standing membranes containing single pore and array of pores. Although the magnetic field force of the neodymium magnet was stronger than that of the electromagnet, the sharp tip of the latter permitted to work inside the silicon window containing the membrane. Moving several particles in free solution accomplished with the electromagnet at 1mm distance and 0.22 mA current through the wire. The same result was achieved to remove a particle from single pores of 800 nm. Instead particles settled in array of pores of more than 650 nm diameter were removed only rarely. This was imputed to the fact that several particles organized in chains due to the generated polarization upon action of a magnetic force instead of been moved away by it. Being settled in large pores, the strength of the magnet may have not been sufficient to remove the block of organized particles from the pores. In support to this hypothesis, there is the experimental observation that when not all the pores of an array are occupied by particles, they could be removed easier without the formation of chains. Moreover it seemed that the solution flux strength in array membranes is higher than the force of the magnetic field to permit it to act on all
4. DISCUSSION

particles at the same time: this is supported by the fact that increasing the volume of solution in the upper chamber the particles were removed with less efficiency or not removed at all from the pores. With pores of smaller diameter, instead, particles were not firmly settled in pores and were easily removed.

The electrophoretic cell set up was conceived to meet these characteristics: transparent polymeric plastic material, unmountable in each component to easy the cleansing, portable. An essential feature was the vertical implant. To accommodate an internal membrane, which could generate two distinct compartments, it was necessary to create two subunits of the cell and to keep them together by pressure exerted on external components. Contemporary the membrane has to be tightly sealed between two o-rings. Mainly for this reason the final setup of the electrophoretic cell are two o-rings patched together inside two rigid plastic pieces. The electrophoretic distinct chambers are simply the holes of the rings. In this electrophoretic cell the loading of buffers is manual. Buffer is in the open air. This solution was preferred to circumvent obstacles, which could origin from closed system: fluidic pumps, channels and loading tips, lodging for electrodes were not necessary.

The silicon nitride membrane was produced by standard lithographic techniques in silicon wafers. To facilitate trans-pore reaction a 100 nm thick membrane was chosen. Because it is very fragile under manipulation the window frame was reduced at 56 µm, dimension which permitted, besides the robustness of the membrane, to easily wash out the particles during the experimental procedure. Lithographic techniques can be applied to production of pores. Advantages are the very polished structures and the perpendicular sides of the pore obtained in respect of silicon nitride surface. Moreover, as a standardized method, it is prone to industrial processing. Disadvantages are the many steps needed and the final dimension of the pore, which with the lithographic technique applied in this project, cannot be inferior to 50 nm. The production of pores by FIB technology is more suitable for laboratory tests because the method is applicable to only one silicon frame at a time. In this project, the effectiveness of the experiment was achieved with pores diameters in the order of hundreds nanometres, following the general concept that a bead of whatever diameter can obstruct a pore of comparable dimensions. However, the diameter of the pore could be reduced until 20 nm, which is the FIB milling limit capacity tested in our former experiments. In case of pore diameter reduction the thickness of the membrane should be reduced accordingly given the
spherical shape of the buoy settled into it. Silicon nitride material was chosen because being an insulator it gave no background signal in electrophoresis thus perturbing negligibly the measure. Repeated polishing with acid solution oxidized very deeply the surface, bringing the material to become a collector of ionic species, while no change in insulator properties was detected. The same acidic solution slightly enlarge the pore diameter: for these two reasons, the durability of the same nanopore membrane was limited to 20-25 reuses. On the other hand, this sort of unwanted chemical etching could be used to control precisely pore dimension after FIB milling. Milling of pores was accomplished with single exposition to single dot mode for different times depending on the diameter required. A good repeatability from one milling action to the other was observed. As shown in fig.3.18b pore distance of less than 3 μm favoured the unwanted clogging of particles. Otherwise, to work with distances of more than 5 μm, is not convenient in terms of number of particles able to be exposed throughout the pores and to react on the other side in relation to the dimension of the membrane (56 μm). A good compromise is the centre to centre distance of 3 μm. Behaviour of the moving solution from top to bottom throughout membrane pores was studied using particles. When a single pore holding membrane was used, particles were conveyed by a solution flux to the pore as expected. When the membrane contains several pores, grouped in arrays of different diameters, the particles are captured first by the solution which moves through larger pores and reach them before the smaller ones. Only when larger pores are occupied, particles are captured by the flux through smaller ones. The promptness with which the closing of the pores was completed, suggested that the origin of the obstruction of the pores by particles was likely the movement of liquid from one side to the other side of the membrane itself rather than electrophoretic power acting on particles. This evidence permitted us to choose the vertical electrophoretic configuration as the best one.

As first implementation of the project the interaction between particles through the nanopore with DNA as linker molecule was developed. In trans-membrane experiments, the capture of molecules and particles by the Dynal particles from the lower chamber was attempted. The trans-membrane capture was also used to test the best diameter pore for through-interacting molecules and beads. Confirming the hypothesis, when two large particles (namely, Dynal and Fluosphere) positioned themselves on the same pore in the trans mode, they reacted each other only when the
pore diameter is larger than 700 nm. During experiments it was observed that, after loading of Fluosphere, they laid in an uniform film on the surface of the membrane, covering completely all the pores. It was after the first washing that almost all the Fluospheres were brought away. The fact that the efficiency of Dynal/Fluosphere interaction was so low, has not been clearly understood. Two are the hypothesis: the particles never react each other, or mechanical shearing broke the DNA linker between particles. Capture of Qdots and of fluorescent streptavidin resulted more efficient. In some experiments, Qdots linked to a portion of the Dynal particle in the upper chamber. In the final project implementation this fact had negative impact on the strand-displacement experiment, where Qdots trespass in the upper chamber has been suspected to block strongly Dynal particles in the pore, making necessary prolonged incubation time for strand-displacement effectiveness.

In parallel with the trans-membrane investigations, tests were carried out on the sensor device including electrophoretic measures and electromagnet operation. The set-up of the electrophoresis detection method was made without particles loading. Preliminary tests with different septums were conducted to characterize the system before trans-membrane experiments. Because of their insulating property, the lowest current value measured with PDMS and PMMA foils and with the Si$_3$N$_4$ coated frame. Clearly, absence of septum or a insulating septum with a punctured pore generated the highest values of current measures. Similar values were obtained using a gold sputtered silicon membrane indistinctly with or without a pore: the measure was conceivably generated by the gold dissolved in the solution. Measures carried out with different materials suggested that the exposed SiO$_2$ surface in the window was the cause of an electrolytic current of 30 nA when measure with Si$_3$N$_4$ membrane was carried out. The value of current measure increased with the size of the pores and the current measure with two distinct pores was about the sum of the current measured for each pore alone. Particles encountering single or multiple pores produced different current decreases at different times after their launch of in the upper chamber. The time required for particles settlement varied from 1 to 60 seconds. The settlement delay was more significant in array membranes.

As verified by optical microscopy, the electromagnet always displaced Dynal particle from single pore. Measures of pore opening by electromagnet confirmed its ability to remove particles, generating a distinct interpretable signal. Repeating cycles of activation
and deactivation of the electromagnet generated ON-OFF signal alternation. The same experiment was conducted on membrane with array of pores: the same behaviour was found except for the fact that at every cycle of activation/deactivation of the magnet the “ON” signal value tent to decrease and the “OFF” signal value to increase. This behaviour may be related to the re-organization of the particles on the array of pores after cycling the electromagnet.

For hybridization and strand displacement trans-membrane assays inside electrophoretic cell, the particle pair Dynal/Qdot gave most consistent results, because the pair Dynal/Fluosphere was found to react only rarely in trans-membrane configuration. While for hybridization detection of matching oligonucleotides the “OFF” signal of hybridization occurred and the “ON” signal of negative control were clearly distinct, removing Dynal particle after incubation for strand displacement often gave a light “ON” signal, lower than the “ON” signal of negative control. If the sensitivity for strand displacement is too low the interpretation of the pore opening could be hindered and generate a false response for the diagnosis. The reason for this lowered sensitivity was imputed to the Qdots probably remaining on the sides of the pore aperture, thus shrinking its lumen and as a consequence the ionic current passing through it. This unwanted effect could be avoided with shorter incubation, but this provided unreliable results on strand displacement.

The project developed in this PhD thesis focuses on some of the several applications envisioned for this complex system. The sensoristic approach was carried to produce a prototype that could lead to develop a portable, simple, highly sensitive and cheap detector for diagnostic purpose. In this first application the entire complex being built was devoted to detect a molecular interaction: the membrane is a septum dividing two compartments, the nanopore is an ON/OFF switch, the particles are carriers and switching means. Although the device is at its first, rough implementation, there are at least three unique prerogatives of this innovative method that are worth considering. First, the system is based on two loadable, membrane separated compartments, with no interacting molecules being linked to the pore or the silicon nitride membrane or other structural parts. This fact implies that on the same device, different targets and different probes can be tested sequentially and independently.

Second, the system gives easily detectable, low noise signals that are dependent on a relatively small threshold number of interacting molecules. According to the binding
4. DISCUSSION

capacity declared by the

manufacturer, a single magnetic bead used in this work (Dynal) binds less than 100 zeptomoles DNA, corresponding to $6 \times 10^4$ molecules; given the geometry of the device, I estimated that about $10^3$ to $10^4$ molecules govern the ability of the pore to remain closed when the electromagnet is operated, i.e. switching between its ON and OFF positions. These characteristics made this system an interesting technology for the implementation of DNA based logics and hybrid computing. Finally, the small size and potential sensitivity of this biosensor make it a candidate for use in intracellular probes. The sensing pore may be conveniently placed on the tip of a needle to puncture a cell, using the cell cytoplasm as the lower chamber.

Before the new technology may find the practical exploitations, several aspects of the device need to be optimized. This involves aspects of nanotechnology, molecular biology and fluidics. As far as the construction of the pore is concerned, several methods are available, such as track-etching in PET foils (Siwy and Fulinski, 2002), micromolding techniques with PDMS (Saleh and Sohn, 2003) or embedding carbon nanotubes into epoxy membranes (Sun and Crooks, 2000). In this work, the simple approach of membrane ion milling was found very reliable and repeatable. Silicon nitride was the material of choice for its insulating properties and low background signal in electrophoresis. The effectiveness of the experiment was easily achieved with pore diameters in the order of hundreds nanometres, because the pore size is not relevant in relation to the dimension of the molecules detected, but only to the particle. In case of pore diameter reduction, the thickness of the membrane should be reduced accordingly, given the spherical shape of the buoy settled. Indeed the thickness of the membrane and its relation with the size of the active molecules and particles was a challenge more serious than pore milling. The sensor strategy presented in this paper includes transmembrane detection through a submicrometric pore, a process that have been successfully attempted to date with an alpha haemolysin proteic pore in a lipid membrane by Nakane et al. (2004); those authors used a molecular anchor linked DNA that crossed the pore, reacted trans-membrane with another DNA complementary to its $3'$ end forming an hybrid that acted as a block in the pore. We adopted a similar approach with a synthetic membrane, but having the focus of the diagnostic in a strand displacement event, that we found more reliable than the capture event. The device is therefore not dependent on the critical and stochastic bead positioning and
transmembrane capture, but by the physically well defined process of displacement and can be interrogated at user will by action on the electromagnet. In the set up used in this work, the probe-carrying beads exposed a considerable part of their surface on the trans side of the Si$_3$N$_4$ membrane in order to interact with particles loaded in the other side, as the nucleic acids linked to the surface were shorter than the thickness of the membrane. Using longer nucleic acids may give limited benefits, as the conditions for maintaining nucleic acid longer than 100 nm in an extended configuration could compromise molecular recognition and interaction. Mixed configurations (extended within the pore, supercoiled outside) have however been recently obtained (Keyser et al., 2006) and could find application in this system. It should be noted, however, that they require very small pores and very long DNA molecules, characteristics that may dramatically affect the reliability and robustness of the device. Moreover, a relatively large pore sustain substantial ionic current even in low salt buffer, thus allowing to tune the salt concentration to the needs dictated by the hybridization or displacement reaction that are critical in governing specificity. High salt concentration may also determine precipitation and clogging of particles. The use of relatively larger pores is therefore advantageous for the mechanical and electrical properties of the device, although it introduced difficulties related to the molecular recognition events. In facts, our preliminary approach consisting in the reaction of beads of similar size, larger than the pore, on both membrane sides (i.e. Dynal and Fluosphere) failed for the scarcity of the encounters between the large bodies, due to the adverse influence of the low diffusion coefficients of large particles. In conclusion, the optimization of the set up including pore size/bead size/membrane thickness still needs further investigations and trials.

Although I applied the biosensor only to detect nucleic acids, the same detection principle can be applied to other interactions occurring between different molecules and in different environments. For example the target object in the trans chamber could be a virus which is captured through the pore by the specific antigen on the magnetic particle. In the same way the system could be interrogated using an enzyme cutting only specific double stranded sequences between the two particles settled in the pore, thus demonstrating that a hybridization has occurred. Alternatively the entire sensor setup could be used to directly detect interactions through a single cell membrane.

A second application implemented in this project was the selective transport of
streptavidin, Qdots and latex particles from the trans-compartment towards the cis-compartment of the silicon nitride membrane using magnetic particles as transporters. In this application membrane and pore are sieving matrices and magnetic particles are selective transporters of molecules from one environment to another. Membrane separation processes have been extensively used for some important industrial purposes, stimulating the progress toward artificial membranes that could attain the purification of specific target molecules in a less energy intensive, more economic and more efficient way than competing methods (Jirage 1999). Despite the noteworthy progress, the separation through artificial membranes remains restricted to facilitated diffusion of relatively simple chemicals, while the selective transport of complex biomolecules such as DNA and proteins has just started to be investigated (Ferraz 2007). In this project, I report a new concept of bio-mechanical device that uses the specificity of biological molecules to selectively pump through pores in a silicon nitride membrane. Several constraints have been identified and addressed in this work and an initial prototype has been set up. First, a simple way to deliver transporters to the pores was developed: in the vertical setup of the electrophoretic cell the solution move from upper to lower chamber by gravity. Particles are captured by the flux established and maintained in the pore by the hydrostatic pressure. Tuning of velocity of delivery was obtained both with increase of the pore diameter and employing solutions with different salt concentration in upper and lower chamber. Second the pore/particle aspect ratio has been addressed by changing the pore diameter in accordance with the trans-membrane interaction and transport investigated. In this project the transporters size is set at 1 µm, so the need to tune the pore diameter came from the transported objects: in particular, biomolecules and Qdots did not influence at all the trans-membrane setup, while larger particles like Fluosphere were the bottleneck of the interaction. Third, removal of transporters from pores was accomplished by an electromagnetic, which was found to manipulate particles in a sufficiently controlled way. Last, the sensor device was used, in an original way, to detect the removal of transporters from pores. This novel approach open new perspectives for the development of lab-on-a-chip applications. Present lab-on-a-chip prototypes relies on fluidic pumps, as reaction products are adsorbed on filters and then eluted to be purified prior to be passed to the next compartment reaction. Alternative approaches that use magnetic particles, still require buffer substitutions. Conversely, the approach presented here, although still in its infancy, is amenable of continuous
operation, as reaction components could be fished from one reaction compartment and used as substrate in another reaction compartment. In a further development, it could be possible to selectively fish an object through a cell membrane and translocate it in a suitable analyzing chip. Other foreseeable applications include the production of devices that can be introduced in cells and selectively remove cellular components without altering other metabolic functions.
PART 5

CONCLUSIONS
CONCLUSIONS AND FUTURE PROSPECTS

Methods which can broaden the comprehension of biomolecular mechanism are developing fast thanks to several innovative approaches, including the development of sub micro-mechanical devices for the study of biomolecules, a field that has been named nanobiotechnology.

As nanobiotechnology is still in its infancy, much work and efforts are needed to define and optimize the general configurations and methods that would give the best performances and be worth to pursue for tomorrow applications. Here I present a novel approach to the general issue of the characterization of biomolecules, and a study on derived practical applications that allowed me to evaluate its constraints, limits, and potential benefits.

In this context, I developed a biosensor that has the potential of reducing detection limits by means of a simple, small and robust instrumentation. In such sensor, an electrical signal is related to the obstruction or aperture of a synthetic nanopore where molecules analyzed have been blocked or removed upon occurrence of biomolecular interaction. This biosensor device can overcome the constraints of other methods based on nanopore-amperometry detection. In fact, while nanopore detectors are severely limited by the transient event triggering of signals, in the sensor presented here the stable permanence of molecules in the nanopore facilitates the detection of the trans-membrane interactions.

The development of the device has faced several constraints to become effective. Single components have been developed separately and then put together, initially in intermediate setups and then in the final implementation of the device. One of the most challenging goals was the molecular interaction through the pore; while different particles and DNA interacted with high efficiency in free solution, a similar result was accomplished only after the tuning of the aspect/ratio of pore/particles, the ionic concentration in buffer solutions and the particles respective size. These experiments of inter-molecular trans-membrane reaction made out of the detection chamber were the preface to the assembled detector device. In the meanwhile, the nanopore detector
feasibility was separately evaluated using a cylindrical electrophoretic cell, which solution was separated in two chambers by the synthetic membrane. Having a rather large (150-900 nm) pore, as compared with other biosensor approaches (Kasianowicz et al. 1996, Li et al. 2001), led to very good signal to noise ratios. In facts, the obstruction of the pore by particles produced a decrease in current of meanly 50% with values two orders of magnitude higher than the background.

Following trans-membrane reactions, the manipulation of reacted components was another major, yet challenging, task. Experimentally, I found that the particles were best removed from small pores, which had to be even smaller if the setup included a membrane with pores array. In the electrophoretic cell, the electromagnet operated properly only with a single nanopore membrane: it was able to remove non-interacting particles settled in pore, while the removal after displacement of the mismatched oligonucleotides was less efficient.

This detail is also important for the second application of this project. The sensor system was applied to the detection of a capturing and releasing event of a trans-membrane through-nanopore interaction. After the interaction occurred through the pore, the magnetic particle was removed carrying, on its functionalized surface, the target molecule; after that, other transporter particles were delivered in the pore. The system recognizes the particle displacement and the second particle settlement as two distinct events: in this way a continuous operation of the transport is feasible.

The method could overcome some common difficulties found in membrane separation which is mostly based on shape or chemical selectivity, while more selective methods, especially in the field of complex biomolecules selective transport, are just at their first investigations (Ferraz 2007). Different kinds of molecules have been captured from the lower chamber of the electrophoretic cell from biotin to Qdots, to 1 µm particles. Particles with size larger than the pore cannot cross it. Even if not tested in this setup, the ability of the sensor could be to detect the target captured in the lower chamber only by its size, because a large molecule cannot cross the pore while a small molecule does. So the pore diameter could be modified in accordance to the size of the target molecule, while the changes on amperometric detection are negligible.

Future developments for the detector system include the reduction of the pore and particle size in accordance with the detection limit envisioned for the interaction. This
will also require a reduction of the cross section of the pore membrane, which is now set at 100 nm. Moreover, long nucleic acids such as a PCR products or a single PCR product could be attached to magnetic particle and elongated through a narrow pore. Particles of intermediate size linked to a larger-than-pore buoy could facilitate the elongation entering the pore while the particle is settled in it and capturing the end of the PCR product. Then, investigation on the trans-pore molecule can be accomplished, such as a tomography by means of laser focus (as an interpretation of Keyser et al. 2005). Before the accomplishment of such an ambitious task, some other improvements have to be carried out on the system. One of those will be the electromagnetic manipulation of the particle/s: a better solution for their control could be a three-four tips magnet or electromagnet, controlled by a piezo-electric micro-positioning. Another part that needs improvements is the electrophoretic cell and its associated electromagnet for their inclusion in microfluidic systems, such as it is required in the case of a lab-on-chip integration of the device.
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