Nanostructured Membranes Functionalized with Gold Nanoparticles for Separation and Recovery of Monoclonal Antibodies

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Giada Soldan

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The thesis of Giada Soldan is approved by the examination committee.

Committee Chairperson: Prof. Suzana P. Nunes
Committee Members: Prof. Mani Sarathy, Prof. Enzo di Fabrizio
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ABSTRACT

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Giada Soldan

The need of purified biomolecules, such as proteins or antibodies, has required the biopharmaceutical industries to look for new recovering solutions to reduce time and costs of bioseparations. In the last decade, the emergent field of membrane chromatography has gained attention as possible substituent of the common used protein A affinity chromatography for bioseparations. In this scenario, gold nanoparticles can be used as means for offering affinity, mainly because of their biocompatible and reversible binding behavior, together with their high surface area-to-volume ratio, which offers a large number of binding sites.

This work introduces a new procedure for purification of monoclonal antibodies based on polymeric membranes functionalized with gold nanoparticles. This novel approach shortens the process of purification by promoting selective binding of antibodies, while separating a mixture of biomolecules during a filtration process. The effects of gold nanoparticles and the surrounding ligand on the proteins adsorption and filtration are investigated.

The results confirm that the functionalization helps in inducing a selective binding, preventing the non-selective one, and it also improves the selectivity of the separation process.
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<th>Full Form</th>
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<tbody>
<tr>
<td>Ac</td>
<td>Acetone</td>
</tr>
<tr>
<td>Au</td>
<td>gold</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Contact Angle</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DOX</td>
<td>1,4-Dioxane</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>γ-ImmunoGlobulins</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>NPs</td>
<td>nano-particles</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PS-b-P4VP</td>
<td>polystyrene-<em>block</em>-poly4-vinylpyridine</td>
</tr>
<tr>
<td>S</td>
<td>Sulfur</td>
</tr>
<tr>
<td>SH</td>
<td>Sulphhydryl (or thiol) group</td>
</tr>
<tr>
<td>-S-S-</td>
<td>disulfide bond</td>
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Chapter 1. Introduction

1.1 Biomolecules separation processes

In the last decades, the pharmaceutical sector has widely progressed in the field of “biopharmaceuticals”, increasing the need of available purified proteins for therapeutic treatments.[1] In particular, the demand of specific antibodies has increased due to their advantage for therapeutic uses and for advanced diagnosis. Among the different types of antibodies (also called immunoglobulins - Ig), the most common is the so-called IgG (immunoglobulins G), which is used in broad applications, moving from science to alternative therapies. As for the medical applications, antibodies can be used as therapeutic agents, for treating inflammatory diseases (like Chron’s disease or multiple sclerosis), asthma, cardiovascular and infectious diseases. [2] Moreover, IgG has been found useful in advanced diagnosis for oncological applications; in this direction, immunoglobulins work as means to carry toxins to the cancerous cells and, at the same time, they are thought to recover the reactivity of the immune system by defeating the cancer[3]. The path into the development of antibodies as therapeutics is long and intricate, but it has been recognized as valid and authentic by national and international authorities: already in 2005, the US Food and Drug Administration (FDA) approved 21 monoclonal antibodies (mAbs) as candidates to treat different types of cancer (like breast cancer, leukaemia, or prostate cancer), rejections after transplants and auto-immune diseases.[4]

While new therapeutic strategies based on monoclonal antibodies are scientifically and medically relevant, at the same time they represent an encouraging big business for the pharmaceutical companies. It was reported by J. M Reichert et al.[4] that, among the products approved by FDA for therapeutics, in 2004 six had global sales over $500 million
each. mAbs for immunological diseases tend to have larger markets, due to their broader use in different treatments, than those covered by oncology mAbs. However, this second category has even higher prices.[4] Therefore, the interest from biotech and pharmaceutical industries is based not only on the possibility of offering a new method to treat diseases, but is also driven by the possible revenue that this development would implicate.

The greatest requirement behind the development of such revolutionary process is embodied in the need of very high purity levels, in order to meet high efficiency and safety standards. Therefore, it is fundamental to have purified antibodies, which can be used for the treatments. The need of purification derives from the production process itself. During the antibodies production, as a general trend, many biological entities (such as proteins, DNA, antibodies) are formed within the same batch and need to be eliminated in order to obtain a purified final product. Whereas the development of biomolecules belongs to the so-called upstream bioprocessing (USP), in industry, the role of separating and purifying a product is assigned to the downstream processing (DSP), which involves the use of many separation techniques (e.g. centrifugation, ultrafiltration and microfiltration, chromatography) in order to isolate each product of interest. Figure 1.1 schematically represents the complete process of mAbs preparation, starting from cell culture. It can be seen that USP only includes the growth in batch or continuous reactors, while DSP involves many more passages. Going into details, the basic steps happening in the downstream processing include: (i) clarification by centrifugation or microfiltration (to remove cells and cell debris), (ii) concentration by ultrafiltration, (iii) selective purification steps, (iv) virus inactivation and removal and, at the end, (v) validation and quality control tests. Besides being a long process, the main drawbacks related to the downstream bioprocessing
are the costs. Whereas the previous (i), (ii) and (iv) steps count only for the 10% of the total downstream costs, the majority of the costs is related to step (iii), the selective purification [2]. Therefore, so far downstream processing represents the bottleneck in obtaining monoclonal antibodies at reasonable costs, thus the major potential for optimization in this market is found in the streamlining of the selective purification, in order to make it more efficient, economic and fast. Downstream bioprocessing development is oriented towards yield and productivity as well as purity and process capacities. Hence, to increase the separation efficiency of single unit operations the best option is not to re-invent a new process, but rather to expand existing facilities and to optimize existing and alternative processes.[5]

![Figure 1. Upstream and downstream processes, schematically represented. Picture reported from [5].](image)

Under these considerations, the aim of the work reported in this dissertation is to offer a support to the development of faster and smarter purification methods. Usually, the
purification is achieved by expensive techniques, as in the case of antibodies by chromatography, which has limitations (in terms of money and costs) for large-scale productions. Therefore, in this work an alternative solution is explored, based on the use of polymeric membranes. Membranes are already being successfully applied in bioseparations, mostly as separating by size-exclusion, thanks to the possibility of tuning their pore size. The methodology described in this work allows the separation of different biomolecules and the recovery of the species of interest (mAbs), through a novel approach.

1.2 Membrane separations

1.2.1 Industrial separations

In industry, many processes require the need of separating products and this can be achieved by taking advantage of the different physical or chemical properties of the components in the mixture. Marcel Mulder, in his book “Basic Principles of Membrane Technology”, summarized different separation processes that are used in industry according to the properties they discriminate (reported in Table 1.1). It is easier and faster to select the property which best helps to differentiate species in a mixture and, therefore, to apply the most appropriate separation method, or a combination of methods. However, in many scenarios, different separation processes could be implemented for the same mixture; in those cases, the way to select the best approach is based on two general criteria: i) the separation must be technically feasible, ii) the separation must be economically feasible. Thus, whenever the separation is known to be technically feasible, the choice of the method to use is based on which is the most convenient in terms of money.
Indeed, the feasibility depends on the value of the product to separate and also on the technology implemented. Whereas the value of the product is generally a fixed one (at least for a certain period of time), a way to decrease the costs of the process is by improving the technique used for separation. In this respect, membrane technology and development have gained lot of attention due to their likelihood of offering comparable performances to current industrial separation processes, but with a lower economic impact.

### 1.2.2 Membrane technology

A membrane can simply be described as a selective layer between two phases, which has the ability of allowing the preferential passage of species. The basic working principles of a membrane is represented in Figure 1.2: the membrane separates a feed stream into two others, the retentate (also called the concentrated stream) and the permeate. According to the purpose of the separation (whether concentration or purification), the products might be found in one or in the two streams. Moreover, membranes can be classified according

<table>
<thead>
<tr>
<th><strong>Physical/chemical property</strong></th>
<th><strong>Separation process</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>size</td>
<td>filtration, microfiltration, ultrafiltration, dialysis, gas separation, gel permeation chromatography</td>
</tr>
<tr>
<td>vapor pressure</td>
<td>distillation, membrane distillation</td>
</tr>
<tr>
<td>freezing point</td>
<td>crystallization</td>
</tr>
<tr>
<td>affinity</td>
<td>extraction, adsorption, absorption, reverse osmosis, gas separation, pervaporation, affinity chromatography</td>
</tr>
<tr>
<td>charge</td>
<td>ion exchange, electrodialysis, electrophoresis, diffusion dialysis</td>
</tr>
<tr>
<td>density</td>
<td>centrifugation</td>
</tr>
<tr>
<td>chemical nature</td>
<td>complexation, carrier mediated transport</td>
</tr>
</tbody>
</table>

Table 1. Analysis of separation processes based on the physical or chemical properties of interest. Reproduced from [6].
to the average pore size ($d_P$), characteristic which corresponds to the applications they will be destined to: hence, we have microfiltration ($d_P > 50$ nm), ultrafiltration ($2 < d_P < 50$ nm) and nanofiltration ($d_P < 2$ nm) membranes. With this description, it is possible to understand the incredible potential of membrane-based technologies, which can offer innovative solutions to the processing industry.

![Schematic representation of a membrane’s working principle.](image)

Figure 1. 2 Schematic representation of a membrane’s working principle.

In terms of benefits, membrane technology seems appealing because of the possibility of being applied in continuous processes, because of the generally low energy consumption and because of their character to adapt to the required situation. In fact, some membranes can be used for very similar applications without requiring modification or even for different applications by simply implementing a different surface functionalization or crosslinking.[6, 7]

**Control parameters**

As it might sound intuitive, a membrane is not classified only by its intrinsic properties, but especially by its performance in a define separation process. Therefore, before going into details, it is necessary to define and understand some key parameters, which are used as terminology when talking about membranes. Here we will consider processes in which
the driving force leading to the separation is a pressure gradient. However, even differences in concentration or temperature can be driving forces for separation processes.

First of all, an important parameter to introduce is the so called permeance, defined as the ratio between the permeability of the material and the thickness of the membrane. The permeability ($P$), which is an intrinsic property of the material constituting the membrane, can be calculated as:

$$ P = \frac{V \cdot l}{A \cdot t \cdot \Delta p} \text{ (barrers)} $$

taking in consideration the thickness ($l$) and area ($A$) of the membrane and the process’s operation pressure gradient ($\Delta p$). $t$ represents a time interval and $V$ the volume of the liquid passing through the membrane during it. The units for the permeability are generally recognized as barrers, where 1 Barrer = $10^{-10} \text{ cm}^3_{\text{(STP)}} \cdot \text{cm} / (\text{cm}^2 \cdot \text{s} \cdot \text{cmHg})$. From this parameter, we can define the permeance ($J$), which is instead a characteristic of the membrane, and can be intuitively derived by normalizing the permeability over the thickness:

$$ J = \frac{V}{A \cdot t \cdot \Delta p} \text{ (GPu)} $$

It is generally measured in GPu, where 1 GPu = $10^{-6} \text{ cm}^3_{\text{(STP)}} / (\text{cm}^2 \cdot \text{s} \cdot \text{cmHg})$, but it is also possible to find it expressed with conventional units, as L / (m$^2 \cdot $h $\cdot$ bar); indeed, in the rest of this manuscript, the second convention will be adopted.

Furthermore, flux ($Q$) is a parameter frequently used to describe the adequacy or efficiency of a membrane for a define process. This parameter is calculated as:

$$ Q = \frac{V}{A \cdot t} \text{ (cm)} / \text{s}. $$
Beyond the “hydrodynamic” properties of a membrane, which give information about its permeability, the system has to be classified according to its ability to discriminate between two molecules/species. A membrane was above characterized as a selective layer separating two phases. Therefore, \textit{selectivity} is one of the most important parameters for classifying the performance of a membrane. Like permeance, selectivity is a membrane property and is calculated as:

\[
\alpha^A_b = \frac{P_A}{P_B} = \frac{J_A}{J_B} = \frac{Q_A}{Q_B}
\]

Researchers in the membranes field aim at high selectivity and high permeability, to have better performing processes and lower costs. However, this achievement is hard to reach because of the trade-off between the two: whenever higher selectivity is achieved, generally this is at the expense of permeability, and vice versa. Therefore, continuous work is oriented towards this goal.

\textbf{1.2.3 How to develop a membrane}

We have talked about the feasibility of dealing with membranes for separation processes, but never mentioned how membrane are developed from scratch. There are different types of synthetic membranes in the market, among which two groups can be classified: polymeric and ceramics. The first class is the most promising and interesting one, as it is much cheaper than ceramic membranes and can be tuned in many different ways to obtain different purposes. Therefore, the focus of this brief overview will be in alignment with the rest of the work, which is oriented towards the use of synthetic polymeric membranes. Polymeric membranes can be \textit{porous} or \textit{nonporous}, according to the application they are created for. The former type is used for micro- and ultrafiltration, thus its separation ability
is mostly related to the tuning characteristics of the pores (to discriminate between particles’ sizes) and the chemistry of the material, whereas the latter corresponds to uses in gas separation and pervaporation, where instead the separation process is dependent only on intrinsic properties of the polymeric material and is determined by the solubility and diffusivity of the species through the materials.

**Phase inversion method**

Synthetic membranes can be obtained by different methods, but among others the most used and developed is the so-called *phase separation method*. It consists in a process, during which a polymer solution is separated in two phases: one with a high polymer concentration and one with a low polymer concentration. The more concentrated phase solidifies immediately after the phase separation, forming the membrane, while the polymer-poor phase will form the pores. There are different techniques for having a controlled phase separation, but mainly four methods have been widely implemented [8]:

- *Thermally induced phase separation* (TIPS), which takes advantage of the fact that solvent’s quality worsens when the temperature is decreased, thus favoring demixing in the polymer solution and inducing phase separation;

- *Precipitation by controlled evaporation*, where the polymer is dissolved in a mixture of more volatile solvent and less volatile nonsolvent. During the evaporation, the composition of the system shifts towards a higher nonsolvent and polymer content, inducing the polymer precipitation.

- *Precipitation from the vapor phase*, in which a cast film, made of polymer and a solvent, is placed in an atmosphere of a nonsolvent saturated with the solvent. Given
the solvent’s saturation, the solvent will not evaporate, whilst the nonsolvent will penetrate and diffuse though the polymer film, forming the membrane;

- **Immersion precipitation**, in which a polymer solution is cast on a support as a thin film and is then immersed in a nonsolvent bath to induce precipitation due to the exchange of good solvent and nonsolvent within the polymer solution.

Beside the first described method, the other three approaches can be classified as *nonsolvent induced phase separations* (NIPS), as it is recognizable that they take advantage of the low solubility of polymers in nonsolvents. Within this group, immersion precipitation has obtained a lot of importance, not only for the feasibility of the method, but also for the convenience that in many systems the nonsolvent role is played by water. Moreover, this technique is the one implemented during the experimental part of this work, therefore, starting from now, our attention will focus towards the understanding of how to predict and obtain membranes through immersion precipitation.

**Thermodynamics**

When dealing with NIPS, three components are part of the same system – polymer, solvent, nonsolvent. In order to estimate whether a certain polymer solution would create a membrane or not, when in contact with a nonsolvent, it is necessary to make use of phase-diagrams, which give information about the system at different compositions. For ternary mixtures, the diagram looks like a triangle, whose corners represent the pure components - polymer, solvent and nonsolvent. A schematic illustration is reported in Figure 1.3 Any
point in one of the sides of the triangle represents a mixture of the two corner components. For example, point A in the figure represents a mixture of polymer and solvent. Instead, any point within the triangle represents a mixture of the three components, like in the case of point B.

The starting point for membrane formation, based on such ternary systems, is to prepare a homogeneous, thermodynamically stable solution. This would generally correspond to point A in the polymer/solvent side of the triangle. However, it should be kept in mind that it is also possible to have an amount of nonsolvent in the starting solution, as long as this does not affect the thermodynamic stability and allow the mixing. The limit between stability and instability can be defined once the binodal line is determined experimentally or calculated from thermodynamics equations. It represents the conditions at which the two phases can co-exist, i.e. the limit between the thermodynamically favorable mixing and demixing (or phase separation). A typical binodal line is represented in Figure 1.4 a), together with the stable and unstable areas. The upper part of the binodal line represents a polymer-rich phase, whereas the lower part represents a polymer-poor phase. Any point within the area delimited by the binodal line corresponds to a system with two phases: a polymer-rich and a polymer-poor phase. The composition of each phase is determined by the tie line, as shown in Figure 1.4 b). Starting from a stable polymer-solvent solution and by adding a nonsolvent, the system moves towards instability, until it reaches the binodal, at which the demixing starts to occur. If the path slowly crosses the metastable region, nucleation and growth is the predominant mechanism. If crossing the binodal is fast or near
the critical point, spinodal decomposition mechanism is predominant. If the major (in volume) separated phase is more concentrated in polymer, a membrane will be formed. If the major phase is diluted in polymer, isolated particles will be formed. [9, 10]

![Phase diagram](image)

Figure 1.4 Phase diagram for a ternary system: a) evidence of binodal and spinodal lines, b) tie line and phase compositions.

The polymer choice

In principle, a broad range of polymers could be used to obtain a membrane; however, according to the chemistry, the applications, the mechanical properties and the versatility of them, only some candidates have been extensively applied in the industry or even explored in research. Moreover, for ultrafiltration and microfiltration applications, the aim is to obtain membranes with a uniform pore size distribution, in order to increase the selectivity of the membrane, therefore its performances. Among others, an emerging class of polymers has gained a lot of interest in the last decade, the *block copolymers*. As the name suggests, they are formed by different blocks, which are covalently bonded in the same polymeric chain. They are very interesting because of the possibility of exploiting blocks with different properties (chemical or mechanical) within the same structure, in order to obtain materials with novel characteristics. Moreover, it was demonstrated that
diblock copolymers spontaneously organize in regular patterns at the nanometer scale[11], self-assembling in many different possible morphologies (e.g. lamella, gyroid, vesicles, spheres). Therefore, by tailoring the environment, it is possible to control the self-assembly and obtain a desired morphology. In particular, our group has been exploring the combination of block copolymer self-assembly and nonsolvent induced phase separation to obtain isoporous membrane[12-16].

Due to their high potential, the current work is oriented towards the implementation of block copolymer membranes and their use for bioseparations. In particular, the polymer chosen as base and starting point for the experimental part is polystyrene-\textit{block}-poly(4-vinylpyridine) (PS-\textit{b}-P4VP), since PS-\textit{b}-P4VP have been highly optimized with very ordered and regular porous structures [14]. Further details and explanations are reported on Chapters 3 and 4, where a deeper understanding of the work is offered to the reader.

1.2.4 Membrane chromatography

An important role in the downstream processing of monoclonal antibodies is played by chromatographic separations[17]. This technique traditionally uses columns or beads, through which the solution to be separated flows. By differences in the compounds’ properties, some species are retained more than others, leading to the desired separation. A drawback of this method is the difficulty to clearly separate species that are similar in a mixture of more than two compounds. Therefore, more separations steps are required in a downstream industry, before obtaining the final desired product. However, chromatography is still a mandatory step in antibodies purification. Affinity chromatography with protein A has been, so far, the best technology developed for
antibodies purification. On the other hand, membranes are being considered with increasing interest by the biotech industry as potential adsorbers for biomolecules, mainly for two reasons: a) they offer a bigger surface area available for binding, thus increasing the efficiency of the process, b) they sustain higher flow rate and productivity, thus increasing once more the overall efficiency. A disadvantage could be that membranes are disposable, meaning that they can increase the cost-effectiveness compared to chromatographic tools[18], but easy replacement could assure a clean sterile separation. Specifically, in the purification of monoclonal antibodies, the mechanisms developed for membrane adsorbers involve affinity and ion exchange adsorption. While the former is used for capturing the biomolecule and requires highly specific interactions between the adsorber and the target species, the latter is used for releasing the molecule from the substrate, creating opposite charges in the mobile and stationary phase. The development of novel membranes presented in this work follows both approaches. An important decision to take during the development of this project was how to create the affinity between the membrane and the antibody, for the adsorption process. This choice strongly depends on the target molecules’ characteristics; in particular, for antibodies, two approaches are generally used: making use of the specificity of the antigen binding or making use of the Fc fragments of the antibody [19]. Whereas the first approach, generally called immunoaffinity, is mainly targeted for diagnostics, as it involves the active sites of the antibody, the second one targets the Fc fragments: on this direction, the most used affinity method so far makes use of Protein A from *Staphylococcus aureus*, whose detailed description is not the purpose of this work, but the reader is invited to have a better understanding by reading the review article of Huse et al.[19] for further details. As for the
development of the current project, the affinity was created by choosing a specific functionalization of the membrane and by working under a precise pH environment. Indeed, proteins are characterized by having different isoelectric points (IEP), functional groups, and hydrophilic-to-hydrophobic ratio, thus offering a variety of possibilities for the interactions between the membrane surface area and each molecule[20].

According to the ion exchange step for the recovery of the antibodies, the methodology consists in creating opposite charges between the membrane and the antibody, such that the two species repel each other and the IgG can be recovered. This step is generally achieved by playing with the pH of the solution (keeping always in mind that denaturation conditions should be avoided). In fact, every biomolecule is characterized by an isoelectric point (IEP), a value of pH in which the net charge of the molecule is equal to zero; below that value the proteins is positively charged, above it is negatively charged. For example, the IEP for IgG is ~7, for BSA it is 4.9. Hence, by working at pH≈6 for example, the BSA is negatively charged and the antibody is positively charged. Therefore, it is possible to have different scenarios, working with the same molecule but changing its environment.

In conclusion, adsorption and recovery of antibodies can be summarized as sequential steps: first the membrane is set for some time in the environment at which the adsorption will take place (equilibration step), in order to stabilize the pore dimensions under the desired pH. Later, the protein solution is put in contact with the membrane and the adsorption process takes place, due to affinity between antibodies and substrate. Once the adsorption reaches equilibrium, the recovery steps can start. First, the membrane undergoes a phase of washing, during which unwanted bonded impurities are washed away, then the final elution step can take place, by changing the pH of the solution and inducing the
recovery of the product through ion exchange. This discussion is further resumed in §4.6, when experimental details are described.

1.3 Nanotechnology in biological applications

The main motivation of this work is the separation and recovery of biomolecules, especially proteins. However, one of the most challenging problems, when working with biomolecules, is the risk of their denaturation. It is critical to avoid this inconvenience, especially when the main purpose is to preserve the activity of the organic molecule. However, the problem of bioconjugation of proteins or antibodies to solid surfaces is not new and similar difficulties have already been faced in applications like sensing and diagnostics. Throughout the past decades many techniques [21, 22] have been proposed and tested, moving from physical entrapment and encapsulation to physical adsorption or covalent immobilization. Nevertheless, the mentioned binding processes are generally non-reversible, then making them incompatible with the recovery process [23].

It is in this scenario that nanotechnology and recent developments in immunosensing contribute to identify a candidate for reversible binding of biological complex. Bioconjugation of biomolecules to nanoparticles simply involves bonding by chemical or biological means, which render them ideal for clinical applications. In particular, it has been proven that gold nanoparticles (Au NPs) are a suitable agent for binding biological molecules[24], because of their good biocompatibility, water affinity and their efficient reversibility, other than showing good signaling and specific adsorption tendency. The coupling and functionalization of NPs with proteins can be achieved by using a variety of methods. These include electrostatics interaction, ligand recognition, chemisorption, and
covalent binding through bi-functional linkers. Under this perspective, it is necessary to underline that antibodies (like other proteins) contain amino acid chains with a number of reactive side groups, which could be utilized for attachment. Examples are -NH₂ (lysine, and N-terminal), -COOH (aspartic acid, glutamic acid, and C-terminal), and -SH (cysteine). The latter one, thiol group, is localized on the side chain of cysteine residues and is present in most proteins. For this reason, it is one of the most interesting groups researchers have been working with. Thiol functionalized nanoparticle is potentially a very useful tool. The presence of the terminal sulfur allows the thiol group of the antibody to undergo oxidation (S-thiolation) when binding to the gold nanoparticles’ ligand and form a reversible –S–S– linkage[25] (Figure 1.5).

Figure 1. 5 Oxidation of an antibody when binding to the sulfur ends of a capped gold nanoparticles, forming the –S–S– bond.

When using gold particles, the ligand surrounding the central gold shell is very important; it gives stability to gold but, at the same time, it must be compatible with the biomolecule. Among others, as previously mentioned, Au NPs provide suitable microenvironment for biomolecule immobilization retaining their biological activity[26], and the combination between thiol ligand and gold nanoparticles increase the strength of the bond with the antibody, creating more stable structures.
Beside the biocompatibility, gold nanoparticles have received great interests because they have several kinds of intriguing properties. Beyond the well-known surface plasmon resonance behavior, typical of metals in the nanometer range, Au NPs have also been proved to have high surface-to-volume ratio and high surface energy, which is the reason for providing a stable immobilization of a large amount of biomolecules retaining their bioactivity. Summing up, the high surface area-to-volume ratio and biocompatibility of gold nanoparticles, which offer a very large number of interaction sites, make Au NPs potential candidates to amplify the sensing surface area and maintain the sensing bioactivity, thereby enhancing the quantity and activity of the biological recognition elements [27]. With different types of signal transducers, the physical or chemical changes that occur during the binding of the analyte to the bioreceptors can be transformed into optical, electrical, or qualitative output signals. These properties sound promising for smart responsive applications and a deeper discussion will follow on Chapter 6.
Chapter 2. Aim and objectives

In the current work, a new approach to antibodies purification is proposed and tested. Supported by the demand of having separation processes less expensive and more efficient, the current proposal tackles the problem by introducing an ultrafiltration membrane, which not only allows the selective separation between IgG and other proteins (like BSA) in solution, but it also promotes the recovery of the antibodies from a filtration stream. The polymeric material chosen is PS-\textit{b}-P4VP, a block copolymer which has been previously demonstrated to form membranes with narrow pore size distribution and pH responsive behavior [14]. This choice is conformed to the need of having isoporous substrate to favor the selectivity and to the presence of the pyridine block which has been found to have affinity with gold nanoparticles [28, 29]. Indeed, the use of gold nanoparticles for membranes application is here exploited to obtain an advanced system for purification, by making use of the biocompatibility of gold at the nanometer scale. Moreover, it is also possible to extend the study to the binding effect between antibody and nanoparticles and understand how the peculiar properties of Au NPs could help in creating smart responsive systems.

In the following paragraphs, the main objectives of this work are listed:

- Demonstrating how the presence of gold NPs influences the adsorption of antibodies on the surface. In particular, the purpose is to obtain a functionalization that promotes specific binding of the antibody and prevents the unwanted binding through the antigen sites. Moreover, this functionalization should create enough binding sites, in order to make the process as efficient as possible;
• Studying a system in which the ligand around the nanoparticles helps in favoring the specific binding. In particular, the approach followed during these tests consisted in using a dithiol molecule as ligand. Dithiols are aliphatic molecules ending with a thiol group, -SH, at both extremities of the chain. The idea of using this type of functionalization is in accordance with the fact that antibodies have terminating –SH group at the end of their main chains at neutral pH (as mentioned in chapter §1.3), thus undergoing S-thiolation by oxidation. As for the nature of the bond, the system is expected to be reversible, therefore suitable for the process under description.

• Understanding how the overall functionalization can affect the process properties and whether the designed filtration process is working as expected.

A graphical summary of the objectives is reported in Figure 2.1: the upper part of the picture represents the membrane functionalization, from left to right first the membrane is functionalized with gold nanoparticles and later the dithiol functionalization is performed. The lower scheme, instead, represents the process itself: the aim is to perform a filtration of a mixture of biomolecules, obtaining the separation between antibodies and other proteins through size exclusion (goal achievable by playing with pH environment). Furthermore, the antibodies are expected to bind to the surface of the membrane (thanks to the increased affinity), from which they can be further recovered.
Figure 2. Representation of how the functionalization of the membrane occurs (upper part) and how the filtration and separation process is expected to happen (lower part), inducing adsorption of the antibodies, which can then be recovered.
Chapter 3. Experimental section

3.1 Synthesis of PS-b-P4VP membrane

As mentioned above, the type of membrane used throughout the whole project is made of PS-b-P4VP [14]. According to different literature resources, which follow the prediction of the ternary diagram, the preparation of the polymer solution was performed by using a solvent system made of dimethyl formamide (DMF), 1,4-Dioxane (DOX) and acetone (Ac), with mass percentages (wt%) reported in Table 3.1.

Table 3.1 Solvent system used for the polymer solution. The numbers represent mass percentages (wt%).

<table>
<thead>
<tr>
<th>PS-b-P4VP</th>
<th>DMF</th>
<th>DOX</th>
<th>Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 %</td>
<td>24 %</td>
<td>44 %</td>
<td>16 %</td>
</tr>
</tbody>
</table>

The components were added in a vial following the order indicated in the table. The system was then left stirring overnight at 80 rpm. Once the solution is clear, and the polymer is completely dissolved, the stirring is stopped, and the solution is left resting for some hours to allow potential bubble of air to disappear from the casting solution and prevent them from compromising the final morphology of the membrane. Later, the solution is cast on a glassy support, which has to be previously washed carefully with water and cleaned further with acetone, to avoid any contaminations. Figure 3.1 illustrates the process of casting. The polymer solution is poured on the glass and, immediately, a casting knife – or doctor blade - (as in the inset in Figure 3.1) is used to form the membrane. Typically, during this work, the gap of the casting knife is set at 200 µm. Once the membrane is cast, in order to undergo complete development, the membrane has to follow the non-solvent induced phase separation (NIPS, as described in §1.2.3) process, thus, after few seconds of evaporation, the membrane is immersed on a Deionized Water bath, which is at 25°C. The membrane
detaches soon from the glass, and it can be collected and stored. The storage is usually done by keeping the membrane inside a water bath in the fridge.

3.2 Gold nanoparticles preparation

Gold nanoparticles (Au NPs) were prepared for functionalization of the polymeric membrane obtained from PS-\(b\)-P4VP. The preparation followed an established procedure, developed first by Turkevich [30, 31] and refined later by Frens [32]. During this project, the gold nanoparticles have been prepared in this way: a 25 mM aqueous solution of tetrachloroauric (III) acid trihydrate (HAuCl\(_4\) \(\cdot\) 3H\(_2\)O) is added to 500 mL of boiling water and, after few minutes, an aqueous solution 50 mM trisodium citrate tribasic dihydrate is added as reducing and stabilizing agent. When the gold salt HAuCl\(_4\) \(\cdot\) 3H\(_2\)O is dissolved in water, it dissociates into ions, H\(^+\) and AuCl\(_4\)^-, like in the representation of Figure 3.2. When the sodium citrate is introduced later, it reduces the gold ions into elemental gold atoms (i.e., passing from Au\(^{3+}\) to Au\(^0\)), which are not as soluble and they agglomerate, forming nano-sized particles. This step is called “nucleation” of the nanoparticles and it is followed by the “growth”, during which the gold atoms still in solution bind to the nucleation site.
Once the solution turned pinkish/purple, it was stored in the fridge at +4°C. The size of the particles was estimated by observing the location of the absorption peak in the UV-Vis. It was found that, by using the above-reported ratio of gold to citrate, the absorption peak is located around 525 nm, corresponding to a size of ~20 - 25 nm. [33] Moreover, by using Beer-Lambert law (A = ε · b · c), the concentration (c) of nanoparticles in solution was calculated to be ~1.5 · 10^{-10} M, using an extinction coefficient (ε) equal to 2.93 · 10^9 M^{-1} cm^{-1}, as reported in the study of Liu et al. [34]

### 3.3 Functionalization of polymeric membranes with gold nanoparticles

According to the purpose of this work, the polymeric membrane was functionalized by incorporation of gold nanoparticles. The process of incorporation is quite simple, it just requires the membrane to be in contact with an aqueous solution of citrate gold nanoparticles for some hours. It was previously reported [28] that gold nanoparticles preferentially deposit in the pyridine coronas and this behavior could be confirmed once more during these experiments when the particles where incorporated in the membranes (Figure 3.3). By following previously reported procedures, two different strategies were attempted in order to functionalize the membrane with gold nanoparticles.
One of them consisted in simply dipping the membrane into an aqueous solution of Au NPs under stirring conditions ("batch"). The second strategy made use of a filtration cell in order to filter the gold nanoparticle through the membrane ("continuous"). Further discussion will be conducted in section §4.2.

### 3.4 Functionalization of membrane with dithiol

The surface modification in this project was mainly performed using dithiol molecules with the objective of obtaining a temporary disulfide bond between the dithiol molecule and the partially reduced antibody, which has a –S– extremity available to form a bond. The dithiol was added after the membrane has been functionalized with gold, in order to gain benefit from the strong interaction between gold (Au) and sulfur (S) to form Au–S bonds. Following the literature[35], the functionalization was performed by dipping the PS-\(b\)-P4VP membrane, holding the gold nanoparticles, into a solution of dithiol in ethanol for 18 hours, followed by rinsing with plenty of ethanol, in order to remove the unreacted thiol.

### 3.5 Characterization section

In order to confirm the structure of the membranes, verify the chemical modifications, and to correlate with the performances of the membranes, different tools were used. The main characterization methods were: SEM (Scanning Electron Microscopy) for morphological...
characterization, FT-IR (Fourier Transform Infrared) spectroscopy for chemical analysis, NanoDrop for adsorption evaluation, permeation line and GPC (Gel Permeation Chromatography) for permeation/selectivity tests, contact angle for hydrophilicity measurements.

3.5.1 Scanning Electron Microscope (SEM)

SEM gives morphological information on the surface of a sample, by scanning it through a focused electron beam. By interacting with the atoms of the material, the electrons are scattered, producing signals which are then translated in images. The microscope used during this project was Zeiss Merlin, characterized by a Gemini II column, where the electrons are emitted, accelerated, focused and deflected, and a Zeiss Smart SEM software for analysis and images collection. During this project, the use of SEM has been fundamental to study the structure of the membrane pre- and post- treatments.

3.5.2 Fourier Transform Infrared spectroscopy

FTIR (Fourier Transform Infrared) spectroscopy was employed to understand whether and which new chemical bonds were formed after the modification with gold nanoparticles and dithiol molecules. FTIR sends infrared radiation through a sample: some of the radiation is absorbed by the sample and some of it is, instead, transmitted. In doing so, the instrument is able to represent the molecular absorption and transmission, such that a unique spectrum (“fingerprint”) of the sample is created. Following this characterization, it has been possible to compare different membrane which have been through different functionalization, such that changes in the molecular structure could be detected.
Specifically, this technique has been useful in characterizing the membrane functionalized with gold nanoparticles or with dithiol molecules. The model used for the current work is a Thermo Scientific™ Nicolet iS10. In Section §4.5 a discussion will follow about how helpful FTIR can be, but also which limitations have been noticed.

3.5.3 NanoDrop 2000c Spectrophotometers

This instrument is generally used to quantify (in terms of concentrations) and assess purity of DNA, RNA and, specifically for the application of this project, proteins. It uses UV-lights, which is absorbed by biological molecules at specific wavelengths. When a measurement of a sample is taken, the instrument records the intensity of light that was transmitted through the sample. By measuring the intensity of the blank solution, it is possible to measure the absorbance as:

\[ \text{absorbance} = -\log \left( \frac{\text{intensity}_{\text{sample}}}{\text{intensity}_{\text{blank}}} \right) \]

Moreover, by using the Beer-Lambert equation we can correlate absorbance and concentration:

\[ A = \varepsilon \cdot b \cdot c \]

where:

- \( A \) = absorbance represented in absorbance units (A)
- \( \varepsilon \) = wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of L/mol·cm
- \( b \) = the pathlength in cm
- \( c \) = the analyte concentration in mol/L or molarity (M)
Therefore, concentrations of proteins in solution is measured. According to which type of protein is used, the extinction coefficient would be different, allowing us to read the correct value of protein’s concentration.

3.5.4 Permeation and rejection tests

As discussed in Chapter 1 (§1.2.2), some of the main features characterizing a membrane are the flux and the permeation. A permeation line, consisting in a set-up of working cells under pressure conditions, was necessary to determine the permeance of the membrane throughout the experiments. Furthermore, for the sake of clarification, it is important to report which type of cells was used during the experimental part. All the flux and rejection measurements were conducted in an Amicon® Stirred Cell, under working conditions of 1 bar and with a stirring speed of 150 rpm.

3.5.5 Gel Permeation Chromatography (GPC)

GPC is a technique that separates and characterizes molecules according to their size. In particular, it consists of a column made of porous rigid voids through which the mixture is forced to pass. The size-exclusion mechanism is based on the principle that lower molecular weights are retained longer into the column, cause their smaller size allows them to be entrapped into the spheres, whereas the higher molecular weights are let passing through the column faster. Therefore, the spectra obtained after a GPC test will report a series of eluted species: the first peak corresponds to the highest molecular weight, which is eluted as first, whereas the last one will correspond to the lowest molecular weight, which has been retained into the column for the longest time and eluted as last.
During this project, the measurement through GPC have been used for having information about the percentage of rejection of the membranes used for the experiments. In particular, the test consists in operating a filtration of a solution containing different molecular weights of polyethylene glycol (PEG) and then collecting its permeate. By analyzing the feed and the permeate at the GPC, it is possible to determine which molecular weights have been rejected from the membrane and which ones instead have permeated; for these ones, then, it is also possible to calculate the percent of permeation or rejection.

3.5.6 Contact Angle (CA) measurements

The contact angle’s measurement is a well-known and simple process, which is used to determine the wettability of a surface, hence its degree of hydrophilicity. By means of a micropipette, a droplet of water is let falling down on a surface of interest. A camera captures the image and the software automatically calculates the angle of contact between the liquid phase and the solid one. If the angle is lower than 90°, the surface is considered hydrophilic, hydrophobic in case of angles greater than 90°.

For the purpose of this work, CA measurements were performed to determine the hydrophilicity of the membranes and the influence of the functionalization on the wettability.
Chapter 4. Results and discussion

The main purpose of this work consists in achieving proteins separation and purification through the use of nanotechnology and membranes. However, studies have also been conducted for understanding the roles of the nanoparticles into the membrane’s structure. Both aspects will be treated in the following data analysis and discussion.

4.1 Effect of evaporation time on membrane porosity

Membranes with different pores sizes and mechanical properties can be obtained by playing with some key factors, among which the evaporation time between the casting and the immersion in the water bath. Different timings have been tried for PS-\textit{b}-P4VP membranes, in order to select the best structure which could fulfill the purposes of this project. Figure 4.1 reports the results of evaporation times of 10, 15 and 20 seconds respectively. It can be noticed that the evaporation time of 10 seconds is not enough to allow the pores to form completely, therefore it is possible to see how part of them is still closed. The pore formation gets better when increasing the evaporation time, but still 15 seconds are not enough. The best structure was found when the casted membrane is let at evaporation for 20 seconds: the pores can form entirely and assume their characteristic rounded shape.

Figure 4.1 Membranes at different evaporation times.
4.2 Incorporation of gold nanoparticles

As mentioned in Chapter 1, the idea of using gold nanoparticles as functionalization for the membrane comes from the consideration that Au NPs offer a possibility of reversible attachment – detachment towards biological molecules. Hence, the first step of the process consisted in obtaining a homogeneous distribution of gold nanoparticles over the surface of a PS-\textit{b}-P4VP membrane.

Following the general procedure described in the experimental part (section §3.2), two main strategies were investigated, the “batch” and the “continuous” ones. The batch process was faster and easier than the continuous one, but it led to a high concentration of gold nanoparticles on the membrane. On the other side, the filtration process (to which we will refer as the “continuous” one) was longer, but it allowed the Au NPs to adhere to the polymer according to a more organized and homogeneous distribution. Hence, both methods showed advantages and drawbacks, but at the end the batch process has been chosen over the continuous one. The decisive reason for this choice came from a practical point of view: the batch mode better conserved the membrane morphology, while the continuous one led to pore deformation and decreased the mechanical stability.

The batch process was then optimized. In order to obtain a homogeneous distribution of particles (like the one observed for the continuous filtration process), the concentration of gold solution in the batch container was varied. Table 4.1 reports some trials of batch incorporations. In particular, the solution used for functionalizing was obtained each time by diluting the gold nanoparticles’ stock (prepared as reported in §3.2). The first row of the table reports the ratios between the concentration of the diluted solutions used for the functionalization and the concentration of gold citrate stock solution initially prepared.
From the table, we see that by using half-diluted solutions (i.e., the concentration is half than the concentration of the stock), many gold nanoparticles are present in the membrane pore walls, without following a really organized distribution over the surface. Instead, a better spatial organization is reached when the original gold solution is further diluted (four times), and the exposure time is left constant (8 hours). Under these circumstances, the particles are more uniformly distributed over the membrane, without creating agglomerations, like in the case of higher concentrations. If the solution is diluted even more (like in the case of the low 12.5% concentration), the exposure time need to be increased in order to reach a comparable situation.

<table>
<thead>
<tr>
<th>% Au NPs (compared to stock)</th>
<th>50 %</th>
<th>25 %</th>
<th>12.5 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hrs)</td>
<td>8</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 4. 1 Different concentrations of gold nanoparticles solution used for functionalization. The SEM images show how a more regular and homogeneous distribution can be achieved by controlling the concentration of the particles in solution and the exposure time of the membrane to the solution.

In order to compare the effect of different gold nanoparticles distributions over the surface, the final functionalization time was set at 8 hours, while three different concentrations of solutions were tested: 12.5%, 25% and 50% of the initial stock concentration. In other words, starting from the stock concentration, the solutions for functionalization were obtained by diluting it 8, 4 and 2 times respectively (Figure 4.2).
4.3 Hydrophilicity

After the modification with gold nanoparticles, the hydrophilicity of the membrane was tested in order to understand whether the gold nanoparticles were giving any change in affinity with water. Contact angle measurements revealed that there is no change in hydrophilicity, as the angle is kept constant around 45°. The results are in harmony with the purpose of the work, which requires a certain degree of hydrophilicity to be adopted in membrane chromatography context.

4.4 Thiol functionalization

The next step after gold incorporation was its functionalization with thiols. Different thiols have been investigated, and in particular two different lengths were used: 1,3-propanedithiol and 1,6-hexanethiol. Their molecular structures are reported in Figure 4.3. The basic structure is constant, while the chain’s length is varied. As mentioned earlier, the goal is to attach one of the two thiol groups to the gold nanoparticles and have the other one free to react with the antibody promoting S-thiolation (as in Figure 1.5). However, the

Figure 4.2 PS-b-P4VP membranes functionalized with different amount of gold nanoparticles. From left to right, the amount of gold nanoparticles in solution decreases, moving from 50% to 12.5% of the original stock. Below, a visual representation of the actual membrane is reported.
main drawback of using a dithiol is that, in principle, both thiols of the same molecule can react with gold, thus inducing a phenomenon that we can call “back-biting”. If this happens, no free thiol is left and the second step of the project cannot be achieved, because there would be no free –SH for the antibody to bind. In order to avoid this situation, some precautions are considered. First, we assumed that, in order to have back-biting, the dithiols molecule have to be “long” enough to bend back. A possibility to escape from this problem is given by using a very short dithiol, like 1,3-propanedithiol, which can decrease the risk of bending. Secondly, we considered that a lack of reactive –SH by full consumption through the back-biting phenomena can be limited by using a concentrated solution of dithiols. In this way, Au-S bonds are formed in excess with different molecules, increasing the probability of having free reactive –SH groups. Bearing this in mind, the experiments were carried out with different concentrations and types of dithiols. A deeper understanding of the process mechanism has been possible with help of FTIR, as reported in the following section.

Figure 4. 3 Dithiol molecules used for functionalization: 1,3-propanedithiol and 1,6-hexanediol.
4.5 FTIR analysis of functionalized membranes

FTIR analyzes the vibration of functional groups upon excitation with infrared rays. This characterization method is very used in membrane science (and many other fields) due to the possibility of studying and comparing surfaces which have undergone chemical modifications. In the current project, FTIR technology was useful for studying the changes in the chemical structure of the membrane, after introduction of nanoparticles and (di)thiol molecules. First of all, the effect of nanoparticles on the membrane was analyzed. Figure 4.4 reports the absorption spectra of the unmodified membrane PS-\(b\)-P4VP and of the modified one with gold nanoparticles. The general adsorption trend is kept in both membranes, but a new broad peak appears in the area between 3500-3200 cm\(^{-1}\) for the gold-modified membrane. This corresponds to the incorporation of –OH bonds, which are contained in the carboxylic groups of the citrate molecules surrounding the gold cores of

![FTIR spectra](image)

Figure 4.4 FTIR spectra of unmodified PS-\(b\)-P4VP (grey) and membrane modified with gold nanoparticles (orange). The evidence of Au NPs incorporation is the increase in –OH content, which is represented by the broad band in the area between 3500 and 3000 cm\(^{-1}\), and which originates from the incorporation of carboxylic groups surrounding the gold.
the nanoparticles. Moreover, a slight increase in the adsorption is also recognizable for aliphatic content: the increase (~3000 cm$^{-1}$) is also due to the small aliphatic chain of the citrate molecules surrounding the gold.

Once the gold functionalization was proven, the second step was faced: the functionalization with dithiol. In order to obtain the functionalization of interest for the purpose of this work, we must use a dithiol molecule which, by reacting with the membrane, will attach to the gold nanoparticles from one side and expose the free –SH group. However, at this point an intermediate step was performed, that helped to characterize the modified membranes; before proceeding to the final goal of the functionalization, it was beneficial to have confirmation that the thiol molecule is reacting with the membrane itself and attaching to the nanoparticles. To reach this goal, a thiol (instead of dithiol) molecule was used as control: dodecanethiol. Briefly, it contains an –OH group at one extremity of the chain, and a –CH$_3$ group at the other end. By testing the thiol molecules, the expectation was to see the –SH group reacting with the membrane (functionalized with Au NPs) and the other extremity free to stretch. In Figure 4.5 the results obtained by FTIR are reported. It is possible to observe that the wavenumber range of interest is ~3000 cm$^{-1}$. Here, the formation of a new peak can be noticed, not present in the previous membranes (unmodified one and gold-modified one): therefore, it is reasonable to conclude that this peak represents the incorporation of the aliphatic chain of the thiol molecule into the membrane. It confirms that the thiol has been incorporated into the membrane through the –SH group. However, it was not possible to find any trace of a new peak corresponding to a bond between the gold atoms and the sulfhydryl end of the
thiol molecules. According to the literature [36], Au – S vibrations appear in small intensity around 650 cm\(^{-1}\). Unfortunately, that area has many overlapping peaks and it would be difficult to reveal any small change. No new peaks are evident in that region. Moreover, it sounds more feasible that the –SH extremity of the thiol molecule interacts with the citrate ions which stabilize the gold core, rather than bonding direct with the gold itself. For this hypothesis, the confirmation would come from a peak at 1600 cm\(^{-1}\), signal corresponding to a S-C-C bond. Some evidence of this peak could be noticed. However, the peak is still not intense, most probably due to the very low concentrations of thiol used.

Despite the difficulties in recognizing whether the sulfur was directly bonding to the gold or to a carboxylic group, the study with simple (mono)thiol gave us the confirmation that the molecule was binding to the membrane through the sulfhydryl group. Following this proof, the next step was to incorporate di-thiol molecules onto the surface of the membrane. The only difference with the thiol is that the dithiol contains –SH groups in both ends, thus the –CH\(_3\) of the (mono)thiol is substituted by a –SH end. The procedure was identical to
the previous one with thiols, and two different types of dithiols were tried: propanedithiol and hexanediithiol. Figure 4.6 illustrates the most relevant peaks which were studied. As expected, the addition of the aliphatic molecule implies the increase of –C(sp²)– H content, which is confirmed by the formation of a new peak in the same area identified for the (mono)thiol incorporation, i.e. ~3000 cm⁻¹. Secondly, we wanted to have proof of the free sulhydryl end. In other words, the expectation is that one –SH group binds to the surface of the membrane, while the other one is left free for binding later the antibody. According to reported spectra and to literature’s discussion[37], -SH bond vibrations happen at ~2500 cm⁻¹, but this peak is absent in the reported spectra. Checking further into literature [37], we confirmed that this peak is generally weak, even when dithiols are used in high concentrations. This fact could have been the explanation of our results, given that our work was dealing with very small concentrations of dithiol (in the order of 50 mM up to 1 M) but it could also be an indication that both sulphydryl groups were reacting. Unfortunately, it is hard to prove one or another theory, because the contemplated
quantities are indeed very small. Therefore, we decided to continue our work by assuming a hypothesis: as long as the dithiol molecule is not long enough, one of the two sulfhydryl end can be considered free. Indeed, if we consider a propanedithiol, its chain’s length is so short that it is improbable to have a “back-biting” of both the extremities (even considering the bond angles and the possible conformations of the three carbons, the chain is still too short to be bent backwards). For this reason, the likelihood of having a free –SH end is higher. Therefore, the further experiments for adsorption have involved the use of propanedithiol only.

4.6 Adsorption of IgG

Once the chemical functionalization was proven, adsorption tests were conducted to know the effect of each functionalization and of the combined one, which should be the final one adopted for the desired separation. The absorption of IgG onto the surface of the membrane was measured. We mentioned before (§1.2.4) that the process of adsorption is one of the four steps taking place during a membrane chromatography separation, whose graphical representation is given in Figure 4.7. This methodology starts with an equilibration step, in which the membrane is immersed in a buffer solution with a pre-chosen pH. After that, the

![Figure 4.7 Schematic representation of affinity and ion-exchange membrane chromatography.](image-url)
membrane is ready to undergo adsorption, during which the protein in solution is attracted by electrostatic charge to the surface of the membrane. Sequentially, a washing step follows, during which impurities are washed away from the membrane, such that the fourth final passage can occur: the elution of the protein from the membrane, which is achieved by using different techniques which will be discussed later (§4.8). The process is quite long and each of the steps is fundamental to reach the final goal. It is necessary to underline the importance of having a controlled volume throughout the whole process. In fact, variations of protein concentrations can be detected by using NanoDrop Spectrophotometer, but in order to get the exact value of the mass of protein that is adsorbed and desorbed, it is fundamental to be working with constant volumes of solution during the four phases of the chromatography.

During the experimental part, the following protocol was established. The equilibration process is fundamental for the membrane to adapt itself to the pH chosen for the experiment, thus this step was usually extended for five hours. For the adsorption, instead, the time was varied during the first trials and it was found that adsorption is no longer changing with time after 24 hours, from the moment in which the membrane is put in contact with the solution of protein. The washing step is useful for cleaning any impurity, which has bound inappropriately to the membrane. It must be mentioned that during our laboratory experiments no impurities have been intentionally introduced, so this step is generally expected to lead to no physical results, but it has always been performed in accordance with the protocol. However, the duration was always limited to two hours. The final step of elution was conducted overnight, as it requires a certain period of time for the protein to detach from the membrane. Therefore, the desorption (or elution) process was
considered finished after 24 hours, but longer times were also tested during the experiments (see section §4.8).

We analyzed the results of adsorption on different functionalized membranes, studying the effect of gold nanoparticles and of dithiol molecules on the membrane. In the following sections, we will discuss the results that were obtained and the observations that could be elaborated.

4.6.1 Effect of gold nanoparticles on the adsorption

This section specifically focuses on the role of gold nanoparticles towards the adsorption of antibodies onto the membrane. Unmodified membranes and membranes functionalized with different percentages of gold nanoparticles are compared. In fact, it was previously reported [28] that gold has preferable interactions with pyridine. Therefore, high amounts of gold nanoparticles should lead to a high number of gold-pyridine interactions and fewer pyridine sites available for further interactions. At the beginning of the project, the role of the pyridine was not considered as problematic in the mechanism of the adsorption. However, when the first tests were conducted, evidence was found that IgG antibodies were binding to the pristine membrane, which had not undergone any modification. This fact, which at first sight seems positive, is not, because the absence of modification is an indication that the binding is happening through non-specific sites. Therefore, these interactions are not reversible and should be avoided for the purpose of this work. To get a confirmation about the role of pyridine on the adsorption process and how the presence of gold could affect it, the adsorption capacities of three different membranes, functionalized with different percentages gold nanoparticles, were compared. The ratios are as mentioned
before (50%, 25%, 12.5%) and were calculated by comparison to the stock solution (for example, ratio 50% means that the concentration of the final solution is half than the concentration of the stock). Table 4.2 shows the three different ratios that were used, together with the resulting membrane morphologies; the size of the membranes to functionalized was chosen as 1.8x2 cm². Moreover, the volume of the gold solution for the functionalization of each membrane was kept constant to 12 mL.

Table 4.2 Influence of gold nanoparticles on adsorption.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>m_{ADS} (mg)</th>
<th>% m_{ADS}</th>
<th>q'_{ADS} (mg/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified</td>
<td>0.43</td>
<td>16 %</td>
<td>0.035</td>
</tr>
<tr>
<td>12.5%</td>
<td>0.8</td>
<td>30 %</td>
<td>0.055</td>
</tr>
<tr>
<td>25%</td>
<td>0.67</td>
<td>25 %</td>
<td>0.046</td>
</tr>
<tr>
<td>50%</td>
<td>0.52</td>
<td>20 %</td>
<td>0.036</td>
</tr>
</tbody>
</table>

The adsorption tests were conducted at a pH=6.5, which is thought to be the most adequate one, for having positive charged antibodies to bind to the membrane, according to their isoelectric point. The PBS (Phosphate Buffer Saline) buffer solution was prepared by using a purchased 10x PBS stock solution and diluting it 10 times until the point in which our buffer is concentrated 1x. The pH was adjusted by using a solution of hydrogen chloride, by monitoring the variation through a pH meter (Fisher Scientific). The steps for the adsorption process were as described in Figure 4.7. The size of the membranes has been recorded as 0.9x1.8 cm² and thickness of 0.09 mm, for a total volume of 18 x 9 x 0.09 mm³.
(=14.58 mm$^3$). The results are reported on Table 4.2, where $m_{\text{ADS}}$ represents the milligrams of antibodies that have been adsorbed by the membrane, $\%m_{\text{ADS}}$ is the ratio between the quantity of antibodies that was adsorbed and the total one that was introduced in the solution at the beginning, $q'_{\text{ADS}}$ is the adsorption capacity of the membrane, which is calculated by considering the adsorbed quantity of IgG over the volume of the membrane. As mentioned before, the table reports that adsorption of IgG occurs even on the unmodified membrane, meaning that something in the membrane has affinity to the antibody in solution. We made the hypothesis that the responsible for this behavior is the pyridine site of the block copolymer membrane. Thus, to demonstrate the role of pyridine we shielded it gradually with an agent, which has strong affinity to it: gold. It is evident that the adsorption capacity of the membranes decreases when increasing the amount of gold. In other words, the more the pyridine sites are covered, the lower is the adsorption of IgG. Therefore, it is confirmed that the pyridine is responsible for the attachment of antibodies on unmodified membranes, causing the main drawback of this procedure: the binding happens non-specifically. Instead, as introduced in the previous chapters, the role of the gold nanoparticles in combination with dithiol segments was studied to increase the specificity of the adsorption, through the main body (heavy chains) of the antibodies. In conclusion, in order to have specific binding (and avoid the non-specific one), it is recommended to use quite high concentrations of Au NPs, in combination with dithiols.

**4.6.2 Effect of dithiol on the adsorption**

The use of dithiol is fundamental to create a sulfhydryl group available to interact with the exposed –SH site of the antibody, which can undergo reduction and form a temporary –S–
The results of FTIR were conclusive that the dithiol is binding to the membrane (revealed by the new peaks in the aliphatic region in the spectra). Moreover, the problem of back-binding (i.e., both the extremities of the dithiol binding to the gold nanoparticles) was solved by using very short chains of dithiol, which in principle should prevent the “back-biting”, due to steric restrictions. Therefore, 1,3-propanedithiol was used as candidate molecule. In order to understand which concentrations of propanedithiol were needed to reach an effective response in adsorption, different membranes functionalized with different amounts of dithiol were tested. Three different batches were tested with different propanedithiol concentrations: 50 mM, 100 mM and 200 mM. The first two batches did not satisfy our expectation, because the values of adsorbed proteins did not vary much from the unmodified membrane to the modified ones. This could mean that the amount of dithiol was not enough to be effective and there were more unmodified sites rather than modified ones. Instead, better results in term of adsorption were recorded when the concentration of dithiol was increase to 200 mM. Once the concentration was optimized, different immersion times were investigated for the adsorption process. In summary, the adsorption test included four membranes: unmodified one, modified with 6 hours of exposure to dithiol, 12 hours, 18 hours. The following Table 4.3 reports the values of adsorbed antibodies on the different membranes. In this case, the size of the membranes was fixed to 1x1 cm\(^2\) and thickness of 0.12 mm, for a total volume of 10 x 10 x 0.12 mm\(^3\) (=12 mm\(^3\)). The parameters \(m_{\text{ADS}}, \%m_{\text{ADS}}\) and \(q'_{\text{ADS}}\) are calculated as described before. The adsorption’s steps and the pH conditions were as previously mentioned. It can be clearly seen that the influence of dithiol is fundamental for the adsorption. Moreover, it is clear that 6 hours are good enough for enhancing the amount of biomolecules entrapped in
the membrane, whereas higher times reduce the adsorption capacity of the membrane (it is not clear why this minor decrease happens though - bearing in mind that anyway it is a very small change). Following the enthusiasm of these results, the next step involved the combination of gold nanoparticles and dithiol functionalization.

### 4.6.3 Effect of gold nanoparticles and dithiol on the adsorption

Supported by previous findings, the “double” functionalization consisted in using different gold ratios but at a fixed amount and time of dithiol incorporation. Therefore, three gold-functionalized membranes (percentages 50%, 25%, 12.5%) underwent a treatment with a 200 mM 1,3-propanedithiol solution for 6 hours. Their properties, in term of adsorption, were compared to the ones of a pristine membrane. All the membranes had the same area 1x1 cm² and same thickness 0.15 mm, therefore the same volume (15 mm³). The adsorption steps were tested once more under pH = 6.5, after an equilibration process of 5 hours, and the solution’ concentrations were measured 24 hours after the beginning of the adsorption.

#### Table 4. 3 Influence of dithiols on adsorption.

<table>
<thead>
<tr>
<th>Time</th>
<th>( m_{\text{ADS}} ) (mg)</th>
<th>% ( m_{\text{ADS}} )</th>
<th>( q^*_{\text{ADS}} ) (mg/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified</td>
<td>0.43</td>
<td>16 %</td>
<td>0.035</td>
</tr>
<tr>
<td>6 hours</td>
<td>0.59</td>
<td>22 %</td>
<td>0.049</td>
</tr>
<tr>
<td>12 hours</td>
<td>0.51</td>
<td>19 %</td>
<td>0.042</td>
</tr>
<tr>
<td>18 hours</td>
<td>0.51</td>
<td>19%</td>
<td>0.042</td>
</tr>
</tbody>
</table>
process. Table 4.4 reports some key values measured during the tests. The results are very interesting, especially if compared to the ones reported in Table 4.2 and Table 4.3. Indeed, each functionalization is contributing to a change. However, when both modifications are performed on the same sample, more attention needs to be paid to understand the final result. From previous discussion, the conclusion was to use membranes functionalized with quite high amount of gold nanoparticles and, later, with dithiol for 6 hours. The data from the table seem in line with the previously hypothesized plan, thus it is possible to notice that the highest adsorption is achieved with 25% and 50% functionalized membranes. However, two observations could be raised by looking at the data: 1) the adsorption capacity of highly modified membranes is not improved if compared to the one of the unmodified membrane; 2) the adsorption capacity of lowly modified membranes (i.e. membranes modified with concentrations ratio 12.5%) is lower than the others whereas, in absence of dithiol, it showed the highest value. The first discussion seems controversial,
but it indeed shows that highly modified membranes and unmodified ones has the same adsorption values. However, we can affirm that the modified ones have better properties: the unmodified membranes are characterized by the absence of specificity, which makes the adsorption process difficult to control. Instead, the highly-modified membranes are tailored for giving specific binding, thus offering a better adsorption. Moreover, the comparable values of adsorption capacity tell us that the shielding of the pyridine sites by gold nanoparticles, followed by dithiol modification, can recover as much adsorption sites as they were before the modification, adding selectivity. In relation to the second observation, membranes functionalized only with low amount gold might have high adsorption capacity, but are non-specific. In conclusion of this section, the adsorption capacity of functionalized membranes has been found to depend on the amount of gold nanoparticles and on the dithiol’s exposure time. Finally, by using high quantities of gold in combination with dithiol’s functionalization, the adsorption process can be specifically tuned to occur through thiol sites of the biomolecule, therefore inducing the thiolation.

### 4.6.4 Adsorption of BSA

Besides the adsorption of IgG, chromatographic tests were conducted also for another (planned) protein in solution, i.e. Bovine Serum Albumin (BSA). As initially discussed, the desired results would be to have modified membranes with selective adsorption of IgG onto the surface, while BSA would freely permeate through the membrane. Therefore, it was necessary to prove that: 1) BSA does not bind to the membrane, 2) BSA passes through the membrane ending in the permeate, without partial (or total) rejection. While the second observation will be treated in the next section (§4.7) the adsorption tests conducted for the
BSA are discussed below. BSA has an isoelectric point at pH = 4.9, thus at working conditions of pH = 6.5 it is negatively charged. Therefore, it should not interact with the membrane, which is also supposed to have a negative charge. The test was therefore conducted as a verification that the theoretical assumptions were valid. As always, an equilibration process at pH=6.5 was followed by the adsorption process, tested for a PS-\textit{b-}\textit{P4VP} membrane modified with a certain amount of gold nanoparticles (at this preliminary test, the exact ration of gold was not fundamental to be known). After 24 hours from the beginning of the adsorption, the concentration of BSA in solution was measured with the NanoDrop, which revealed that no adsorption had taken place. In other words, all the free BSA was still in solution and did not attach to membrane. The promising result not only confirmed the preliminary assumptions, but it also suggested that it is more probable that the IgG adsorption is caused by the external \textit{–SH} sites and it is not due to partial denaturation of the antibody. In fact, even the BSA has some dithiol bonds within its structure, but they are in the internal part, and therefore not accessible. The same conclusion can be adopted for the IgG structure and dithiol bonds, confirming once more the assumptions made for the adsorption of IgG.

\subsection*{4.7 Filtration measurements}

Once it was proved that the adsorption process occurs as expected, then it was necessary to test the filtration performance. In particular, it is fundamental to define whether the protein of interest (BSA) passes through the membrane and whether the adsorption of IgG onto it affects the flux. Therefore, measurements were performed in terms of rejection and cut-off, flux and flux recovery.
When talking about rejection, we define cut-off as the minimum value of molecular weight, which is rejected with a percentage of at least 90%. It is important to determine or, at least, have an estimation of this number, because it gives an approximate information about which is the size of the macromolecules that can, in principle, pass through the membrane. First, polyethylene glycol (PEG) rejection measurements were conducted. A common procedure consists in using many PEG polymers with different molecular weights, dissolved in a water solution with a concentration of 1 g/l. The solution is then filtered through a filtration cell containing the membrane and the permeate is collected. Furthermore, feed, permeate and retentate samples are taken for GPC analysis. In this way, by comparing the values of feed and permeate it is possible to calculate how much each molecular weight is rejected. For the unmodified membrane, the molecular weight cut-off was estimated as ~190 kDa. From the literature, it is convenient to retrieve the molecular weight of the proteins of interest, as 66.5 kDa for BSA, and ~150 kDa for IgG. Hence, in principle, both proteins could pass through the membrane, even though the desired process requires only the Bovine Serum Albumin to reach the permeate side. Will be the functionalization enough to retain the γ-globulin in the feed side? Before moving to the results, two remarks should be underlined: first, it must be remembered that molecular weight and hydrodynamic volume are two different parameters. They are related to each other, but whereas the first one is independent of the environment, the second one strongly depends on the interactions of the macromolecule with the solvent. Secondly, despite the fact that the functionalized membranes are modified on purpose to favor the adsorption of antibodies, it must be kept in mind that the previously reported tests (§4.6.1) showed how the pyridine coronas offer strong adsorption sites to the IgG.
The membranes were tested under operational conditions to have an estimation of which is the flux of Phosphate Buffer Saline at pH=6.5, which is the working environment of interest. It is important to record this value, because as known from literature, PS-4b-P4VP has responsive pH behavior, thus the flux of an unmodified membrane at neutral pH will not be the same at more acidic conditions, for the same membrane. From water flux measurements (neutral pH), unmodified and modified membranes have similar permeances, respectively ~900 (± 20) L/m²·h·bar and ~880 (± 20) L/m²·h·bar. The difference is very subtle, so it is reasonable to think that the two values are comparable, meaning that the presence of gold does not compromise the membrane performance. In terms of permeation at pH=6.5, the values are slightly different, with an permeance of ~800 (± 20) L/m²·h·bar for the unmodified PS-4b-P4VP and ~815 (± 20) L/m²·h·bar for the membrane functionalized with gold nanoparticles. As expected, the flux in both cases is decreasing while decreasing the pH, because the pores of the block copolymer membrane tends to close when moving to acid conditions. In fact, it has been reported (Figure 4.8) that the pore sizes change as a function of pH, due to stretching of protonated P4VP segments. Therefore, the flux through the membrane is also influenced. However, what is new in this study is the comparison with the modified membranes. Until this moment, it was unclear whether the presence of nanoparticles would have influenced the pH responsive behavior of the pyridine system or not, especially in relation to the system’s working conditions. The results reported above give a final
understanding that also in the presence of gold nanoparticles, the responsiveness is kept. Moreover, the numbers say that the drop in permeance in not that drastic when moving to pH=6.5, thus operational boundaries are still respected. Once the flux was measured, BSA and IgG rejection test were performed at pH of interest, i.e. 6.5. It was successfully recorded that BSA mostly permeates through the membrane (rejection was lower than 8%), whereas IgG is mostly retained (>90%), proving the feasibility of the hypothesized separation process.

### 4.7.1 Flux recovery

The membrane operation was then tested in relation to the flux recovery. We studied how the permeance of the membrane changes after a single filtration process of an IgG solution. Both membranes (functionalized or not) were tested. The followed protocol included PBS (pH=6.5), IgG in PBS (pH=6.5, concentration of 0.5 g/L) and again PBS (pH=6.5). In terms of permeance of PBS at pH=6.5, the values between the two membranes were comparable (~800 L/m²⋅h⋅bar for unmodified and ~815 L/m²⋅h⋅bar for the one modified with gold nanoparticles). When the protein solution was then filtered, the flux substantially dropped, reaching values between 20 and 30 L/m²⋅h⋅bar. The purpose of the test was then to determine how much the flux was recovered after this drastic drop. Both membranes showed a recovery in permeance, but the value did not get back to the previous ones of the first PBS filtration. In any case, the recovered flux reached 200 L/m²⋅h⋅bar. It must be noticed that, during the first measurements, the recovery did not happen instantaneously, but it increased during the filtration itself. This could mean that some protein is still entrapped in the pores or bound to the surface. Therefore, the explanation of the reduced
flux lies on the binding of some IgG to the membrane, but further membrane application is possible.

4.7.2 Flux and rejection at different pH

While discussing the possibility that modified membranes could maintain the same response to pH as the PS-\textit{b}-P4VP, the interest towards understanding the behavior of the system at extreme low pH pushed towards the study of flux recovery, after the membrane was taken to very acid conditions. By doing this, the goal was to understand if the structure’s recovery takes place in the functionalized membrane as well as in the unmodified PS-\textit{b}-P4VP. Indeed, the copolymer system was shown to undergo reversible changes in structure, meaning that if the pores close when moving to low pH, the structure is however recovered when increasing the pH. While this is happening in the unmodified membrane, no literature is reported regarding a block copolymer membrane modified with gold nanoparticles. Thus, it was not known whether the NPs play a role in the shape-recovering step. To perform the necessary tests, a protocol was set-up, consisting in the following steps: filtration of water, filtration of PBS (pH=6.5), filtration of PBS (pH=3.5), filtration of PBS (pH=6.5). At this point, SEM images were collected to determine whether

![Figure 4. 9 Permeation data during the filtration steps (system 1). SEM images of the membranes before and after the filtration.](image-url)
there was any difference in the structure of the two membranes (modified and non). We studied two different systems: the first one compared the unmodified membrane with a functionalized one with gold concentration 12.5%; the second one compared the unmodified with a functionalized one with a higher concentration of the gold solution, 37.5%. For the first set-up, the values of the fluxes through the whole process (reported in Figure 4.9) were comparable and followed the same trend. The structure of the membranes was checked by SEM characterization. The images show how the unmodified PS-\textit{b}-P4VP completely recovers even after being exposed to extremely low pH. Regarding the modified membrane, most of the pores are reformed, thus meaning that gold nanoparticles do not compromise the responsive behavior or the pyridine coronas. However, not all the parts of the membrane show recovered pores, and it seems that some pores are smaller or completely absent. Despite this observation, the flux of the membrane is not lowered. It seems that the treatment of the membranes led to the formation of a more selective membrane, without compromising its permeability. However, in order to confirm it, some rejection tests need to be performed. For this purpose, a solution of BSA and a solution of IgG of known concentration were prepared, and the amount of these two proteins in the permeate feed, along with time, was measured. The rejection of BSA was almost constant with time for both membrane, with a slighter increase for the modified membrane (10% against the 5% of the unmodified membrane). Better rejection was proved for the IgG filtration, for which both membranes performed well and rejected almost all the protein. The difference occurred in terms of trend with time: in both cases the amount of IgG in the permeate decreased with time, following an exponential decay, however for the modified membrane the decay was steeper, meaning that more protein was rejected since the
beginning. The difference, though, was not as great as one could expect. Therefore, the next step involved the study of a membrane functionalized with a higher amount of gold, in order to understand how relevant the role of gold could be in “blocking” the complete recovery of the pores size. During the second experiment, the unmodified PS-b-P4VP was functionalized with a solution with concentration 37.5% compared to the stock solution. The steps of the protocol were performed as before, and some differences could be noticed during the process. Figure 4.10 reports the trend observed during the permeability tests. First of all, the permeance through the membrane is lower than the previous one, but these variations are due to the process’ formation of the membrane itself which determines the small differences in performances. However, the trend is consistent with the previous one described, where it is evident that some decrease happens while changing from pure water to different buffers, but mostly while passing to very low pH.

In particular, we noticed that, while moving from pH=7.5 to pH=3.5, the membrane functionalized with gold nanoparticles takes longer to decrease its permeance, while for the unmodified membrane the jump in permeance happens almost instantaneously. For the modified membrane, the decrease happens slowly and never reaches a value as low as the

![Permeation data during the filtration steps (system 2). SEM images of the membranes before and after the filtration.](image)
one reached by the pristine membrane. Therefore, it seems that the nanoparticles are playing a role in opposing the membrane’s shrinking. However, when the pH is again increased, the membrane recovers similarly as the unmodified membrane, showing a comparable trend. To have a clearer understanding, we focused once more on the SEM characterization to get information about the modification of the structure (Figure 4.10). We could notice that the modified membrane mostly does not recover the pore shapes after treatment at low pH, thus indicating a possibility of having a membrane with higher selectivity. However, the characterization technique did not give a complete and clear understanding of the final membrane’s structure, thus the recovery of the pore’s size was investigated by rejection’s measurements. In other words, by checking which sizes are rejected by the membrane during the filtration process, we could understand whether the pores’ size is completely recovered after the pH switching or not. As described before, the molecules used are BSA and IgG. While the rejection for BSA was comparable in both cases and, anyway, lower than 10%, more interesting results were obtained from the rejection of IgG. Indeed, the rejection for the unmodified membrane reached 90% only after 15 minutes from the filtration process, due most probably to concentration polarization effects, while the rejection for the modified membrane was above 95% since the beginning, reaching 100% very soon. Given that the solution of IgG was the same in both contexts, there is no doubt that the difference is attributed to the size of the pores through which the biomolecule needs to pass.
4.8 Desorption of IgG

The previous sections described how the designed system worked in the adsorption and filtration processes, thus satisfying the fundamental requirements for the membrane application. Despite these results, the final step to verify is the desorption of the antibody from the surface of the membrane. Once this is proved, the system can be taken to its second stage, when the polymer is substituted with a redox-responsive one and the nanoparticles can be used for their incredible properties rather than just for their high surface to volume ratio (more details about it in Chapter 6). Therefore, this last part of the Results and discussion section will concentrate in the attempts followed to detach the antibodies from the active adsorption sites.

4.8.1 Effect of ion strength

Generally, in membrane chromatography applications, one of the most used approach is to work through the so-called Ion-Exchange Chromatography, in which proteins bind to surfaces due to electrostatic interactions. In these cases, the desorption process (elution) occurs through the use of a salts (i.e., an ionic compound) which has a stronger ionic strength than the protein solution. By ionic strength we mean a measure of the concentration of ions in that solution, which occurs when dissolving a salt in a buffer solution where the membrane is kept. In this way, by increasing the ionic strength, competition for charged groups on the ion exchanger (membrane) arises, and the interactions between the membrane and the proteins are reduced. Therefore, the result is the elution of the biomolecule.
The process just described does not seem to fit in the system we are dealing with: despite the fact that we are using specific pH to have attraction between antibodies and membrane, the real interactions happen through creation of a –S–S– bond, thus preventing the attraction to be only of an electrostatic type. Nevertheless, we tried to see whether the use of salt in solution was able to cause any change in the interaction forces between molecules and membrane. The protocol requires the use of the same buffer, at the same pH, used throughout the “ion-exchange” process. Then, 1M of NaCl is dissolved on it. The membrane is immersed in the freshly prepared solution and stirred for 24 hours. The concentration of IgG in solution is then measured using NanoDrop. The results gave a negative response to the treatment, meaning that no detachment was recorded after the exposure time. The same procedure was repeated for an increased concentration of NaCl salt (2M), in order to increase the ionic strength in solution, but neither this procedure allowed the antibody to detach from the surface of the membrane. Therefore, in the view of above, we concluded that the use of salt does not influence the binding capacity of the antibodies. Following the reasoning, we investigated whether the influence of pH values was playing a role in determining the binding strength of the antibodies.

4.8.2 Use of buffers at higher pH

As mentioned earlier, the elution step is carried out by affecting the ion strength of the solution, generally by increasing it with the use of a salt. However, the ion strength of the immobilized species can be modified even by the use of a different pH than the one used for the affinity or the binding. In fact, the biomolecule would be affected through the change on its charge, which will then cause a different attraction to the stationary phase
According to our system, the choice of pH = 6.5 agrees with the acquisition of a positive charge and the partial reduction on the antibody at those conditions, which are fundamental to obtain the binding site. Therefore, a strategic way to induce detachment from the antibody consists in using a buffer solution of a higher pH, such that the biomolecules obtains a negative charge and detaches from the membrane due to charge repulsion. Moreover, the change in the environment would prevent the partial reduction of the sulfhydryl group, thus avoiding the binding. This methodology was tested for pH around 8.5, because in this range the biomolecules are known to be above their isoelectric point, thus acquiring a negative charge, which opposes to the one of the membrane. Despite the theoretical expectations, the experiments revealed that there is no elution of antibodies even when an increase of pH is accompanied by the use of NaCl. Therefore, it is possible to conclude that the binding between biomolecule and membrane occurs through formation of relatively strong disulfide bond which are not easy to break and are not mainly based on electrostatic interactions. However, pH still plays a role for the adsorption steps, when it favors the partial reduction of the sulfhydryl group, creating a –S– site available for the formation of the bond with the membrane.

4.8.3 Use of a reducing agent

The formation of a disulfide bond corresponds to the oxidation of two sulfhydryl group, therefore a way to break this bond selectively is by using a reducing agent which is sensitive to –S–S– sites. From the literature[38], dithiothreitol (DTT) appears as a redox reagent that attacks disulfide bonds, breaking them into two thiol groups. It is considered as an agent for purifying proteins, when the unfolded structure is preferred to the folded one. Hence,
the main drawback of this agent is that it is generally used for denaturing biomolecules. Indeed, almost any protein contains disulfide bonds, which are part of their typical folded structure. However, for antibodies these disulfide bonds are located between the two heavy chains, thus they are considered as “buried” and not too easy to access. The idea is to try to use DTT as a reducing agent for breaking the disulfide bonds between the IgG and the membrane, but without destroying the internal structure of the antibody itself (Figure 4.11). A way to achieve this goal is by using very low concentrations of DTT. In doing so, only the most exposed disulfide bonds (i.e., the ones between the IgG and the membrane) will be involved by the chemical reduction, without compromising the overall structure of the antibody. Therefore, the solution for the elution was prepared by dissolving a minimum amount of DTT (0.1 mg/mL) in a buffer solution at the same pH of the one used throughout the chromatography process. The results obtained are satisfactory and confirm the reducing power of DTT in detaching the antibody from the membrane into the solution. After 24 hours of stirring, the measurements through NanoDrop revealed concentrations of IgG in solutions (m_ELU). In Figure 4.22 it is recognizable that the adsorbed amount of antibodies (m_ADS, same values as reported in Table 4.4) could not be recovered completely (most probably due to the low concentration of DTT used), but the overall achievement of partial recovery was anyway considered as satisfactory.

Figure 4.11 Working principle of DTT molecules for the current case study (left) and recovery’s results for the three different functionalized membranes (adsorption values as reported in §4.6.3; the numbers represent the percentages of recovery for each of the membranes).
Chapter 5. Conclusions

The aim of this work was to study the effect of a novel functionalization on a PS-\textit{b}-P4VP membrane to achieve selective separation between two different biomolecules and the specific adsorption of antibodies on it. Furthermore, a recovery step is thought to release the adsorbed species. The main difficulties to overcome consisted in: i) obtaining a homogeneous functionalization over the surface, ii) inducing a specific binding of IgG, such that it does not compromise its active sites, iii) achieving a good separation of the two biomolecules, which could in principle be difficult because of possible interaction between the protein to filter (e.g. BSA) and the membrane.

We successfully functionalized the desired membrane with different content of gold nanoparticles to study how the binding was influenced by increasing the amount of Au NPs. The results demonstrated that the binding to the membrane decreased when the content of gold increased, because the gold was preventing the non-specific binding to the pyridine sites. The loss in adsorption recorded after the first functionalization step was partially recovered by implementing the functionalization with dithiol. Therefore, we proved that, through the double functionalization, the binding was made not only reversible but also increased. Furthermore, we tested the membrane under operational conditions and we successfully demonstrated that there are no interactions between BSA and PS-\textit{b}-P4VP and that BSA is almost completely filtered through the membrane, whereas IgG is retained. Moreover, we evaluated that the use of a reducing agent is necessary to break the disulfide bond between the antibody and the membrane, allowing the recovery.

In conclusion, we proved the feasibility of the designed filtration process and of the selective adsorptions.
Chapter 6. Future work

On section §1.3, gold nanoparticles were described along with their peculiar properties, derived from the scaling-down to the nanometer range. For the purpose of this work, they were mostly used for their biocompatibility and tendency to offer selective binding; however, the use of gold nanoparticles is thought into a wider utilization, which explores other properties of Au NPs.

Since their discovery in the last centuries, gold nanoparticles appeared as a novel material to use for development of traditional applications and, in the latest decades, they have been highly studied because of their several intriguing properties. Beyond the well-known surface plasmon resonance behavior, typical of metals in the nanometer range, Au NPs have also been proved to have high surface-to-volume ratio and high surface energy; moreover, they have been recognized as signal amplifiers and transducers, transforming chemical stimuli into optical, electrical, or qualitative output signals. It is in this direction that future work is aimed to develop, making use of the incredible features gold nanoparticles have.

The first idea is to take advantage of the redox reaction to manipulate the reversibility of the $-S-S-$. By inducing a reduction, the bond can in principle be broken and the antibody can be detached from the membrane. The second aim is to obtain a smart responsive system which can change its response according to redox stimuli. In particular, by using a redox responsive polymer for the membrane, it could be possible to have pores which open when an antibody attaches to the membrane (thus it undergoes thiolation, i.e. oxidation): the gold nanoparticle would act as a charge carrier and transport the electrons to the polymer, which by reduction shrinks and opens its pores. What just described is not a pure imaginary belief,
as a candidate has already been selected for the role of redox responsive polymer (poly[3-carbamoyl-1-(p-vinylbenzyl)pyridinium chloride] – PCVPC [39] [40]), due to its known properties of shrinking and relaxing when undergoing redox reactions. Moreover, the gold nanoparticles are known for behaving like charge carriers in these contexts, thus the whole system appears as a promising one. Furthermore, the system is thought towards the development of a smart responsiveness. In fact, the aim is trying to have redox responsive behavior to control the pore sizes, but at the same time to have control over a continuous process of filtration. In details, the signal transducing power of gold nanoparticles is intended to work as piezoelectric sensors revealing the binding of each single antibody. The main advantage of this approach lies in the possibility of setting a target, at which the amount of antibodies bound to the membrane could be considered as satisfactory and the recovery step could be started automatically. The throughput of this vision is to avoid cycles, in which the number of attached antibodies is so low that recovery would be insignificant and would only generate more costs. In combination with this application, gold nanoparticles work also as electron-transfer agent, carrying electrons from the antibody side to the membrane, in order to promote reduction of the polymer and enlargement of pores.


