
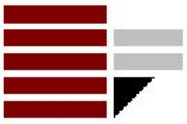


Università della Calabria

Dottorato di Ricerca in Ingegneria Chimica e dei Materiali

SCUOLA DI DOTTORATO "PITAGORA" IN SCIENZE INGEGNERISTICHE

Ciclo XXVIII

Tesi

FUNCTIONALIZED POLYMERIC MEMBRANES FOR DEVELOPMENT OF BIOHYBRID SYSTEMS

Settore Scientifico Disciplinare CHIM/07 – Fondamenti chimici delle tecnologie

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A.A. 2014-2015

ACKNOWLEDGEMENTS

First I desire to mention my family, and in particular Rossella that always supports me.

I would like to express heartfelt gratitude to my supervisors Dr. Lidietta Giorno and Prof. Enrico Drioli for providing me instruction, dedication, support, and encouragement through the entire PhD studying. Theirs outstanding passion and dedication to the field of membrane science have really positively impressed me. I would also like to thank Dr. Rosalinda Mazzei who helped me during these years. A special thanks to my colleagues and friends at Institute on Membrane Technology, Alessandra, Fabio, Leo, Giuseppe, and Francesca. Finally, I would also like to thank Prof. Dr. Mathias Ulbricht for giving me the opportunity to carry out part of my project at University of Duisburg-Essen (Germany) and D. Büning which cooperated with me during this experience.

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SOMMARIO

Le proprietà di superficie di una membrana sono di grande importanza per la sua funzione. Mediante tecniche di funzionalizzazione chimica è possibile ottenere membrane con gruppi funzionali in grado di adempiere nuove e diverse funzioni che rendono la membrana funzionalizzata un dispositivo in grado di svolgere funzioni multiple trovando applicazione in vari impieghi.

Le membrane funzionalizzate, infatti, trovano impiego nei processi di separazione, nei settori che richiedono l'uso di membrane biocompatibili, e nell'immobilizzazione di biomolecole che a sua volta trova applicazione nella preparazione di biosensori e bioreattori a membrana. Questi ultimi sono particolarmente interessanti poiché sfruttano l'alta superficie specifica della membrana e permettono di integrare il processo di separazione con quello catalitico.

Il presente lavoro di tesi ha riguardato lo sviluppo di membrane polimeriche biofunzionalizzate per la decontaminazione di acque da sostanze tossiche quali i pesticidi organofosfati. Il lavoro è stato focalizzato sullo studio di diverse tecniche per l'ingegnerizzazione di membrane polimeriche aventi differenti caratteristiche chimico-fisiche. L'impatto dei diversi tipi di funzionalizzazione è stato valutato considerando il grado di legame e le proprietà catalitiche di biomolecole immobilizzate sulle membrane funzionalizzate. I polimeri utilizzati per l'immobilizzazione delle biomolecole sono stati il fluoruro di polivinilidene (PVDF) e il polietersulfone (PES), materiali ampiamente usati in sistemi di filtrazione. La proteina sieroalbumina bovina (BSA) e l'enzima lipasi da *candida rugosa* (LCR) sono state selezionate quali biomolecole modello per lo studio della capacità di legame e le proprietà catalitiche delle membrane ingegnerizzate.

Le condizioni ottimali di funzionalizzazione e immobilizzazione sono state poi impiegate per lo sviluppo di sistemi bioibridi contenenti l'enzima fosfotriesterasi (PTE), un enzima in grado di operare la detossificazione di organofosfati.

Al fine di migliorare le performance degli enzimi immobilizzati sul PVDF è stato sviluppato un nuovo approccio di ingegnerizzazione. Esso ha riguardato la sintesi di nanoparticelle colloidali a base di poliacrilammide e il loro utilizzo, dopo opportuna funzionalizzazione, come vettori per l'immobilizzazione covalente di enzimi sul PVDF. La nuova strategia di immobilizzazione ha permesso di mantenere il microambiente idrofilo a livello dell'enzima immobilizzato migliorandone di conseguenza le proprietà catalitiche. La strategia allo stesso tempo ha consentito di preservare l'idrofobicità della membrana. Tale proprietà è necessaria per lo sviluppo di sistemi operanti nella decontaminazione di aria.

I risultati hanno mostrato che l'enzima fosfotriesterasi immobilizzato sul PES mantiene un'attività residua maggiore rispetto a quella dell'enzima immobilizzato sul PVDF. La membrana biocatalitica in PES è risultata idonea per la decontaminazione di organofosfati in fare acquosa.

OBIETTIVO DELLA RICERCA

L'obiettivo del presente lavoro di tesi è stato quello di studiare metodi per la biofunzionalizzazione di membrane polimeriche a base di PVDF e PES per lo sviluppo di sistemi bioibridi in grado di operare la decontaminazione da pesticidi organofosfati.

L'obiettivo sopra menzionato è stato perseguito tramite i seguenti studi:

- Sviluppo di una strategia di funzionalizzazione di membrane in PVDF
- Immobilizzazione di biomolecole su membrane in PVDF funzionalizzato
- Determinazione delle proprietà di legame e delle proprietà catalitiche delle membrane in PVDF biofunzionalizzato
- Sviluppo di strategie per il miglioramento delle proprietà catalitiche di enzimi immobilizzati su membrane in PVDF
- Funzionalizzazione di membrane in PES
- Immobilizzazione di biomolecole su membrane in PES funzionalizzato
- Determinazione delle proprietà di legame e delle proprietà catalitiche delle membrane in PES biofunzionalizzato

Le strategie di funzionalizzazione studiate per la produzione di membrane bioibride, potranno essere utilizzate nello sviluppo di dispositivi e sistemi basati sulle membrane bioibride quali ad esempio bioreattori a membrana, sistemi di separazione e biosensori.

CONTENUTI DELLA TESI

La tesi è organizzata in due sezioni principali, la prima incentrata sull'analisi dello stato dell'arte e la seconda sull'attività sperimentale. L'analisi dello stato dell'arte è stata finalizzata al consolidamento delle conoscenze generali raggiunte nel campo oggetto di studio. In particolare, sono state studiate le tecniche per la funzionalizzazione di membrane polimeriche e le stategie utilizzate per l'immobilizzazione di biomolecole (**Capitolo 1**). L'attività sperimentale è stata suddivisa in diversi capitoli come riportato di seguito:

- Nel **Capitolo 2** è riportato lo studio condotto su membrane in fluoruro di polividene (PVDF) al fine di creare su queste membrane gruppi reattivi adatti all'immobilizzazione di biomolecole.

- Nel **Capitolo 3** è discussa la caratterizzazione e la biofunzionalizzazione di membrane a base di polietersulfone (PES).

- Nel **Capitolo 4** è riportata la caratterizzazione dell'enzima fosfotriesterasi e la sua immobilizzazione su membrane in fluoruro di polivinilidene e polietersulfone. L'impatto sulle performance dell'enzima immobilizzato dei due diversi materiali è stato studiato.

- Nel **Capitolo 5** è fornita una breve introduzione sulla tecnica della polimerizzazione a emulsione inversa, che è stata usata nella sintesi di carrier per immobilizzazione enzimatica.

- Nel **Capitolo 6** è stata riportata la tecnica studiata e messa a punto per l'immobilizzazione di enzimi su membrane idrofobiche (PVDF) tramite carrier idrofili al fine di migliore le performance dell' enzima immobilizzato.

SUMMARY

The surface properties of membranes are critical to their application. Chemical functionalization of membranes is one of the efficient techniques that can provide the membranes with novel functionality-properties and transform them into valuable finished devices for specific applications.

Functionalized membranes are used in the pressure driven separation processes, for improvement of biocompatibility, for immobilization of biomolecules, preparation of biosensors and application in membrane bioreactors due to their interesting properties of high specific surface area and the possibility to combine separation with chemical reaction.

The present research work has been directed towards the development of functionalized polymeric membranes for detoxification of streams containing organophosphate pesticides. The impact of different types of functionalization techniques, such as functionalization of different membrane materials, on loading and catalytic properties of different biomolecules has been studied.

Membranes based on two different kinds of polymers such as polyvinylidene fluoride (PVDF) and polyethersulfone (PES) were used for covalent immobilization of biomolecules after functionalization by wet chemical methodologies. The protein bovine serum albumin (BSA) and the enzyme lipase from *candida rugosa* (LCR) were employed as model biomolecules for testing the binding capacity and the catalytic properties of the modified membranes.

The optimized functionalization and immobilization conditions were applied to develop biohybrid systems containing the enzyme phosphotriesterase (PTE), an enzyme able to detoxify organophosphates.

In order to improve catalytic activity of the biofunctionalized PVDF membrane an alternative approach for enzyme immobilization on the hydrophobic membrane was developed. Polyacrylamide (PAAm) nanogels were synthesized in the colloidal range and after functionalization, used as carrier for enzyme immobilization on the functionalized PVDF membrane. Improvement of catalytic activity was obtained using this new strategy which allowed immobilizing the enzyme kept in hydrophilic microenvironment without affect the hydrophobicity of the PVDF membrane. The hydrophobicity is a valuable property for the membrane when the goal is develop systems for air decontamination.

The results showed that PES membrane has superior performance than PVDF membranes basing on both, PTE activity and membrane permeability, in liquid phase decontamination.

RESEARCH OBJECTIVE

The research focus was on the development and characterization of methods for bio-functionalize polymeric membrane based on PVDF and PES in order to obtain biohybrid systems able to detoxify organophosphate pesticides.

In order to achieve the aim the following studies were carried out:

- Development of a strategy for functionalization of PVDF membrane
- Biomolecules immobilization on the functionalized PVDF membrane
- Evaluation of catalytic properties and binding capacity of biofunctionalized PVDF membrane
- Investigation of strategies to improve specific activity of biomolecules immobilized on PVDF membrane
- Functionalization of PES membrane
- Biomolecules immobilization on the functionalized PES membrane
- Evaluation of catalytic properties of biofunctionalized PES membrane

The strategies investigated for production of biohybrid membranes, may be used to design systems and devices based on the biohybrid membranes, such as membrane-based bioreactors, systems for separation and biosensing.

DISSERTATION OUTLINE

The thesis is organized in two main sections, the first one covering the analysis of the state-of-the-art and the second one covering the research activity. The analysis of the state of the art aimed at consolidating the knowledge achieved in the field present in the open literature. In particular the methods for polymeric membrane functionalization and biomolecules immobilization were studied (**Chapter 1**). The research activity is organized in several chapters as reported below:

- Development of a functionalization method in order to introduce on polyvinylidene fluoride (PVDF) membrane reactive groups suitable for biomolecules immobilization (**Chapter 2**).
- The characterization and biofunctionalization of membrane based on polyethersulfone (PES) is reported in **Chapter 3.**
- The characterization and immobilization of a thermophilic phosphotriesterase and the impact of materials having different chemical-physical properties such as PVDF and PES on its performance is reported in **Chapter 4**.
- A brief introduction on the inverse miniemulsion polymerization technique, is provided in **Chapter 5**. This technique was used for the synthesis of hydrophilic carrier for enzyme immobilization.

In order to improve the performance of enzyme immobilized on hydrophobic membrane (PVDF), a strategy for enzyme immobilization, by means of hydrophilic carrier was developed; it is presented in **Chapter 6**.

WORK STRATEGY

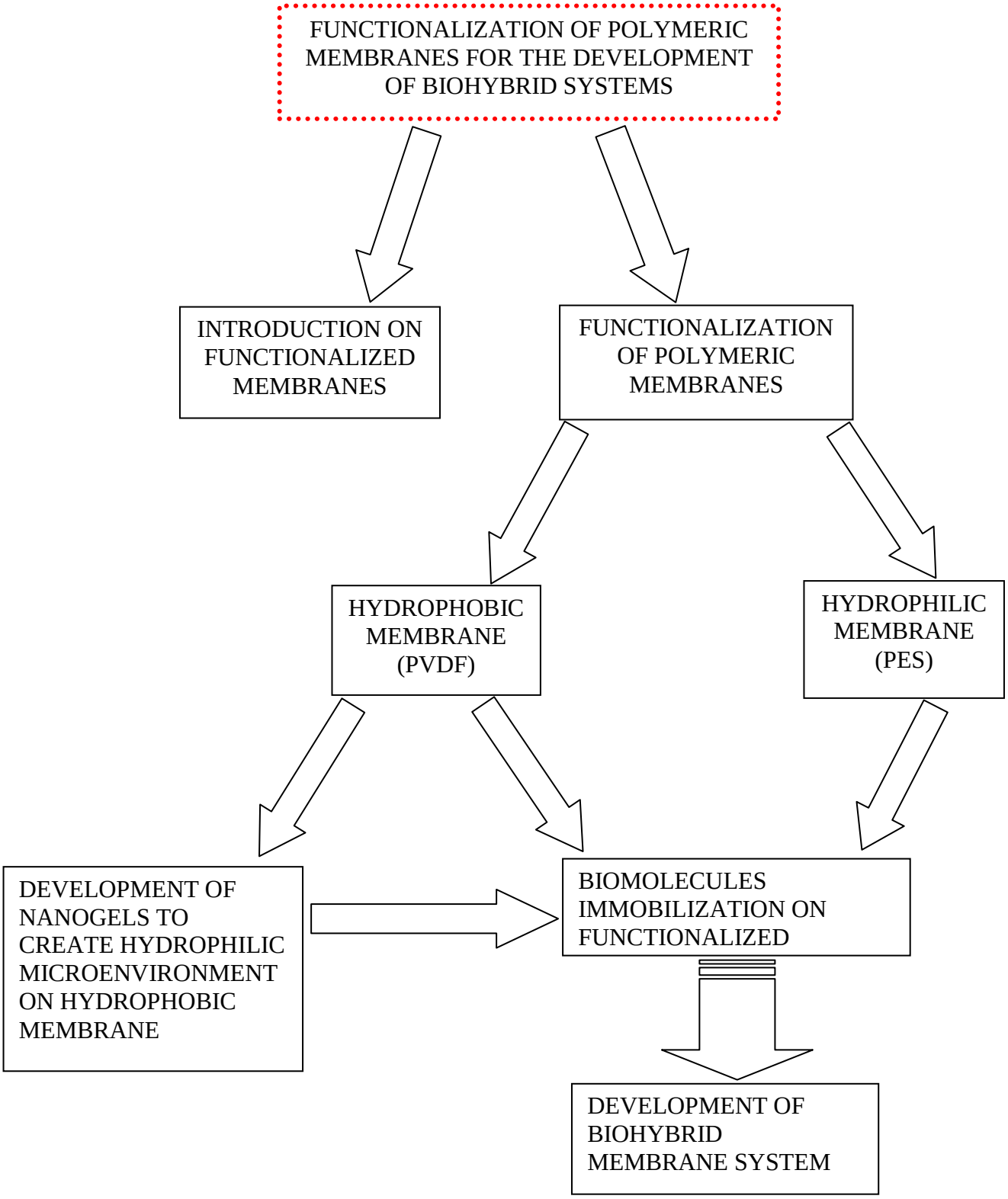


Fig. 1. Flow sheet illustrating the overall strategy of the work carried out

Chapter 1

Introduction on functionalized membranes

1.1. MEMBRANE AND MEMBRANE PROCESSES

A membrane can be in generally defined as a barrier which separates two phases and allows selective transportation of species under the influence of a certain driving force. The driving force is generally due to the difference in chemical or electrical potential in two sides of the membrane, and is expressed in terms of pressure or electrical potential gradients. The pressure driven membrane processes are reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), microfiltration (MF), pervaporation (PV) and membrane gas separation (GS). The concentration gradient driven membrane processes are dialysis and membrane extraction, while an electrical potential driven membrane process is electrodialysis (ED).

An important point should be considered with respect to the definition of a membrane: a membrane is defined by what it does, in other words by its function, not by what it is [1]. Therefore, a wide range of material can be used as membranes. A membrane can be homogenous or heterogeneous, symmetric or asymmetric in structure, solid or liquid, can carry a positive or negative charge, or can be neutral or bipolar [2].

Membranes can be made of polymers, metals, inorganic compounds, carbon, ceramic and liquids. Although a large number of novel polymers have been developed at a lab scale, so far less than twenty have been used as materials for industrially established membranes. They include cellulose, polyvinylidene fluoride, polyethersulfone, polypropylene, polyethylene, polyacrylonitrile, polyamide, polyimide, etc. (see Figure 1.1). These polymers have different properties which make the corresponding membranes suitable for certain separation processes [3]. The commercial application of membranes started since 1960s. One of the most important discoveries in the membranes field was that of Loeb and Sourirajan [4]. They developed the first asymmetric integrally skinned cellulose acetate

reverse osmosis membrane. The concept of a thin skin layer of separating barrier on the top of a highly porous polymer support is associated with a much lower pressure drop than symmetric membranes. This development stimulated the interest about membrane based separation techniques, either in industrial processes or in academic field. Membranes have been used extensively in water desalination, waste water treatment, biotechnology, biomedical industries, pharmaceutical industries, food industries and gas separation [5].

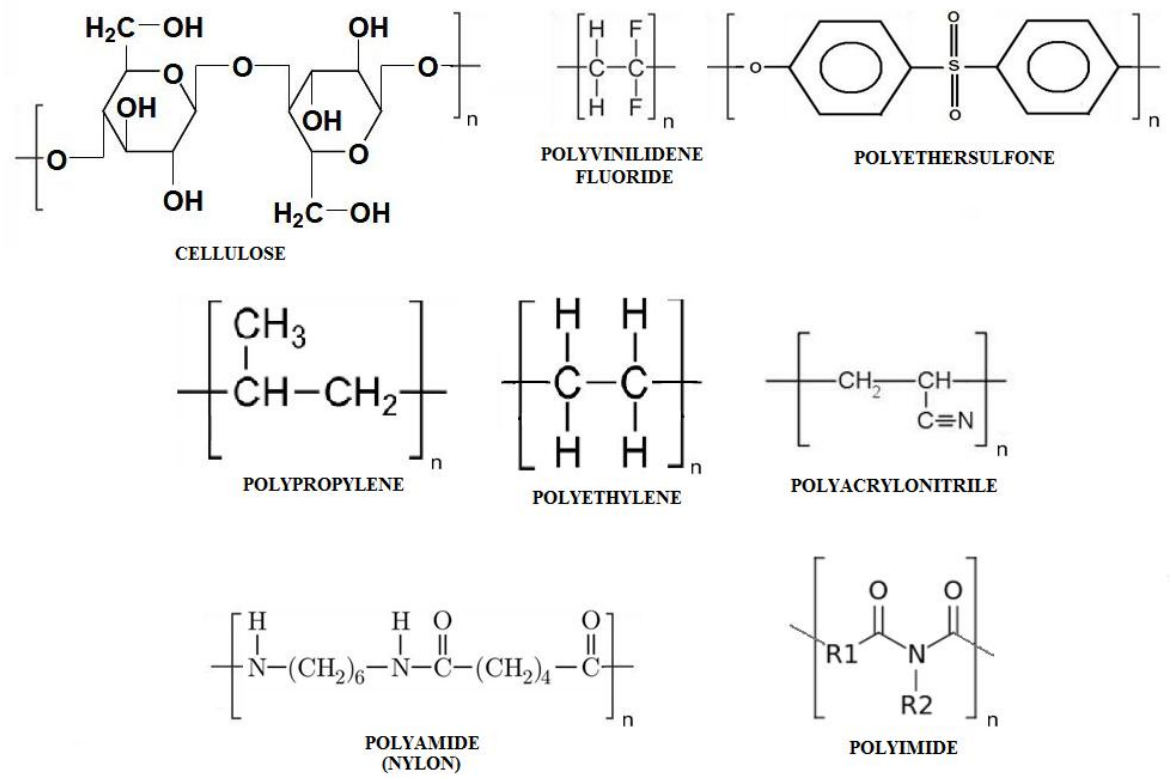


Fig. 1.1. Chemical structures of some polymers used for membrane production.

The most important advantages provided by membrane technology as compared with other unit operations in biochemical engineering are due to this unique separation principle that is the transport selectivity of the membrane. Separations performed using membranes do not require additives; in addition they can be performed isothermally at low temperature

and compared to other thermal separation processes at low energy consumption. Also, up scaling and downscaling of membrane processes and their integration into other separation or reaction processes are easy [6].

However, with the progress of functionalization chemistry and availability of new materials and technologies, it was realized that membrane has more potential than just being used as a separation media. Then, the concept of functionalized membranes was conceived and researchers started modifying membranes to incorporate various kinds of functionality to use them in different fields of research. This research work involves experimental and studies with functionalized polymeric membranes. Background studies of the important research areas related to this thesis are discussed in following sections.

1.2. FUNCTIONALIZED MEMBRANE: BASIC CONCEPTS

The in-service performances of a polymer membrane mainly depend on the chemistry of the membrane material, the structures and properties of the porous layer, and the microstructures and surface properties of the top layer. Surface modification of polymer membranes has emerged in recent years. The main advantage of functionalized membranes is the versatility of the active groups. It can be tailor-made to impart the desired functionality from a variety of active groups or molecules depending on the applications. Functionalized membranes are widely used in various fields, such as, biotechnology, food and pharmaceutical industries [3, 7]. Functionalized membranes are created by attaching active groups in the base polymer of the porous membranes (UF or MF membranes). Porous membrane compared to non porous membrane, offers advantages related to the higher specific surface area. The active groups can be either incorporated during the formation of the membrane, or after membrane formation using functionalization methods.

The surface functionalization of existing membranes is preferred in many cases, as for example, when functionality of interest cannot be introduced by adding the hypothetical monomer formally recognized in the repeating unit during polymerization.

Functionalized membranes are generally required in membrane processes based on developing technologies, including those for biocatalysis, sensing and bioseparation. In addition the performances of these technologies strongly depend on the functionality of the membrane surface [8]. Based on the strategies adopted for surface modification, a functional membrane surface can be prepared. A functional membrane surface, immobilized with enzyme, can be applied in an enzyme-membrane bioreactor as well as in biosensors, in which the activity retention and stability of the immobilized enzyme are essential. In these systems the choice of membrane material and functionalization strategy influences the enzyme performance.

1.3. DEVELOPMENT OF FUNCTIONALIZED POLYMERIC MEMBRANES

The most widespread approach for the preparation of functionalized membranes is by surface modification of the preformed membranes with desired functional groups. This field has progressed rapidly in recent years and has become an important point in membrane science and technology. Thought these strategies the performance of preformed polymeric membranes can be improved either because undesired interaction, such as adsorption or adhesion, can be minimized (e.g. obtaining significant improving for what concern membrane fouling), or by introducing additional interactions (affinity, responsiveness or catalytic properties) for new applications. The approaches to surface functionalization of polymeric membranes are summarized in following sections.

1.4. OVERVIEW ON ENGINEERIZATION OF POLYMERIC MEMBRANES

The surface properties of polymeric membranes such as biocompatibility, anti-fouling, hydrophilicity, hydrophobicity, antibacterial properties and conductivity can be improved by surface modification methodologies without affect the bulk properties of the membrane. In order to achieve these improvements several approaches have been proposed, e.g. coating, self-assembly, chemical treatment, plasma treatment and surface graft polymerization.

1.4.1. Coating

Coating is a simple but effective method for membrane surface modification, and it has been widely adopted to tailor the surface properties of RO, NF, UF and MF membranes [9-12].

It is a modification method based on the addition of an extra layer on top of the native membrane surface. The materials are physically deposited on the membrane surface via one of the following mechanisms:

- Adsorption-adhesion, the functional layer is only physically attached on the base polymer; the binding strength can be increased via multiple interactions between functional groups in the macromolecular layer and on the solid surface;
- Interpenetration by mixing between the added functional material and the base polymer in an interphase;
- Entanglement of an added material layer and the pore structure of the membrane.

Owing to the coating of these functional materials on the membrane, the surface property of the membrane could mutate from hydrophobic or non-biocompatible to hydrophilic and biocompatible. However, this method cannot gain a stable surface, for the materials

absorbed on the membrane surface run away easily due to the relatively weak interaction [13].

1.4.2. Self-assembly

Self-assembly is a relatively new technique for membrane surface modification. The techniques involves self-assembled monolayer (SAMs) and layer by layer assembly (LbL) which enables the fabrication of "engineerable" highly selective membrane top layers. SAMs are highly ordered molecular assemblies formed by the adsorption of functionalized alkanes onto the solid surface [14]. It leads to a structure which is at, or close to, thermodynamic equilibrium, and thus tends to self-healing/defect rejection and leads to a closely packed, well-ordered, and stable configuration on the surface [15]. The LbL technique is extremely versatile in that uniform thin films with a wide range of properties can be prepared onto various substrates, including membranes [16]. The LbL process is simple and can be performed in mild conditions using environmentally friendly reagent. It has become a primary tool for thin film fabrication. The basic process involves dipping a charged (e.g. cationic) substrate into a dilute aqueous solution of an anionic polyelectrolyte and allowing the polymer to adsorb and reverse the charge of the substrate surface. The negatively charged coated substrate is rinsed and dipped into a solution of cationic polyelectrolyte, which adsorbs and re-creates a positively charged surface. Sequential adsorptions of anionic and cationic polyelectrolytes allow the construction of multilayer films [17]. The unique advantages of the method are that a wide variety of materials, both organic and inorganic, can be incorporated into LbL thin films and that the film architecture is largely controlled by the deposition sequence.

1.4.3. Chemical Treatment

Wet chemical treatment is one of the most common methods for functionalize membrane. Usually the modifications allow retaining mechanical properties of the membrane changing the interfacial properties. In wet chemical surface modification, a membrane is treated with liquid reagents to generate reactive functional groups on the surface. This approach to surface modification can be conducted in most laboratories because does not require expensive or special equipments. This method is also more capable of penetrating into the membrane pores compared to plasma and other energy source surface modification techniques [7]. Chemical treatments include oxidation, addition, substitution and hydrolysis. Oxidative treatment is the dominant means for chemically modified reactions, which generally involve dry or wet oxidation. In the case of dry oxidation, a gaseous oxidation agent, like oxygen, ozone or carbon dioxide is often used. Oxygen-containing groups such as hydroxyl and carboxyl are introduced onto the membrane surface by flame treatment or corona discharge in these gases. These groups can also be introduced onto a membrane by wet oxidation. Wet chemical oxidation involves the use of nitric acid, sulfuric acid, phosphoric acid, alone or in combination with hydrogen peroxide, sodium hypochlorite, permanganate, chromate or dichromate of potassium, transition metal nitrates, etc. [8].

1.4.4. Plasma Treatment

The excitation with plasma is very surface selective [18]. Plasma surface modifications exhibit multifunctional chemistries such as oxidation, degradation, changes in the carbon/hydrogen ratio which cause cross-linking and structural changes occurring in the surface layer without substantially changing the bulk structure. Thus, these chemistries

occurring only at the contact point between plasma and surface of the solid materials result in changed physicochemical properties of the surface. The complexity of the chemistries is due to the complexity of plasma, which contains electrons, ions, radicals, metastable excited species and vacuum ultraviolet radiations [19]. Plasma treatment for the surface modification of membranes includes plasma sputtering, etching, implantation, and spraying.

1.4.5. Surface Graft Polymerization

Grafting methods can usually be divided in two classes, *i.e.* "grafting to" and "grafting from" processes. In the case of the "grafting-to" process incorporation of polymer chains bringing reactive groups can be tailor-made. The "grafting-from" method involves the incorporation and polymerization of monomers using an active species on the membrane surface. The main advantage of this technique is that the membrane surface can be modified or tailored to acquire the desired properties by the choice of suitable grafting monomers, but at the same time the bulk properties can be maintained. However, another important advantage of the graft polymerization methodology compared with the physical modification methods such as coating, is the long-term chemical stability of the modified surface, ensured by the covalent attachment of polymer chains onto the membrane surface which avoids detaching.

1.5. MEMBRANE MODIFICATION FOR BIOMOLECULES IMMOBILIZATION

The type of polymer used for membrane preparation, strongly influence the characteristics of a polymeric membrane. Often such polymeric membranes that have excellent physical-chemical bulk properties do not possess the appropriate surface properties required for

particular applications, like for example biomolecules immobilization. In fact, biomolecules immobilization is only possible on a surface provided of enzyme binding sites (functionality) which are the points where the membrane manage to form chemical bonds with the biomolecules. The membrane modification techniques therefore can be used to create these desired functionalities on the membrane surface [20]. In the case of enzyme immobilization, the requirements for these modifications are either dependent on the enzyme or on the membrane, because enzyme–membrane interactions play an important role in maintaining the enzyme activity. A brief introduction on the most common polymeric membrane materials that are being used to prepare membranes with active groups, are presented below.

1.5.1. Polymeric membrane materials

Several membrane forming material have been investigated as target of functionalization procedure in order to introduce binding sites. Applications of cellulose [21], regenerated cellulose (RC) [22], cellulose acetate (CA) [23], polyacrylonitrile (PAN) [24], polysulfone (PS) [25], nylon [26], PVDF [27], polypropylene (PP) [28] and PES [29] have been reported. This dissertation deals with PVDF and PES membranes, in the next sections the strategies, reported in literature, used for functionalization of both PVDF and PES membranes are presented.

1.5.2. Polyvinylidene fluoride (PVDF) membrane modification

Polyvinylidene fluoride is a popular engineering polymer; membranes based on PVDF have been used either for scientific research or industrial application in particular micro and ultrafiltration processes. The great interest to PVDF membrane is due to its

extraordinary thermal, mechanical and chemical resistance in addition to membrane forming properties. Its structure consisting of repeated –(CH_2-CF_2)$_n$ - units confers to PVDF these good properties, but makes not possible use it for particular application like for example biomolecules immobilization by covalent bond. This disadvantage can be overcome performing PVDF functionalization, in order to introduce on the polymeric membrane structure, reactive groups that can covalently couple with biomolecules functional groups. Surface modification of existing PVDF membrane can be performed by coating or grafting. Coating modification is preferred when only an improved hydrophilicity of the membrane surface is required, but it is not useful as anchor point for enzyme immobilization because of the instability of the coated layer which interacts with the membrane merely by physical adsorption. More interesting for enzyme immobilization is grafting modification which can introduce on the PVDF backbone functional chains that can be used for enzyme immobilization, in addition acting on degree of grafting and type of functional chain introduced it is possible affect or preserve the membrane bulk properties as for example hydrophobicity [30]. Surface grafting can be performed by high energy radiation techniques like plasma treatment, ozonation and UV photo irradiation or by wet chemistry in harsh basic environment. Plasma treatment is an efficient way to introduce the functional graft chains on the surface of PVDF membrane without affects the bulk properties of the membrane. The thickness of the modified layer can be controlled up to the angstrom levels. Hydrogen, argon, helium, oxygen, nitrogen and fluorine-containing plasmas are frequently used to modify the membrane surface [31]. The ionized species abstract hydrogen atoms from PVDF and produce the initiating sites on the membrane surface to initiate the polymerisation of functional monomers as for example acrylic acid (AAc). However, plasma treatments for PVDF membranes are usually used when the goal

of the modification is the surface hydrophilization. The main disadvantage of plasma is that it requires a vacuum system, which increases the cost of the operation. PVDF membrane prepared by thermally induced graft copolymerization of AAc with ozone-preactivated PVDF molecules dissolved in an organic solvent (*N*-methyl-2-pyrrolidone (NMP)) has been reported by Ying et al. 2002 [32]. Using this strategy a bulk-modified PVDF was obtained, afterwards, the carboxyl groups of the AAc side chains were used for the direct immobilization of the enzyme glucose oxidase. Another technique mostly used for improve the antifouling properties of PVDF membrane is the one that exploits the UV photo irradiation method. The photo-initiator benzophenon is usually needed to initiate the photo grafting polymerisation process [33].

In the present work the activation of PVDF membrane for biomolecules immobilization was performed by wet chemical strategy, under basic condition, using a diamine as carrier of amino groups.

1.5.3. Polyethersulfone (PES) membrane modification

Membranes based on polyethersulfone (PES) are widely used membranes in ultrafiltration processes due to their high performance and low cost profile. PES membranes have been used also in more sophisticated application such as carrier for immobilization of bioactive molecules in membrane bioreactors. For this aim effort have been done to introduce reactive groups for biomolecules immobilization without affect the overall bulk properties. Because the traditional application of PES membrane are the pressure driven processes the main problem correlated with the use of pristine PES is due to its high hydrophobicity. This feature lead the native PES membrane to be easily affected by fouling problem, therefore the early modification strategy, reported in literature, for PES membrane

modification were developed in the aim of increase its hydrophilicity. These modifications can be carried out in several ways. Physical or chemical membrane modification processes of existing membrane create more hydrophilic surfaces. Such modification processes can be classified as follow [34]:

- graft polymerization that chemically attaches hydrophilic monomers to the membrane surface;
- plasma treatment, that introduces different functional groups to the membrane surface;
- physical preadsorption of hydrophilic components to the membrane surface.

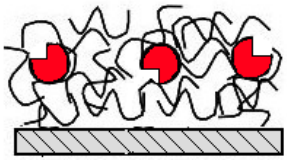
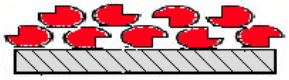
When the aim is the biomolecules immobilization, photochemical surface modification of existing poly (arylsulfone) membranes (PES and PS) is a popular technique for introduction of reactive groups. Yamagishi et al. [35] firstly applied the UV irradiation induced grafting method to modify PES membranes. It was discovered that the poly (arylsulfone) are intrinsically photoactive and that no photo initiators were required for this process making possible to perform the modification in mild reaction condition [36]. For example in the work of Ulbricht et al. [37] acrylic acid was photo grafted in this way on PS membrane and then activated for enzyme immobilization using 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC).

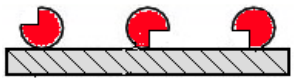
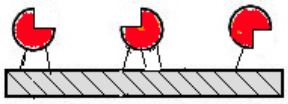
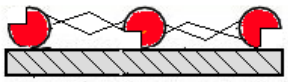
1.6. IMMOBILIZATION OF BIOMOLECULES ON MEMBRANE

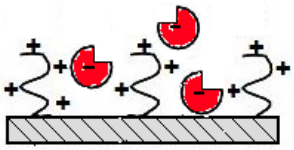
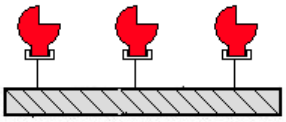
Once the membrane is modified, further attachment of groups or molecules depends on the final use of the membrane. Since this dissertation mainly deals with biomolecules, further discussion concerns those molecules only. Several techniques of biomolecules immobilization on membrane or solid support have been reported in literature, they can be

broadly classified as physical, where weak interactions between support and biomolecules exist, and chemical, wherein covalent bonds are formed with the biomolecules [38]. A schematic diagram showing different types of interactions is presented in Table 1.1.

Table 1.1. Strategies used to immobilize biomolecules on membranes

METHOD	**CHARACTERISTICS**	**EXAMPLE OF IMMOBILIZED BIOMOLECULE**	**TYPE OF MEMBRANE**
Entrapment 	The entrapment of biomolecules can be performed during membrane preparation, by immobilization between membrane layers or by cross-flow filtration of the biomolecule solution through an asymmetric membrane so that the biomolecules are entrapped in the pores. Blockage of membrane pore due to the biomolecules and leakage are the main problems.	β-glucosidase [39]	Polysulfone
Gelification 	Gelification is based on one of the main drawbacks of membrane processes: concentration polarization phenomena and subsequent protein precipitation upon reaching a critical concentration at the membrane interface. An important drawback of this method is biocatalyst leakage during the process.	DNase [40]	UF capillary membrane

Adsorpion 	The adsorption can be performed also on unmodified membrane; it is a method of preparing a biocatalytic membrane in which the interaction of the biomolecule and the membrane happens through weak and nonspecific bonds, promoted by van der Waals forces, hydrogen bonds or hydrophobic interaction. The method is cheap and very simple but stability can be low.	Lipase [41]	Polypropilene
Covalent linking 	The covalent linking is the most stable biomolecule immobilization technique. This strategy exploits active groups present on the membrane such as aldehyde, amine, epoxide, anhydride and carboxylic acid. The main advantage is that the covalent bond between the biomolecule and the membrane is irreversible therefore no leakage of biomolecules occurs.	Glucose oxidase [42]	Cellulose
Crosslinking 	Crosslinking is an immobilization technique which is performed by intermolecular crosslinking of the enzymes, either to other protein molecules or to functional groups present in an insoluble support matrix, by using a di-functional agent such as glutaraldehyde. A drawback is loss of active properties of immobilized biomolecules	α-chymotrypsin [43]	Polytetrafuoro -ethylene

Ionic binding 	This technique consists in the interaction of a charged biomolecules with the membrane that has opposite charge.	Catalase [44]	Chitosan
Site-specific 	This technique is performed by introduction of a spacer molecule, or in the case of glycoproteins by orientating the attachment though the carbohydrate moieties. Other strategies are based on gene fusion, on site direct mutagenesis introducing unique cystein molecules on biomolecule and on biotin-avidin strategy. This technique retains the activity of the biomolecule along with the benefits of traditional covalent bonding but it is expensive and complex.	Xanthine oxidase [45]	Textile

1.7. APPLICATIONS OF FUNCTIONALIZED MEMBRANES

The functionalized membranes have been employed in many membrane fields (Fig. 1.2), like separations processes, biocatalysis, sensing and so on [46]. The applications of functionalized membranes can be classified in the following different categories on the base of the membrane role:

- Functionalized membranes used in separation processes, where the functionalized membrane permit selective permeation of one of the species and can be used in continuous mode.
- Functionalized membrane used in sorption processes, in this case the functionalized membrane have the function of adsorbents, leading to separation and capture of analytes of interest.
- Functionalized membranes used for catalytic application, in this case functional groups, enzymes or immobilized nanoparticles perform catalysis.

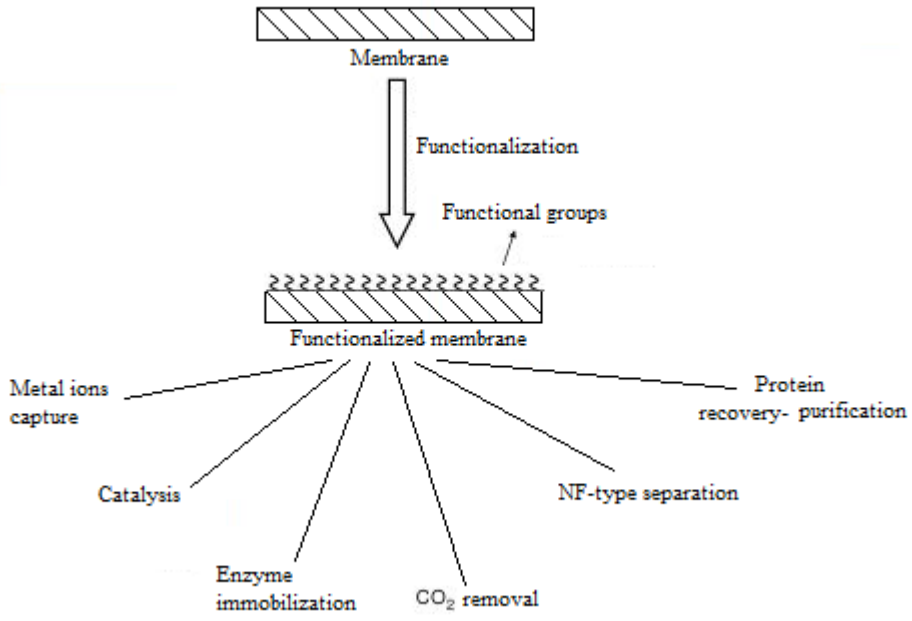


Fig. 1.2. Applications of functionalized membranes.

This thesis work has been directed towards development of biohybrid polymeric membranes for sensing, separation and bioconversion. Therefore, background studies of these three research areas are presented below.

1.7.1. Functionalized membranes for sensing systems

A chemo or biosensor is described as a device, which consists of a receptor coupled with a transducer to a detector, thus enabling the conversion of a (bio)chemical signal binding to the receptor into a physical signal. The difference between a biosensor and a chemosensor is that the former contains a biological entity such as enzyme, antibody, bacteria, etc. as recognition agent, whereas the later does not contain these agents [6, 47]. Many sensor devices involve functionalized membranes which either participate in sensing mechanisms or immobilize the component responsible for sensing the analyte. There are many advantages leading to use membrane as component of sensor device. For example the membrane can perform the function of barrier between the sensor system and its environment, allowing selective access (e.g. of the analyte only) to the receptor or/and protecting the receptor from disturbing influences of the environment. In addition if the transducer is a separate chemical species, the membrane either used as support for the immobilization of the receptor or tool for bringing it close to the detector, can allow to integrate the sensing system.

1.7.2. Functionalized membranes for separation processes

Modification of polymeric membrane can be performed in the aim of minimize undesired (secondary) interaction like adsorption or adhesion which decrease the performance of the membrane. For example surface modifications of hydrophobic membranes in order to reduce biofouling problems are often conducted by one of the functionalization techniques reported previously [48]. Membrane modification strategies can be also conducted for improving the selectivity of the membrane or creating a new separation property. For example modification of membranes with immobilized functional polyelectrolytes has

received attention for its potential in the development of ion-exchange materials and tunable membrane separations. In the work of Ritchie et al. [49] cellulose membrane were functionalized with poly-(α,β)-DL-aspartic acid in order to recover heavy metal (Cu^{++}) demonstrating the importance of the creation of a mobile condensation zone in the membrane pores by charged polyelectrolytes. The immobilization of suitable functional groups inside membrane pores allows selective transport of species. An example is the functionalization of membrane by stimuli responsive polypeptides that was adopted in the work of Smuleac et al. [50]. In this work the performances of a functionalized polycarbonate membrane where, evaluated in terms of both permeability and retention capabilities, which can be reversibly modulated by changing the pH.

1.7.3. Functionalized membranes for bioconversion processes

Functionalized membranes have the potential to contribute significantly toward the improvement of catalytic applications by providing alternative support for catalyst immobilization. Membrane-supported catalytic applications not only mitigate the need for dispersion of the catalyst and its subsequent removal from reaction mixture, but also provide very high mass transport conditions [46]. Since this dissertation mainly deals with biomolecules, further discussion concerns functionalized membrane with biocatalysts (enzymes) only.

1.8. ENZYMES

Enzymes are a group of proteins produced in tiny quantities by all living organisms (bacteria, plants, and animals) and functioning as highly selective biochemical catalysts in converting one molecule into another. The reaction involving

enzymes is known as biocatalysis and the reactant that is being catalyzed with a high degree of specificity is called the substrate. Enzymes catalyzes reactions in mild conditions (such as temperature, pH, etc.), that involves in energy saving and reduced manufacturing costs compared to conventional reactions. They are used in field such as fine-chemical and pharmaceutical synthesis, food processing, detergent applications, biosensors fabrication, for degrading of harmless compounds, etc. [51]. Lipase from *candida rugosa* (LCR) and a phosphotriesterase (PTE) are the enzymes that have been used in this research. Additional background information about these two enzymes are provided below.

1.8.1. Lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases of particular physiological importance and industrial potential that can catalyze several reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Fig. 1.3). Lipases are used in the dairy and food industries, in the production flavor and aroma components, in oleochemical industry and in medical application [52]. In addition, they are used in the leather and detergent industry, in the production of surfactants and for analytical purposes such as lipase-based potentiometric biosensors for detection of organophosphate pesticides [53]. One of the most promising fields of lipases application is the production of optically active compounds for the pharmaceutical industries [54]. This ability and other special properties such as their capacity to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents has meant that the interest in lipase research increased over the past decades. These properties allow lipase to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure.

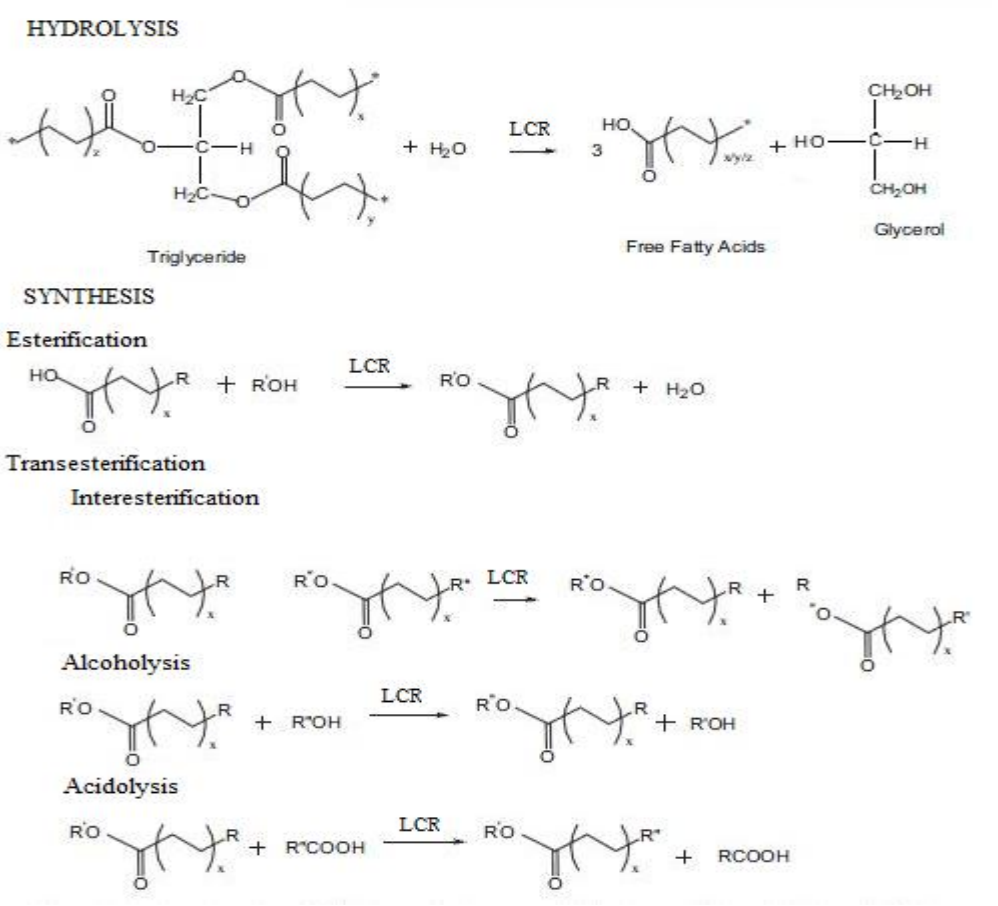


Fig. 1.3. Reaction mechanism of reactions performed by LCR.

Lipases are special enzymes also because their activity is higher against water-insoluble substrates (such as emulsions) and enhanced at the substrate (oil)-water interface, this phenomenon was first observed in 1936 by Holwerda et al. [55] and named "interfacial activation". In 1990 F. K. Winkler at al. [56] determined the 3D structure of human pancreatic lipase. They showed that a surface loop, i.e. a lid domain, covers the active site, which is not accessible to substrates. It was postulated that this loop had to undergo a conformational change in the presence of a lipid-water interface and this might explain the phenomenon of interfacial activation.

Despite all the advantages, application of lipases in industrial level is still underdeveloped because of drawbacks of the extensive use of lipases and biocatalysts in general compared

to classical chemical catalysts can be found in the relatively low stability of enzyme in their native state as well as their prohibitive cost. Lipase immobilization can be a solution to these drawbacks because of enabling reusability, operational flexibility and ease of product recovery from the enzyme. Different immobilization methods for lipase have been reported in literature and it even showed an increased activity when immobilized on the right support with the appropriate method [57].

1.8.2. Phosphotriesterase

This class of enzymes catalyse the hydrolysis of synthetic organophosphate compounds (OPs) i.e the nerve agent (Sarin, Soman, VX) and the pesticide classes (paraoxon, parathion, etc.). In the last few years the environmental decontamination of OPs has attracted an overwhelming interest. OPs are toxic compounds for all vertebrates because they irreversibly inhibit acetylcholinesterase, a key enzyme of the nervous system. They have been distributed globally since the end of World War II and their toxic properties have also been exploited for the development of chemical warfare agents such as sarin, soman and VX as well as for the production of agricultural insecticides [58]. Enzymatic detoxification of OPs has become the subject of many studies because alternative methods of removing them, such as bleach treatments and incineration are impractical due to high costs or environmental concerns. The best known bacterial phosphotriesterase identified so far has been isolated from the soil bacterium *Pseudomonas diminuta* (PTE: E.C. 3.1.8.1). Structurally PTE is a homodimeric $(\beta/\alpha)_8$-barrel with a binuclear metal centre located at the C-terminal end of the barrel [59]. The catalytic centre is composed of two closely spaced divalent cations ligated to the protein via direct interactions with four histidines, one aspartate and a carboxylated lysine residue.

The catalytic mechanism for the hydrolysis of organophosphate triesters has been well studied. The best substrate known for PTE is the pesticide paraoxon but the substrate specificity is very broad and covers a wide range of organophosphates, thiophosphates, and phosphorothiolates. The Model for the chemical-reaction mechanism of the bacterial PTE has been proposed by D. Aubert et al. [60], it is presented in Fig. 1.4.

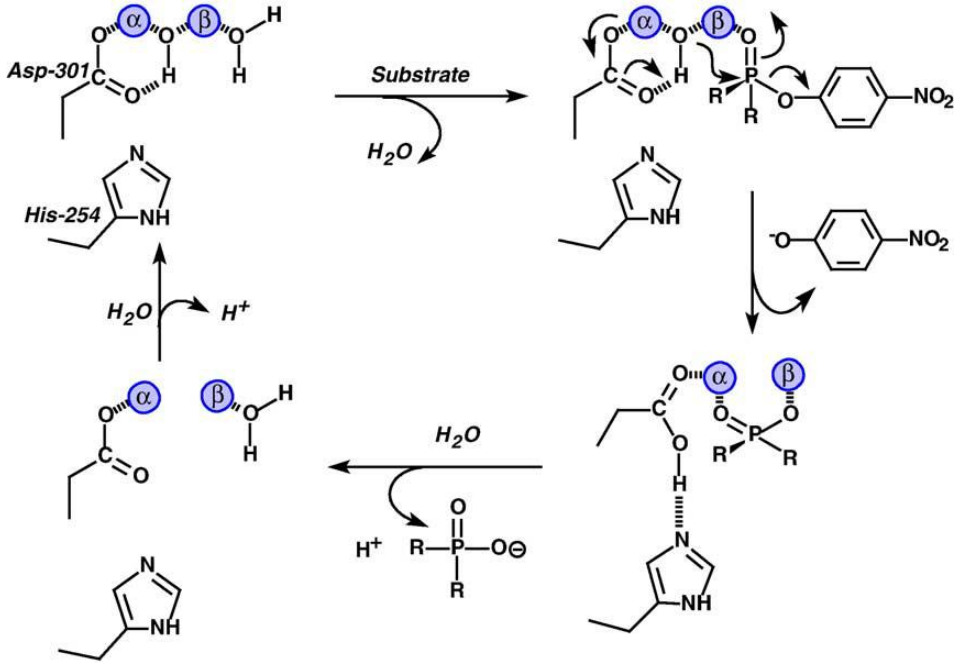


Fig. 1.4. Catalytic mechanism for hydrolysis of paraoxon by PTE proposed by D. Aubert et al.

Unfortunately, mesophilic PTE often suffers problems of low stability due to sensitivity to changes in pH or temperature also after immobilization. The PTE which is subject of the present work, is a mutant of the thermophilic Phosphotriesterase-like lactonase (PLL) isolated from the hyperthermophilic archaea *Sulfolobus solfataricus* (*Sso*Pox) [61]. This enzymes has been chosen because its outstanding intrinsic thermal stability which is a property of valuable interest for applications in any detoxification system, in fact it is well

known that a high thermal stability involves a high resistance to harsh condition (solvents, detergents, etc.) and long time stability.

1.9. CONCLUDING CONSIDERATIONS

From the detailed background study of the research areas relevant to this thesis, some interesting observations can be made.

1.9.1. PVDF functionalization

Various studies have been done on PVDF functionalization in particular in order to decrease the fouling problems due to its hydrophobicity. In the literature it is reported that PVDF membrane functionalization is usually carried out by high energy radiation techniques like plasma, ozonation and UV. Only few examples of existing PVDF membranes, modified by wet chemistry are reported, principally in harsh basic or acid environment. However, partial or none information about degree of functionalization, membrane damaging and amounts of attached biomolecules compared to the number of reactive groups introduced on PVDF membrane have been reported in these few studies. Therefore, in the present work have been investigated, for the first time, the process parameters to achieve a tuned control of the degree of membrane modification using the chemical grafting method under alkaline conditions.

1.9.2. Phosphotriesterase immobilization

Various systems PTE-based have been used in literature for organophosphate decontamination. The main problem of all these systems is the low stability of PTE. To our best knowledge, this is the first study reported on the development of immobilized

thermostable PTE and the characterization of the resulting biohybrid membrane for organophosphates pesticides degradation.

References

[1] kaushik Nath. Membrane separation processes. PHI Learning Pvt. Ltd. 2008.

[2] H. Strathmann, L. Giorno, E. Drioli. An introduction to membrane science and technology. 2006

[3] L. S. Wan, Z. M. Liu and Z. K. Xu. Surface engineering of macroporous polypropylene membranes Soft Matter, 5 (2009) 1775-1785

[4] S. Loeb, S. Sourirajan, The preparation of high-flow semi-permeable membranes for separation of water from saline solutions 1964.

[5] D. Bhattacharyya, D.A. Butterfield. New Insights into Membrane Science and Technology: Polymeric and Biofunctional Membranes. Membr. Sci. Technol. Ser. 8 (2003).

[6] M. Ulbricht. Advanced functional polymer membranes. Polymer 47 (2006) 2217–2262

[7] J.M. Goddard, J.H. Hotchkiss Polymer surface modification for the attachment of bioactive compounds. Prog. Polym. Sci. 32 (2007) 698–725

[8] Z. Xu, X. Huang, L. Wan Surface Engineering of Polymer Membranes. Advanced Topics in Science and Technology in China 2009.

[9] J.S. Louie, I. Pinnau, I .Ciobanu, K.P. Ishida, A. Ng, M.R einhard. Effects of polyether-polyamide block copolymer coating on performance and fouling of reverse osmosis membranes, J. Membr. Sci. 280 (2006) 762–770.

[10] I.C. Kim, K.H. Lee. Dyeing process waste water treatment using fouling resistant nanofiltration and reverse osmosis membranes, Desalination 192 (2006) 246–251.

[11] D.A. Musale, A. Kumar, G. Pleizier, Formation and characterization of poly (acrylonitrile)/chitosan composite ultrafiltration membranes. J. Membr. Sci. 154 (1999) 163–173.

[12] X.Y.Wang, C.Chen, H.L.Liu, J.Ma. Preparation and characterization of PAA/ PVDF membrane-immobilized Pd/Fe nanoparticles for dechlorination of trichloroacetic acid, Water Res. 42 (2008) 4656–4664.

[13] G. Kang, Y. Cao. Application and modification of polyvinylidene fluoride (PVDF) membranes – A review. J. Membr. Sci. 463 (2014) 145–165.

[14] H. A. Biebuyck, Christoph Gerber, E. Delamarche, B. Michel. Golden Interfaces: The Surface of Self- Assembled Monolayers. Adv. Mater. 8 (1996) 719.

[15] E. Ruckenstein, Z.F. Li. Surface modification and functionalization through the self-assembled monolayer and graft polymerization. Advances in Colloid and Interface Science 113 (2005) 43– 63

[16] D. Lee, Z. Gemici, M. F. Rubner, R. E. Cohen. Multilayers of Oppositely Charged SiO2 Nanoparticles: Effect of Surface Charge on Multilayer Assembly Langmuir 23 (2007) 8833-8837

[17] W. Chen, T. J. McCarthy. Layer-by-Layer Deposition: A Tool for Polymer Surface Modification. Macromolecules 30 (1997) 78-86

[18] F.S. Denes , S. Manolache. Macromolecular plasma-chemistry: an emerging field of polymer science. Prog Polym Sci 29 (2004) 815-885.

[19] P. Jochems, Y. Satyawali, L. Dielsa and W. Dejonghe, Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs). Green Chem. 13 (2011) 1609

[20] J. Deng, L.Wang, L. Liu, W. Yang. Developments and new applications of UV-induced surface graft polymerizations. Prog. Polym. Sci., 34 (2009) 156–193.

[21] C. Tyagi, L. K. Tomar, H. Singh. Surface modification of cellulose filter paper by glycidyl methacrylate grafting for biomolecule immobilization: Influence of grafting parameters and urease immobilization J. Appl. Polym. Sci. 111 (2009) 1381–1390.

[22] C. Chen, C. Chen, C. Huang, Y. Liu. Simultaneous purification and immobilization of penicillin G acylase using bifunctional membrane. J. Membr. Sci. 298 (2007) 24–29.

[23] D. Brady, J. Jordaan. Advances in enzyme immobilization. *Biotechnol. Lett.*, 2009, 31, 1639–1650.

[24] T. Godjevargova, R. Dayal, I. Marinov. Simultaneous covalent immobilization of glucose oxidase and catalase onto chemically modified acrylonitrile copolymer membranes. *J. Appl. Polym. Sci.* 91 (2004) 4057–4063.

[25] M. Ulbricht, M. Riedel. Ultrafiltration membrane surfaces with grafted polymer tentacles: preparation, characterization and application for covalent protein binding Biomaterials 19 (1998) 1229-1237

[26] V. Smuleac, D. A. Butterfield, D. Bhattacharyya, Layer-by-Layer-Assembled Microfiltration Membranes for Biomolecule Immobilization and Enzymatic Catalysis. *Langmuir* 22 (2006) 10118–10124.

[27] C. Jolivalt, S. Brenon, E. Caminade, C. Mougin, M. Ponti. Immobilization of laccase from Trametes versicolor on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater. *J. Membr. Sci.* 180 (2000) 103–113.

[28] M. Becker, N. Provart, I. Lehmann, M. Ulbricht, H.-G. Hicke. Polymerization of Glucans by Enzymatically Active Membranes. *Biotechnol. Prog.* 18 (2002) 964–968.

[29] B. Fang, Q. Ling, W. Zhao, Y. Ma, P. Bai, Q. Wei, H. Li, C. Zhao. Modification of polyethersulfone membrane by grafting bovine serum albumin on the surface of

polyethersulfone/poly(acrylonitrile-co-acrylic acid) blended membrane. *J. Membr. Sci.* 329 (2009) 46–55

[30] Y. J. Byuna, J. H. Kimb, S. S. Kima. Surface modification of PVDF membranes for water treatment via hydrophilic thermal cross-linking method Desalination and Water Treatment 51 (2013) 5371–5378

[31] S. Kaur, Z. Ma, R. Gopal, G. Singh, S. Ramakrishna, T. Matsuura. Plasma induced graft copolymerization of poly(methacrylic acid) on electrospun poly(vinylidene fluoride) nanofiber membrane. Langmuir 23 (2007) 13085–13092

[32] Lei Ying, E.T. Kang, K.G. Neoh. Covalent immobilization of glucose oxidase on microporous membranes prepared from poly(vinylidene fluoride) with grafted poly(acrylic acid) side chains. Journal of Membrane Science 208 (2002) 361–374

[33] N. Hilal, V. Kochkodan, L. Al-Khatib, T. Levadna. Surface modified polymeric membranes to reduce (bio)fouling: a microbiological study using E. Coli. Desalination 167 (2004) 293–300.

[34] B. Van der Bruggen. Chemical Modification of Polyethersulfone Nanofiltration Membranes: A Review Journal of Applied Polymer Science. 114 (2009) 630–642

[35] H. Yamagishi, J.V. Crivello, G. Belfort. Development of a novel photochemical technique for modifying poly(arylsulfone) ultrafiltration membranes. *J Membr. Sci*. 105 (1995) 237–47.

[36] C. Zhao, J. Xue, F. Ran, S. Sun. College of Modification of polyethersulfone membranes – A review of methods. Progress in Materials Science 58 (2013) 76–150

[37] M. Ulbricht, M. Riedel, U. Marx. Novel photochemical surface functionalization of polysulfone ultrafiltration membranes for covalent immobilization of biomolecules. J Membrane Sci 120 (1996) 239–59.

[38] Y. Wang, J. Zhang, J. Yin. Progress of enzyme immobilization and its potential application Desalination and Water Treatment 1 (2009) 157–171

[39] R. Mazzei, L. Giorno, E. Piacentini, S. Mazzuca, E. Drioli. *Journal of Membrane Science* 339 (2009) 215–223

[40] L. Giorno, E. Drioli. Biocatalytic membrane reactors: applications and perspectives. Trends in Biotechnology 18 (2000) 339-349

[41] F.X Malcata, H.S. Garcia, C.G. Hill, C.H. Amundson. Hydrolysis of butteroil by immobilized lipase using a hollow-fiber reactor: Part I. Lipase adsorption studies. *Biotechnol. Bioeng*. 39 (1992) 647–657

[42] U. Bora, P. Sharma, K. Kannan, P. Nahar. Photoreactive cellulose membrane a novel matrix for covalent immobilization of biomolecules. J. Biotechnol. 126 (2006) 220-229.

[43] R. A. Sheldon. Enzyme immiobilization the quest for optimum performance. Adv. Synth. Catal. 349 (2007) 1289–1307

[44] G. Bayramoglu, M. Y. Arica. Reversible immobilization of catalase on fibrous polymer grafted and metal chelated chitosan membrane. *J. Mol. Catal. B: Enzym.* 62 (2010) 297–304

[45] M. Amounas, V. Magne, C. Innocent, E. S. Dejean, P.Seta. Elaboration and chemical reactivity of enzyme modified ion exchanging textiles. *Enzyme Microb. Technol.* 31 (2002) 171-178

[46] E. Drioli, L. Giorno. Comprehensive Membrane Science and Engineering. Newnes 2010.

[47] D. G. Pijanowska, W. Torbicz. Ion Selective and Semi-permeable Membranes for Biosensors in Biomedical Applications. *Biocybernetics and Biomedical Engineerin*g 28 (2008) pp. 11–19

[48] H. Ma, C. N. Bowman, R. H. Davis. Membrane fouling reduction by backpulsing and surface modification. *Journal of Membrane Science* 173 (2000) 191–200

[49] S. M. C. Ritchie, L. G. Bachas, T. Olin, S. K. Sikdar, D. Bhattacharyya. Surface Modification of Silica and Cellulose-Based Microfiltration Membranes with Functional Polyamino Acids for Heavy Metal Sorption. *Langmuir* 15 (1999) 6346-6357

[50] V. Smuleac, D. A. Butterfield, D. Bhattacharyya. Permeability and Separation Characteristics of Polypeptide-Functionalized Polycarbonate Track-Etched Membranes. *Chem. Mater.* 16 (2004) 2762-2771

[51] O. Kirk, T. V. Borchert C. C. Fuglsang. Industrial enzyme applications. *Current Opinion in Biotechnology* 13 (2002) 345-351

[52] Z. D. Knežević, S. S. Šiler-Marinković, L. V. Mojović. Immobilized lipase as practical catalysts. *APTEFF* 35 (2004) 1-280

[53] F. Kartal, A. Kilinc, S. Timur. Lipase biosensor for tributyrin and pesticide detection. *Int. J. Environ. Anal. Chem.* 87 (2007) 715–722.

[54] L. Giorno, E. D'Amore, E. Drioli, R. Cassano, N. Picci. Influence of –OR ester group length on the catalytic activity and enantioselectivity of free lipase and immobilized in membrane used for the kinetic resolution of naproxen esters. *J. Catal.* 247 (2007) 194–200.

[55] K. Holwerda, P. E. Verkade and A. H. A. de Willigen Vergleichende Untersuchungen über die Verseifungsgeschwindigkeit einiger Einsäuriger Triglyceride unter Einfluss von Pankreasextrakt. I. Der Einfluss des Verteilungszustandes der Triglyceride auf die Verseifungsgeschwindigkeit Recueil des Travaux Chimiques des Pays-Bas 55 (1936) 43–57

[56] F. K. Winkler, A. D'Arcy, W. Hunziker. Structure of human pancreatic lipase. Nature 343 (1990) 771-774

[57] G. Pugazhenthi, A. Kumar. Enzyme membrane reactor for hydrolysis of olive oil using lipase immobilized on modified PMMA composite membrane. *Journal of Membrane Science* 228 (2004) 187–197

[58] E. Porzio, L. Merone, L. Mandrich, M. Rossi and G. Manco. A new phosphotriesterase from Sulfolobus acidocaldarius and its comparison with the homologue from Sulfolobus solfataricus. *Biochimie* 89 (2007) 625-636

[59] M.M. Benning, H. Shim, F.M. Raushel, H.M. Holden. High resolution X-ray structures of different metal-substituted forms of phosphotriesterase from Pseudomonas diminuta. *Biochemistry* 40 (2001) 2712-2722

[60] S. D. Aubert, Y. Li, F. M. Raushel. Mechanism for the Hydrolysis of Organophosphates by the Bacterial Phosphotriesterase. *Biochemistry* 43 (2004) 5707-5715

[61] L. Merone, L. Mandrich, E. Porzio, M. Rossi, S. Müller, G. Reiter, F. Worek, G. Manco. Improving the promiscuous nerve agent hydrolase activity of a thermostable archaeal lactonase. *Bioresource Technology* 101 (2010) 9204-12

Chapter 2

Biofunctionalization of PVDF membrane

2.1. Introduction

Numerous polymeric membranes that have excellent physical and chemical bulk properties do not possess the appropriate surface properties required for particular applications. For this reason, surface modification of polymeric membranes has been of prime importance in various applications from the advent of membrane-involving industries. Incorporation of novel functionalities is a versatile means for surface modification of polymeric membranes. Functionalized membranes are used for immobilization of enzymes [1], for improvement of biocompatibility [2], preparation of biosensors [3] and application in membrane bioreactors [4] due to their interesting properties of high specific surface area and the possibility to combine separation with chemical reaction [5].

Polyvinylidene fluoride (PVDF) is a hydrophobic polymer that because of its outstanding properties (high mechanical strength, chemical resistance, thermal stability, ultraviolet weathering resistance), has received great attention as a membrane material compared to other commercialised polymeric materials. For this reason, PVDF has been largely applied in microfiltration (MF) and ultrafiltration (UF) membranes processes [6].

Owing to the well-known low reactivity of PVDF, biomolecules are unable to couple with its surface in a covalent manner, so for this aim the grafting of functional groups is required. Different surface modification of PVDF membranes have been proposed in literature. The functionalization is usually carried out by high energy radiation techniques like plasma, ozonation and UV [6]. Only few examples of PVDF modification by wet chemistry are reported principally in harsh basic or acid environment [7,8,9]. In this contest one of the main issues to be addressed is the control of the PVDF degradation with loss of mechanical properties. Also in the case of PVDF modification for biomolecule attachment few example are reported in which wet chemical approach was used [7,10].

Surface grafting is one of the most promising methods to modify the membrane surface through the covalent bonding interaction between the grafted chains and the membrane [11]. The grafting method proposed by Kuo et al. [12] which consists of an introduction of amino groups on PVDF is one of the most interesting because it is economic and no expensive equipments are required. Starting from this method the functionalization of PVDF membrane under basic condition was investigated. In the literature it is reported that when PVDF is treated with an alkaline solution an elimination reaction occurs so fluorine and hydrogen are eliminated and double bond created in the PVDF chains [13], subsequent attack of nucleophilic molecules can lead to their grafting into PVDF chains.

In particular, the aim of the present work was the study of the conditions to functionalize PVDF membranes by graft of 1,5-diamino-2-methylpentane (DAMP) thus creating a reactive outer surface for biomolecules immobilization.

For the preparation of polyvinylidene fluoride grafted DAMP (PVDF-DAMP) membrane surfaces, possibilities for adjusting the degree of grafting (DG) by variation of functionalization conditions such as DAMP concentration, reaction time and reaction temperature were investigated. The new modified membrane surfaces were in detail characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, ninhydrin test and mechanical resistance test.

After DAMP grafting, subsequent covalent binding of biomolecules to the membrane can be performed by using a coupling reagent, like glutaraldehyde (GA) [14]. This methodology is simple and it is known that reactions between the carbonyl group of glutaraldehyde and the amino functions of the enzyme take place yielding a Schiff base [15]. This strategy can improve enzyme stability due to multipoint interaction between enzyme and membrane [10,16].

Immobilization efficiency test of PVDF-DAMP-GA activated membrane were performed using as model two biomolecules with different structural properties: the protein bovine serum albumin (BSA) and the enzyme Lipase from candida rugosa (LCR). Lipases are serine hydrolases of considerable physiological significance and industrial potential that can catalyze numerous reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis [10].

The method carried out in this work is particularly usefull in systems in which the hydrophobic properties of inert membrane are highly needed to improve membrane stability in multiphasic membrane reactors.

2.2. Experimental

2.2.1. Materials and chemicals.

Lipase from *Candida rugosa* (E.C.3.1.1.3, 65 kDa), BSA protein (65 kDa), ninhydrin, ethanol, glycine, glutaraldehyde and 1, 5-diamino-2-methylpentane were purchased from Sigma. Lipase and BSA were dissolved in 50mM phosphate buffer pH 7.

Flat sheet polyvinylidene fluoride (PVDF) membranes were used as support for biomolecules immobilization; they were kindly supplied by GVS Spa (Italy) and have an average pore size of 0.2 μm.

The membranes had hydrophobic properties and composite structure consisting of a selective PVDF layer on a nonwoven polyester (PE) support.

2.2.2. Instrumentation

Samples for scanning electron microscopy (SEM) were analyzed using an EVO MA 10, Zeiss, Assing. FT-IR spectra were recorded using the Spectrum One System by Perkin

Elmer Instruments in the range of 4000-650 cm^{-1}. Mechanical properties were carried out on a Zwick/Roell Universal Testing machine (single column, model Zwick Z2.5, Ulm, Germany) equipped with a 50 N maximum load cell and with pneumatic clamps. Colorimetric tests were performed using an UV spectrophotometer Perkin-Elmer Lambda EZ 201.

2.2.3. Surface modification of PVDF membrane.

The PVDF membrane cut into disk (d: 47 mm, pore size: 0.2 μm, thickness: 200 μm) was soaked into 20 mL of DAMP solution (1 M carbonate buffer, pH 11) in order to graft the DAMP onto the membrane surface [17]. After that the membrane was washed with ultrapure water.

Then the PVDF-DAMP membrane was treated for 2 hours with GA solution at 25 °C to graft the GA onto the PVDF-DAMP membrane (PVDF-DAMP-GA). Various GA concentrations: 0.1-1-5-10-20 % (v/v) were tested in order to obtain a complete coupling of amino groups. After that the membrane was thoroughly washed with distilled water.

2.3. Characterization of functionalized membrane

2.3.1. Determination of the degree of grafting

Ninhydrin method [18] was used in order to check the amount of grafted amino groups on the PVDF membrane surface. Ninhydrin reacted with the amino groups grafted on the PVDF surface to form a purple-coloured compound (Ruhemann's purple) in solution, the absorbance of this reaction solution was proportional to the number of grafted amino groups. The determination was performed adding to a small pieces of PVDF-DAMP membranes (0.25 cm^2), 1 mL of ninhydrin reagent in a tube and heating in boiling water

bath. Thereafter the reactants were cooled in a cold water bath and diluted with ethanol in a ratio 1:1 (v/v). The absorbance was measured at 570 nm. Glycine was used as standard, to obtain a calibration curve for the quantification measurement of the amino groups. The correlation coefficient between absorbance and concentration was 4000.

2.3.2. Chemical physical and morphological characterizations

Fourier transform infrared (FT−IR) spectra performed with an attenuated total reflectance method (ATR) was used in order to investigate chemical modification introduced on the active filtration layer of PVDF membrane as effect of the functionalization treatment.

The samples to be analyzed by scanning electron microscope (SEM) were prepared by breaking specimens in liquid nitrogen, and after sputtering them with gold.

2.3.3. Mechanical test

Mechanical properties of native and PVDF-DAMP modified membranes were carried out on membrane samples cut into strips of 10 mm width. The effective membrane strips length was 30 mm (i.e., the distance between the clamps). The strips thickness were measured with a digital micrometer (Carl Mahr, Germany) in five points and the average value was used. The test speed was 7 mm/min, the stress-strain curves were recorded and elaborated by the Zwick/Roell Master TestXpert software. The average value and the standard deviation of the Young's modulus, the tensile strength, and the maximum deformation were determined on a series of four samples, for each membrane type.

2.3.4. Biomolecules immobilizations

PVDF-DAMP-GA membrane were immersed in a BSA (1 g L^{-1}) or LCR (0.8 g L^{-1}) solution (25 mL; in 50 mM phosphate buffer pH 7) at 25°C for 24 h under gentle stirring. Intense rinsing with buffers at different pH (7 - 5.5 - 8.5) followed, after that the membranes with immobilized BSA (PVDF-DAMP-GA-BSA) or LCR (PVDF-DAMP-GA-LCR) were obtained. The rinsing at different pH values (5.5 or 8.5) was carried out on the membranes functionalized with BSA or LCR in order to remove biomolecule not covalently linked on the surface (e.g. adsorbed by electrostatic interactions). However, no BSA or LCR traces were detected in the washing solutions by UV-Vis analyses, indicating that these macromolecules were immobilized only by covalent bonds.

In order to eliminate unreacted aldehyde groups which can block active site of immobilized LCR, the PVDF-DAMP-GA-LCR membrane was immersed in a glycine solution (25 mL, 1 M, in 50 mM phosphate buffer pH 7) at 25°C for 2 h. Thereafter the membrane was washed with water.

In order to evaluate the quantity of BSA or LCR immobilized on the membrane, the protein concentration in the initial, final, and washing solution was measured by BCA test kit (Sigma). The amount of immobilized protein was determinated by mass balance according to equation 1:

$$C_i V_i = C_f V_f + C_{ws} V_{ws} + m \quad (1)$$

Here, m indicates the immobilized protein mass in the membrane, C and V represent the concentration and volume, respectively; the subscripts i, f and ws indicate the initial, final, and washing solutions, respectively. The immobilized mass was then normalized by the membrane area to estimate the immobilized enzyme amount related to membrane surface.

2.3.5. Lipase Activity measurements

The activity of free lipase was studied by measuring the amount of fatty acids produced by the hydrolysis of triglycerides in a batch reactor. A Mettler DL25 automatic titrator was used. The reaction mixture was formed by 19 mL phosphate buffer 50 mM, 1 mL of olive oil and 1 mL of enzyme solution prepared by dissolving 7 g $_{\text{raw powder}}$/l. The mixture was stirred at 300 rpm by a magnetic stirrer to produce an oil–water interface.

The activity of immobilized lipase with triglycerides was monitored by titration of fatty acid extracted into the aqueous phase in a biphasic membrane bioreactor [19] depicted in Fig. 2.1.

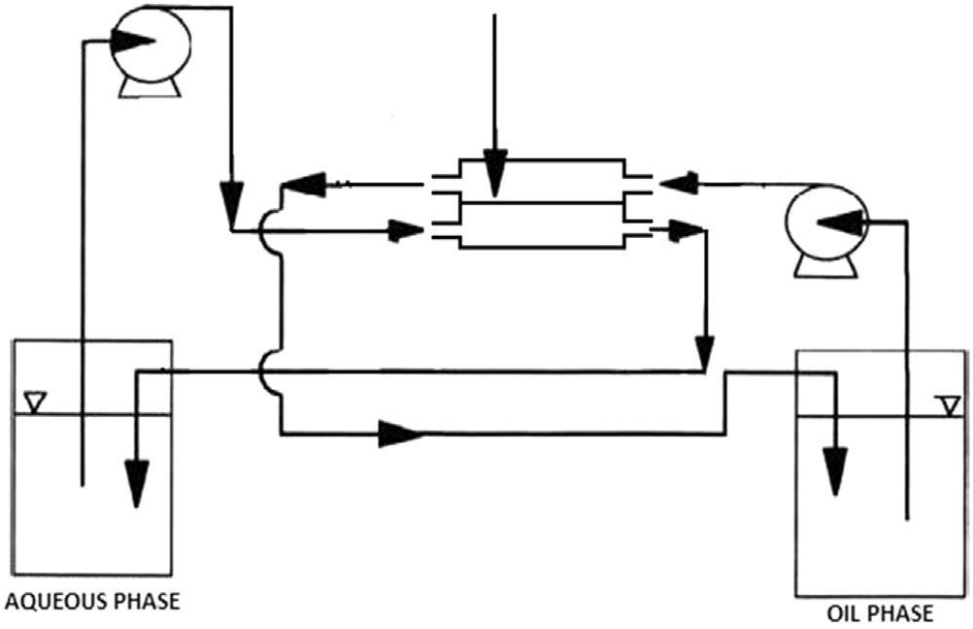


Fig. 2.1. Biphasic membrane bioreactor scheme

It consisted of two compartments with an arrangement for holding the PVDF-DAMP-GA-LCR membrane between them, the effective surface area of the membrane was 9.62 cm^2. The substrate (200 mL of oil) was circulated in the compartment facing the immobilized membrane side. In the other compartment the aqueous phase (200 mL of 50 mM phosphate buffer pH 7) was circulated. The axial flow rate in the two compartments was 200 mL/min,

while the applied pressure from the organic phase was 9 kPa and from the aqueous phase 18 kPa to prevent oil passage through the membrane. The resulting trans-membrane pressure was 9 kPa. Gear pumps were employed to supply the organic and aqueous phases to the reactor. Two control panels, with valves, flow meters and pressure gauges, were used to control, separately, the two circuits. A water bath with a thermostat was used to keep the temperature at 30°C (temperature optimum for the enzyme activity).

In heterogeneous systems the overall reaction mechanism depends on the immobilization site of the enzyme in the microenvironment and the type of mass transfer of the reactants from the bulk solution to the enzyme and vice versa for the products [20].

The mass transfer in biphasic membrane reactor, from and to the biocatalytic membrane microenvironment is due to the diffusion [21].

In this work, the performance of this membrane reactor has been studied on the basis of the "observed specific activity" (e.g., moles of product obtained per hour per gram of enzyme). This parameter depends on mass transport and catalytic activity of the enzyme, which means that fluid properties and hydrodynamics near the interface of the membrane should be taken into account.

2.4. Results and discussion

2.4.1. Preparation of PVDF-DAMP membrane and characterization

In order to graft amino groups on the surface of PVDF membrane DAMP was used in basic environment. The PVDF polymer consists of $-CH_2CF_2-$ groups, but when it is treated with DAMP at very high pH (>10) -CH=CF- groups are formed due to HF elimination. Based on the surface modification mechanism of PVDF in aqueous-alkaline medium, proposed by G.J. Ross et al. [13], a possible mechanism of DAMP grafting on PVDF is

proposed in Fig. 2.2. In the proposed mechanism only the nucleophilic attack of amino group in C5 (the less sterically hindered) it is shown for clarity. However is not possible to exclude the nucleophilic attack of amino group in C1, as well as the reaction of both amino groups forming a bridge between two polymer chains.

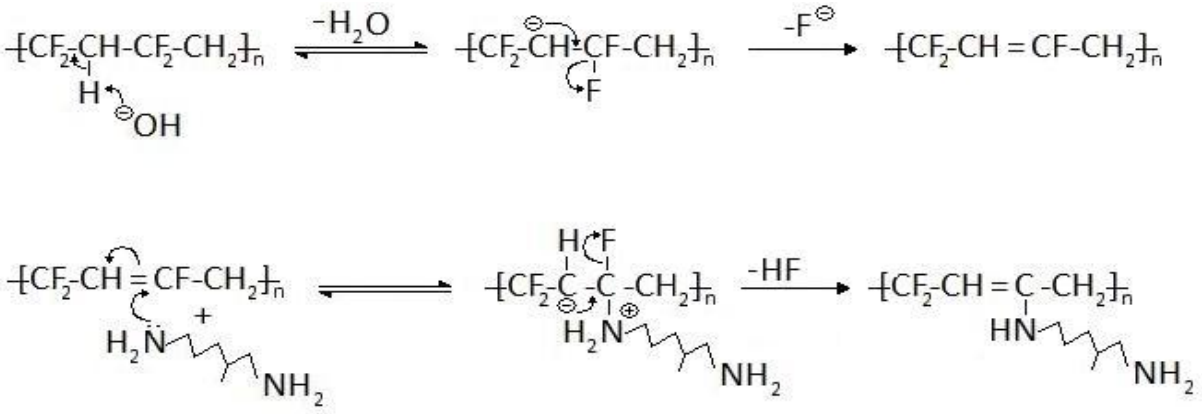


Fig. 2.2. Possible mechanism of alkaline-induced DAMP grafting on PVDF (only the nucleophilic attack of amino-group in C5 is shown for clarity).

In order to graft the DAMP onto PVDF membrane three different parameters were varied: DAMP concentration, reaction temperature and reaction time. A summary of the modification conditions tested (columns A – G) and their characterizations by ninhydrin tests were reported in Table 2.1.

Table 2.1. Operative condition tested for DAMP grafting on PVDF membrane and relative positive or negative results of ninhydrin test

	A	***B***	***C***	***D***	***E***	***F***	***G***
REACTION TEMPERATURE	25	25	25	35	35	50	50
[DAMP] (M)	1	1	2	1	2	2	2
REACTION TIME (h)	12	24	24	24	24	12	24
NINHYDRIN TEST	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE

Based on the ninhydrin test (Table 1) no amino groups were grafted on the PVDF membrane at room temperature (25°C). The test was positive for modification conducted at 35°C and 50°C for all DAMP concentration and reaction times investigated.

Various degrees of modification of PVDF membranes were achieved changing reaction temperature and reaction time. The influence of reaction temperature was evaluated in the range 25-50 °C using DAMP solution 2 M and reaction time 24 hours (Fig. 2.3).

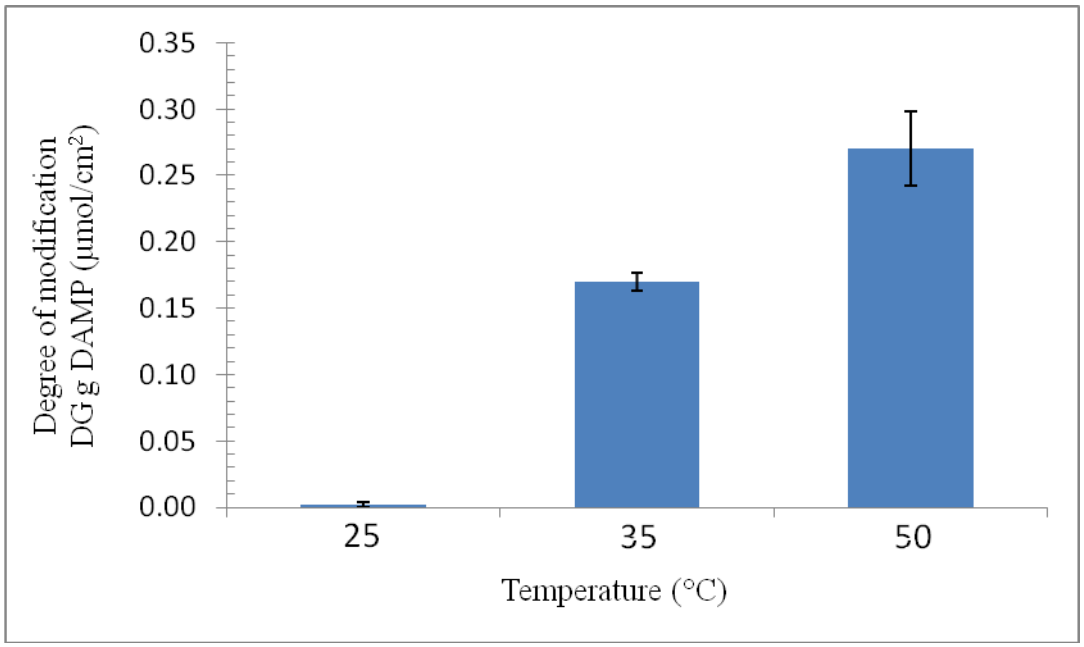


Fig. 2.3. DAMP degree of grafting (DG–DAMP) on PVDF membrane (in terms of mmol of amino groups per membrane cm^2) as function of reaction temperature using DAMP 2M and reaction time 24 hours.

Based on ninhydrin test and FT-IR spectra the functionalization conditions reported in column G (Tab. 1) were chosen for further investigations. The influence of reaction time on the degree of grafting (DG) was quantified keeping the other parameters unchanged (Fig. 2.4).

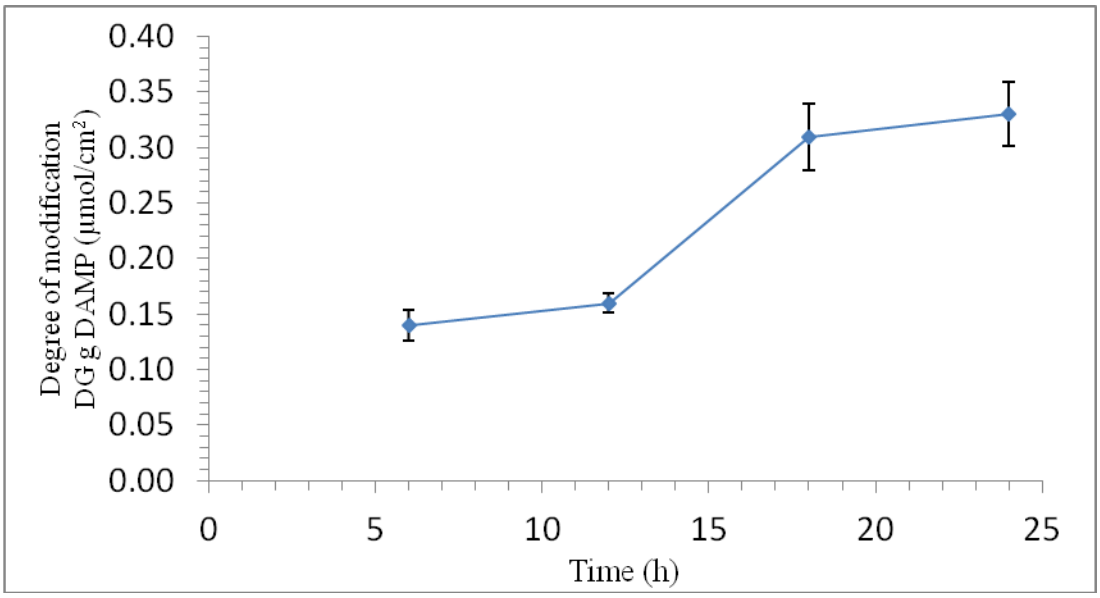


Fig. 2.4. DAMP degree of grafting (DG–DAMP) on PVDF membrane (in terms of mmol of amino groups per membrane cm^2) as function of reaction time using DAMP 2M and reaction temperature 50° C.

The amount of amino groups present on the membrane increases with treatment time and reached a plateau at about 0.3 µmol/cm^2.

Modified membranes were also characterized by FTIR-ATR spectroscopy. An interesting data emerged from ATR-FT-IR analyses, it was the presence of a band at 1739 cm^{-1} due to -CH=CF- groups present in the native PVDF membrane, that were probably formed as defects by HF elimination during the polymer synthesis. After the grafting with DAMP the intensity of this band increased and a new intense IR absorption was detected at 1632 cm^{-1} and assigned to N-H bending.

The FT-IR analysis performed on membranes treated increasing reaction times, showed increased peak intensity (Fig. 2.5), in a good correlation with ninhydrin test showed in Fig. 2.4.

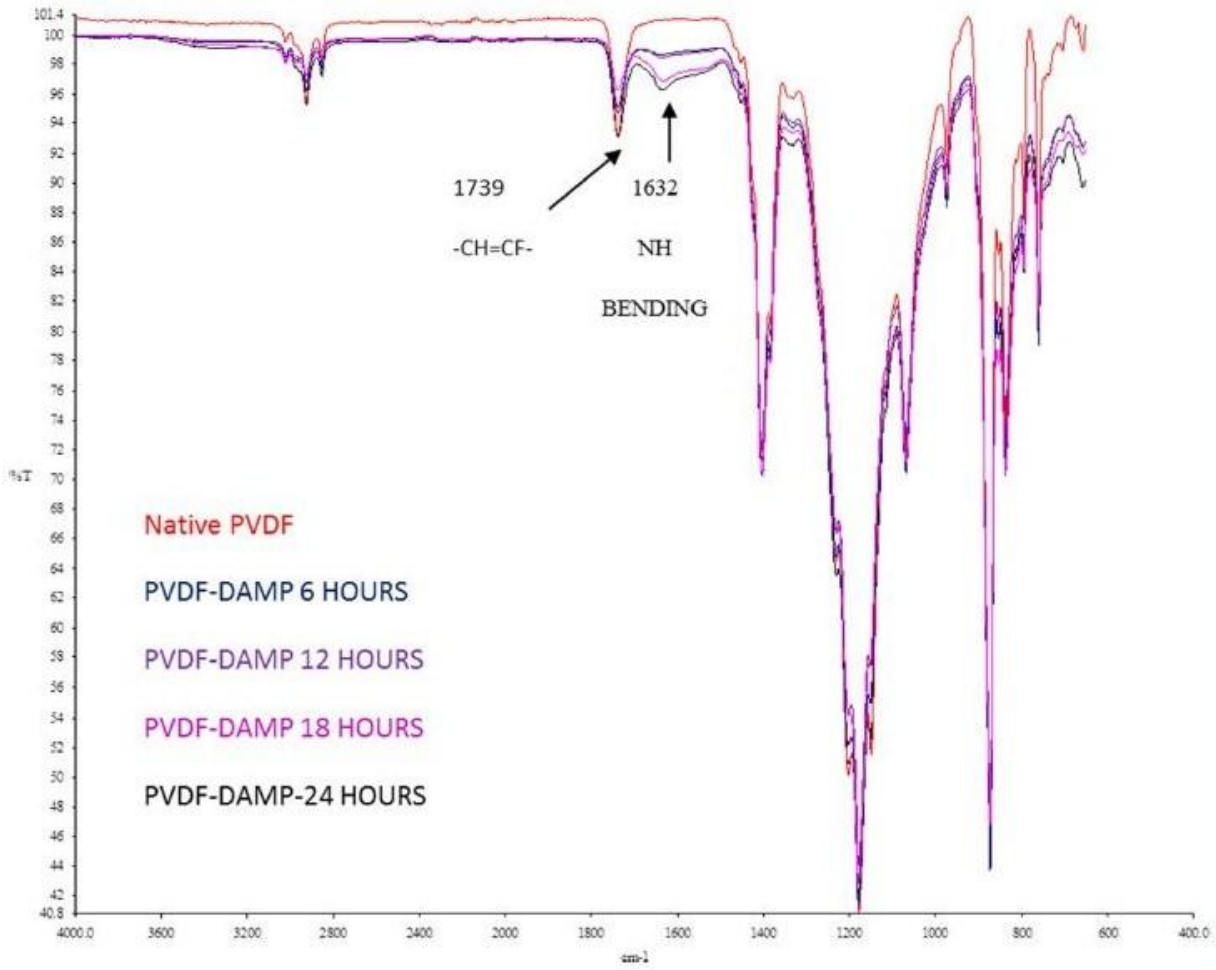


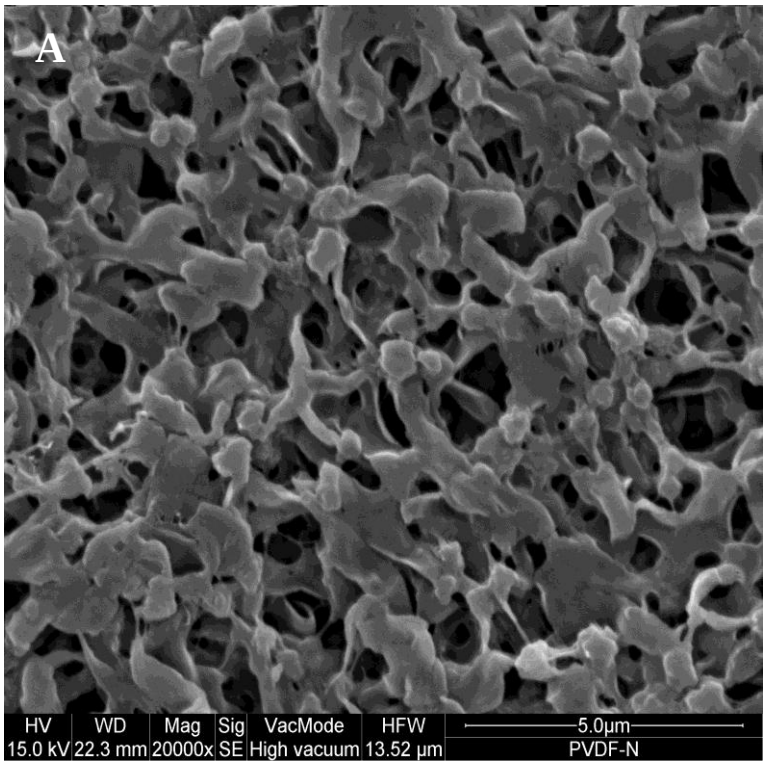
Fig. 2.5. FT-IR spectra : (a) PVDF membranes treated with DAMP 2M at 50° C for different reaction times.

Since the membrane used is composite and our purpose was to modify just the PVDF layer, the PE support was removed from the membrane, by dissolving the PVDF layer in DMA (PVDF is soluble in DMA, while PE is stable in this solvent). The remaining PE support was then treated as previously reported for the membrane, but as expected, from ATR-FT-IR and ninhydrin test no chemical modification were observed. Then the PVDF-DAMP membrane was activated for covalent immobilization of biomolecules, by glutaraldehyde treatment. Glutaraldehyde as well as DAMP increase the distance between membrane surface and biomolecule, thus working as a spacer, they are able to increase the

biomolecule degree of freedom. In the case of the enzyme, allows better interaction with the substrate.

Ninhydrin test was used in order to check the effect of GA concentration (0.1, 1, 5, 10, 20 %) and time contact (2, 12, 24 hours) on coupling of amino groups with GA. A treatment of 2 hours with GA 10 % (v/v) at 25°C was found to be enough to obtain complete coupling of amino groups.

No relevant differences were observed by SEM analyses on membrane morphology before and after functionalization of PVDF membrane (compare Fig. 2.6a with Fig. 2.6b).



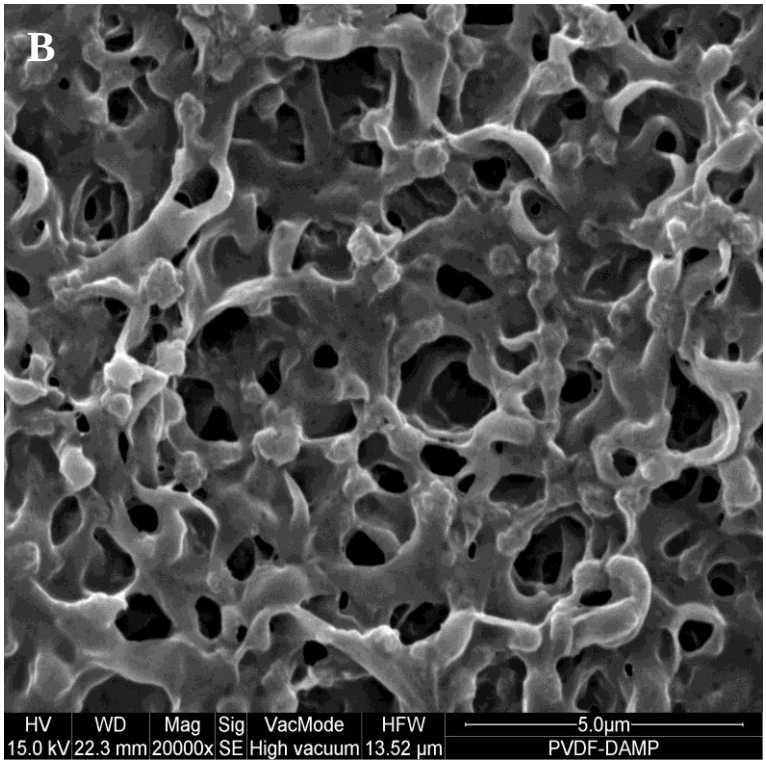


Fig. 2.6. SEM images of native PVDF membrane (A) and PVDF–DAMP (B).

2.4.2. Mechanical properties of modified membrane

The mechanical properties of the membranes were evaluated by stress-strain elongation tests conducted on native and functionalized samples in dry state. The effect of the different functionalization time on the elastic modulus of the membranes is plotted in Fig. 2.7.

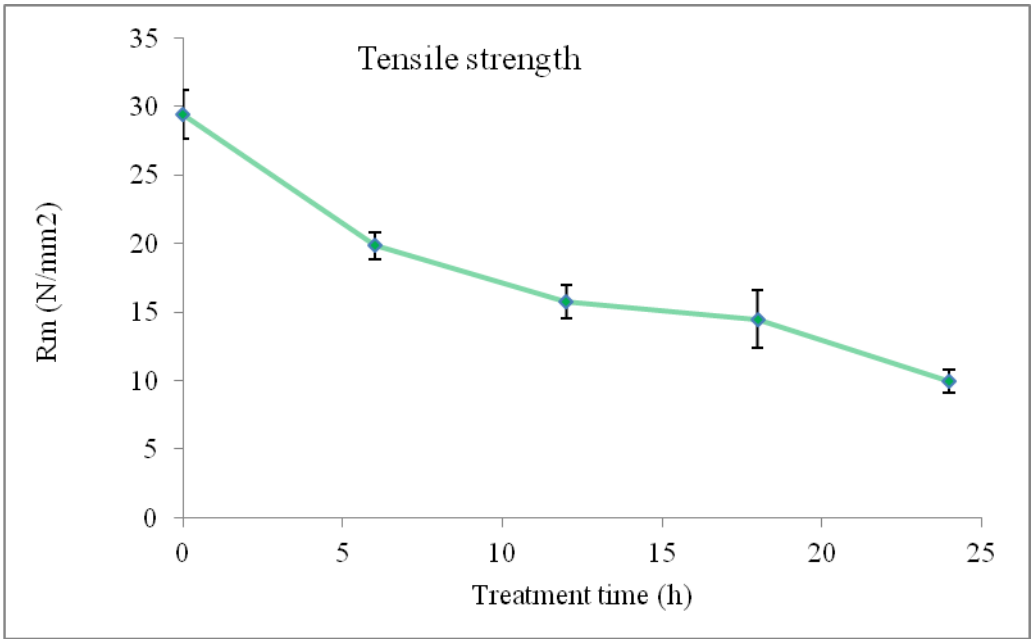


Fig. 2.7. Tensile strength as function of reaction time in the PVDF–DAMP membranes.

As we can appreciate from the graph, the tensile strength of the membrane decreases with the treatment time. As a consequence, an opposite effect of the treatment time on the degree of grafting and mechanical properties of the membrane was observed. In fact, after 6 hours of DAMP treatment a decrease in tensile strength of about 30% was found, in respect to reference membrane. The value increased to 58% with the sample treated at 24 hours. However, going from 6-12 hours to 18-24, the degree of grafting increases of about 50% (see Fig. 2.4). The evaluation of treatment may be identified also on the basis of immobilization efficiency, as discussed in the next sections.

The reduction in the tensile strength with the increasing of the reaction time (Fig. 2.7) was probably due to the chemical damage of the polymer chains occurring after long exposition to highly alkaline chemicals [22, 23]. Moreover, an excessive number of double bonds on the polymer chains can induce an increase of membrane stiffness increasing membrane fragility.

2.4.3. Biomolecule immobilization and enzyme activity

After membrane functionalization the immobilization of biomolecules was carried out. A model protein (BSA) and a model enzyme (LCR) were selected as biomolecules for immobilization experiments. These two proteins have different properties in terms of hydrophilicity/ hydrophobicity, in particular data on 3D structure of lipases indicated that they were fairly hydrophilic and exposed uncharged polar residues in aqueous solution. However this protein also contains an important hydrophobic core located in the catalytic site that is activated in the presence of emulsion interface [24,25]. BSA chemical structure is basically composed of a low content of apolar amino acids and a high content of charged amino acids and the most outstanding property of BSA was the ability to interact with different types of ligands [26]. The study of the immobilization method with these two proteins may allow to verify the influence of hydrophilic/hydrophobic interaction on the loading efficiency.

The amounts of BSA and LCR immobilized onto the PVDF-DAMP-GA and native PVDF (control) membranes are shown in Fig. 2.8, for a contact time DAMP/membrane of 24 hours.

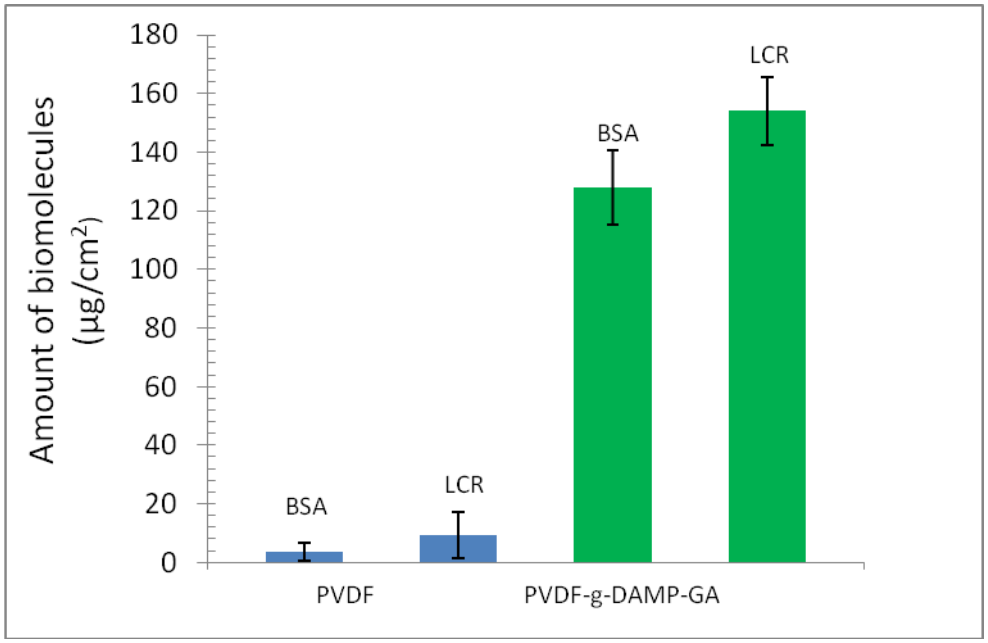


Fig. 2.8. Immobilized amount of BSA and LCR on PVDF and PVDF–DAMP–GA membranes.

The grafting yield of BSA on the surface of the PVDF-DAMP-GA-BSA membrane (128 ± 12.6 µg/cm^2) was about 35 fold higher than for the unmodified membrane. The grafting yield of LCR on the surface of the PVDF-DAMP-GA-LCR membrane (154 ± 11.5 µg/cm^2) was about 18 fold higher than for the unmodified membrane. The different degree of immobilization between the two proteins could be attributed to the difference nature lipophilic/hydrophilic for lipase and prevalently hydrophilic for BSA as well as the different degree of protein aggregation [7]. The degree of lipophilicity/hydrophilicity can influence the protein concentration at the interface membrane/protein solution: the less hydrophilic protein (lipase) will have an higher concentration at the membrane (hydrophobic) interface with respect with the more hydrophilic (BSA), favouring the enzyme loading.

The specific activity of free and immobilized lipase was evaluated in the batch reactor and in the biphasic membrane bioreactor (described in section 2.3.5) respectively. The enzyme activity was reported in terms of units/mg enzyme. One unit is defined as a rate of production of 1 μmol of fatty acid per minute. The specific activity of free lipase was 10 (± 0.98) U/mg.

As previously reported in section 2.4.2 (see also Fig. 2.7), the degree of grafting increases of about 50% if the DAMP/membrane contact time was increased from 12 to 18 h, while the tensile strength have higher decrease. So, in order to check the effect of DAMP/membrane contact on enzyme immobilization/activity the experiment was focused considering 6 and 24 hours.

The amount of immobilized BSA was 87.6 μg/cm^2 and 128 μg/cm^2 after 6 and 24 hours of DAMP/membrane contact time, respectively. Same trend was observed for lipase; in fact 66.3 μg/cm^2 and 154 μg/cm^2 of immobilized enzyme were obtained. However for what concerns lipase activity, after 6 h of time contact between DAMP/membrane 3.68 U/mg was obtained, while 2.30 U/mg was obtained using the longer contact time. In addition, with the first membrane the observed specific activity remains the same after 4 cycles of measurement, for a total time of about 20 days.

The reduction of enzyme activity with the increase of enzyme amount might be explained by enzyme crowding on the surface that could result in the blocking of the active site or protein denaturation [8, 28]. This could cause higher rigidity of the biocatalyst, thereby, inhibiting the ability of the enzyme to bind the substrate. In the work of Kuo et al. [12] same trend was obtained about the relationship between amount of immobilized enzyme/specific activity, although the lipase had different degree of purity and was used with a different substrate.

A good compromise between observed specific activity, amount of immobilized enzyme and membrane stability was obtained by using a contact time DAMP/membrane of 6h.

2.5. Conclusions

In this study a method to functionalize hydrophobic PVDF membrane was developed. Although PVDF is a chemically inert material, the possibility of using it as support for covalent biomolecules immobilization after appropriate treatments has been demonstrated. Parameters such reaction temperature, time of treatment and DAMP concentration have been tuned to control the total degree of modification. The mechanical properties of the membranes were reduced proportionally to the increase of the degree of modification. A suitable compromise between membrane functionalization and biocatalytic efficiency has been identified.

The observed specific activity of lipase immobilized on PVDF was about 40% of the activity of free lipase which is quite good considering that this observed value is strongly influenced by mass transfer properties and therefore underestimated.

References

[1] K.K. Sirkar, P.V. Shanbhag, A.S. Kovvali, Membrane in a reactor: a functional perspective, Ind. Eng. Chem. Res. 38 (1999) 3715–3737.

[2] J. Yuan, J. Zhang, X. Zang, J. Shen, S. Lin, Improvement of blood compatibility on cellulose membrane surface by grafting betaines, Colloids Surf. 30 (2003) 147–155.

[3] V.G. Gavalas, N.A. Chaniotakis, Lactate biosensor based on the adsorption of polyelectrolyte stabilized lactate oxidase into porous conductive carbon, Mikrochim. Acta 136 (2001) 211–215.

[4] L. Giorno, E. Drioli, Biocatalytic membrane reactors: applications and perspectives, Trends Biotechnol. 18 (2000) 339–349.

[5] V.C. Gekas, Artificial membranes as carriers for the immobilization of biocatalysts, Enzym. Microb. Technol. 8 (1986) 450–460.

[6] N. Fu Liu, H. Awanis, L:Yutie, M.R. Moghareh Abed, K. Li, Progress in the production

and modification of PVDF membranes, J. Membr. Sci. 375 (2011) 1–27

[7] P. Jochems, Y. Satyawali, L. Diels, W. Dejonghe, Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs), Green Chem. 13 (2011) 1609

[8] G. Kang , Y. Cao, Application and modification of poly (vinylidene fluoride) (PVDF) membranes – A review Journal of Membrane Science 463 (2014) 145–165

[9] G.J. Chen, C.H. Kuo, C.I. Chen, C.C. Yu, C.J. Shieh and Y.C. Liu, Effect of membranes with various hydrophobic/hydrophilic properties on lipase immobilizer activity and stability, Journal of bioscience and Bioengineering 113 (2012) 166-172

[10] S. Gupta, A. Bhattacharya, C.N. Murthy, Tune to immobilized lipases on polymer membranes: techniques, factors and prospects, Biocatalysis and Agricultural Biotechnology 2 (2013)
171–190

[11] L. P. Zhu, J. Z. Yu, Y.Y. Xu, Z.Y. Xi, B.K. Zhu, Surface modification of PVDF porous membranes via poly(DOPA) coating and heparin immobilization, Colloid Surf. B 69 (2009) 152−155

[12] C.H. Kuo, G.J. Chen, Y.K. Twu, Y.C. Liu, C.J. Shieh, Optimum Lipase immobilized on diamine-grafted PVDF membrane and its characterization, Ind. Eng. Chem. Res 51 (2012) 5141−5147

[13] G.J. Ross, J.F. Watts, M.P. Hill, P. Morrissey, Surface modification of poly (vinylidene fluoride) by alkaline treatment: 1. The degradation mechanism, Polymer 41 (2000) 1685–1696

[14] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: behaviour in aqueous solution, reaction with proteins and application to enzyme crosslinking, Biotechniques. 37 (2004) 790-802.

[15] D.H. Lee, C.H. Park, J.M. Yeo, S.W. Kim, Lipase Immobilization on Silica Gel Using a Cross-linking Method, J. Ind. Eng. Chem. 5 (2006) 777-782.

[16] A. A. Mendes, R. C. Giordano, R. D. L. C. Giordano, H. F. De Castro, Immobilization and stabilization of microbial lipases by multipoint covalent attachment on aldehyde-resin affinity: Application of the biocatalysts in biodiesel synthesis, J. Mol. Catal. B: Enzym. 68 (2011) 109−115.

[17] R. Mazzei, G. Vitola, E. Fontananova, L. Giorno, "Immobilization of enzyme on polymeric membranes by molecular recognition", 29th EMS Summer School, Essen, Germany, 22nd-26th July 2013.

[18] S. W. Sun, Y. C. Lin, Y. C. Weng, M. J. Chen, M. J. Efficiency improvements on ninhydrin method for amino acid quantification, Journal of Food Composition and Analysis 19 (2006) 112–117

[19] L. Giorno, E. D'Amore, R. Mazzei , E. Piacentini , J. Zhang , E. Drioli , R. Cassano , N. Picci, An innovative approach to improve the performance of a two separate phase enzyme membrane reactor by immobilizing lipase in presence of emulsion Journal of Membrane Science 295 (2007) 5–101

[20] J. Zhang, G. Barbieri, F. Scura, L. Giorno, E. Drioli, Modeling of two separate phase enzyme membrane reactors for kinetic resolution of naproxen ester, Desalination 200 (2006) 514–515

[21] L. Giorno, R. Molinari, M. Natoli, E. Drioli, Hydrolysis and regioselective transesterification catalyzed by immobilized lipases in membrane bioreactors, Journal of Membrane Science (1997) 177–187

[22] V. Puspitasari, A. Granville, P. Le-Clech, V. Chen, Cleaning and ageing effect of sodium hypochlorite on polyvinylidene fluoride (PVDF) membrane, Separation and Purification Technology 72 (2010) 301–308

[23] N. A. Hashim, Y. Liu, K. Li, Stability of PVDF hollow fibre membranes in sodium hydroxide aqueous solution, Chem. Eng. Sci. 66 (2011) 1565–1575

[24] P. Reisa, K. Holmberg, H. Watzke, M.E. Leser, R. Millerc, Lipases at interfaces: a review, Adv. Colloid Interface Sci. 147–148 (2009) 237–250.

[25] P. Grochulski, Y.G .Li, J.D. Schrag, F. Bouthillier P. Smith, D. Harrison, B Rubin, M. Cygler, Insights into interfacial activation from an open structure of Candida rugosa lipase, J. Biol. Chem. 268 (1993) 12843–12847.

[26] J.R. Brown, P. Shockley, Serum albumin: structure and characterization of its ligand binding sites, in: P.C. Jost, O.H. Griffith (Eds.), Lipid–Protein Interactions, John Wiley, NewYork, 1982, pp. 25–68.

[27] Y. Wang, J. Zhang, J. Yin, Progress of enzyme immobilization and its potential application, Desalin. Water Treat. 1 (2009) 157–171.

Chapter 3

Biofunctionalization of PES membrane

3.1. Introduction

Polyethersulfone (PES) is a polymer that shows excellent oxidative, thermal and hydrolytic stabilities as well as good mechanical and film-forming properties. Owing to these factors, membranes based on PES are widely employed in processes such as microfiltration (MF) and ultrafiltration (UF) [1]. However, the disadvantage for applying PES is their intrinsic hydrophobicity, which increases membrane fouling in MF and UF processes [2]. The low wettability of PES membrane can cause troubles also in more sophisticated applications such as biomolecules immobilization for production of bio-hybrid membrane. In addition the presence of binding site is required to immobilize covalently the biomolecules on PES. Owing to these reasons, commercial membrane made of a non standard grade (NSG-PES) functionalized with a positively charged polyelectrolyte layer bringing free amino groups was selected as hydrophilic membrane provided of functional groups suitable for biomolecules immobilization. The model biomolecules (the enzyme Lipase and the protein BSA) were covalently immobilized on the membrane after glutaraldehyde activation of the amino groups. The pristine and glutaraldehyde-activated membranes were characterized by static water contact angle measurements and ninhydrin test. The new bio-hybrid membranes were characterized in terms of binding efficiency and catalytic performances of immobilized lipase.

3.2. Experimental

3.2.1 Materials and chemicals.

Lipase from *Candida rugosa* (E.C.3.1.1.3, 65 kDa), BSA protein (65 kDa), ninhydrin, ethanol, glycine, BCA test kit and glutaraldehyde were purchased from Sigma.

The NSG-PES membranes were supplied by GVS Spa (Italy) and have a pore size equal to 0.2 µm, a thickness equal to 110 (± 10) µm and bubble point higher than 4.3 bar.

3.2.2. Instrumentation

Static water contact angle (SCA) measurements were performed using a CAM 200 device (KSV Instruments, Ltd.). A drop of water (5 µL) was injected using a micro-syringe with automatic dispenser onto the membrane surface. Data for 4 drops on different locations were averaged to obtain the contact angle of each membrane sample. Colorimetric tests were performed using an UV spectrophotometer Perkin-Elmer Lambda EZ 201.

3.2.3. Surface modification of NSG-PES membrane.

The NSG-PES membrane brings free amino groups which can be easily converted in reactive groups suitable for enzyme immobilization. The NSG-PES membrane cut into disk (Ø 47 mm) was treated with glutaraldehyde solution at 25°C. Various glutaraldehyde concentration (1-5-10% v/v) were tested for one or two hours in order to obtain a total coupling of amino groups and generate reactive aldehyde pendant groups onto the membrane (NSG-PES-GA). After that the membrane was thoroughly washed with distilled water and stored at 4°C.

3.3. Characterization of membrane

3.3.1. Chemical physical characterizations

The content of the amino groups on the NSG-PES membranes was determined by the ninhydrin method [3], using glycine as standard. The membranes were cut in small pieces (0.25 cm^2) and reacted with 1 mL of ninhydrin reagent in a tube heated in boiling water bath. Thereafter the reactants were cooled in a cold water bath and diluted with ethanol in a ratio 1:1 (v/v), measuring the absorbance at 570 nm. The wettability of the membranes was investigated by SCA measurement.

3.3.2. Biomolecules immobilizations

NSG-PES-GA membrane were immersed in a BSA (1 g L^{-1}) or LCR (0.8 g L^{-1}) solution (25 mL; in 50 mM phosphate buffer pH 7) at 25°C for 24 h under gentle stirring. Rinsing with buffers at different pH (7-5.5-8.5) followed, after that the membranes with immobilized BSA (NSG-PES-GA-BSA) or LCR (NSG-PES-GA-LCR) were obtained.

In order to evaluate the quantity of BSA or LCR immobilized on the membrane, the protein concentration in the initial, final, and washing solutions was measured by BCA test kit. The amount of immobilized biomolecules was determinated by mass balance according to equation 1 reported in chapter 2. The immobilized mass was then normalized by the membrane area to estimate the immobilized biomolecules amount related to membrane surface.

3.3.4. Lipase Activity measurements

The activity of free lipase and immobilized LCR was measured using olive oil as substrate. The protocols used have been already reported in section 2.3.5 (chapter 2). Briefly the

NSG-PES-GA-LCR membrane was placed in the two compartments cell of the biphasic membrane bioreactor depicted in figure 2.1. The organic phase (oil, 200 mL) was circulated in the compartment facing the immobilized membrane side, while the water phase (200 mL of 50 mM phosphate buffer pH 7) was circulated in the other compartment.

3.4. Results and discussion

3.4.1. Preparation of NSG-PES-GA membrane and characterization

The amount of primary and secondary amino groups present on the NSG-PES membrane was quantified by ninhydrin method. The amino groups on the NSG-PES membrane were reacted with ninhydrin to form a purple-coloured compound (Ruhemann's purple) in solution, the intensity of this reaction solution was proportional to the amino groups amount. The amino content of the NSG-PES membrane was 0.1 ± 0.015 μmol/cm^2. Afterwards the NSG-PES membrane was activated for BSA and LCR immobilization by GA treatment. The ninhydrin test was used in order to check the effect of GA concentration (1, 5, 10 %) and time contact (1-2 hours) on quenching of amino groups by GA, with the production in the case of positive results of a colourless solution, see Table 3.1. After a treatment of 2 hours with GA 5% (v/v), amino groups were not detected by ninhydrin method, this means that it is enough to obtain complete coupling of amino groups.

Table 3.1: Condition tested for GA of NSG-PES membrane and relative positive or negative ninhydrin test

Membrane	GA Concentration (% v/v)	Treatment time (h)	Ninhydrin test
NSG-PES	1	1	Positive
	5	1	Negative
	10	1	Negative
	1	2	Positive
	5	2	Negative
	10	2	Negative

The wettability of NSG-PES membrane wasn't affected by GA treatment, in fact for the NSG-PES membrane a contact angle of 33° ± 1 was detected whereas for the NSG-PES-GA membrane a contact angle of 36° ± 3 was detected. Therefore the NSG-PES membrane before and after GA treatment are highly hydrophilic membrane.

3.4.2. BSA and LCR immobilizations

Proteins with different structural properties and similar molecular weight, such as Lipase and BSA, were selected as models for immobilization on the NSG-PES-GA membranes. The 3D structure of lipases indicated that they expose uncharged polar residues in aqueous solution [4], whereas BSA chemical structure is basically composed of a low content of apolar aminoacids and a high content of charged aminoacids [5].

The amounts of BSA and LCR immobilized onto the NSG-PES-GA membranes are shown in Fig. 3.1.

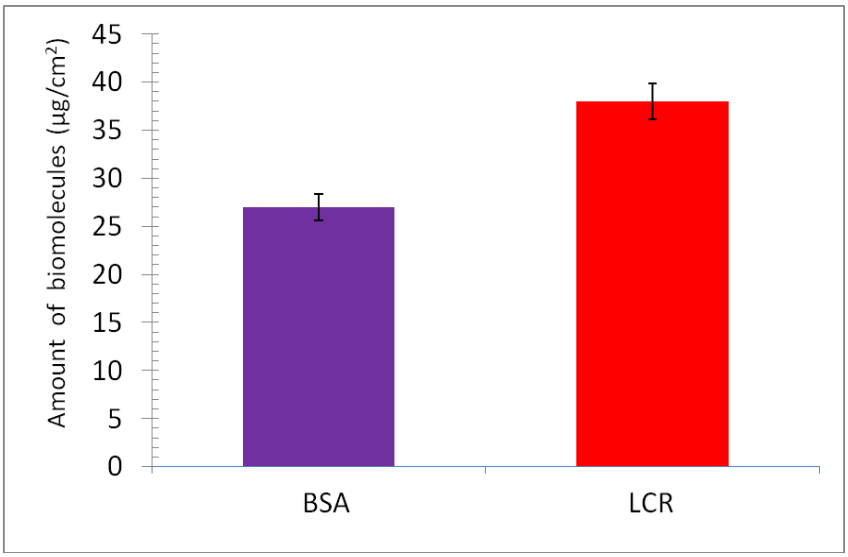


Fig. 3.1 Amounts of BSA and LCR immobilized on NSG-PES-GA membranes

The difference in terms of total immobilized amount between Lipase (38 ± 2 µg/cm^2) and BSA (27 ± 2 µg/cm^2) was not significantly high on NSG-PES-GA membranes in good agreement with the finding on PVDF-DAMP-GA membrane (Fig. 2.8). However, compared to BSA, in both case LCR is more prone to be immobilized on the membrane. This could be explained considering the chemical structure of lipase characterized by uncharged polar and apolar residues that reduced the interaction with water molecules. This aspect can influence the protein concentration at the interface membrane/protein solution: the more hydrophobic protein (lipase) will have a higher concentration at the membrane interface with respect with the more hydrophilic (BSA). A higher concentration of the lipase at the interface is expected to favour the lipase immobilization.

3.4.3. LCR activity assays

The most important characteristic of lipases is the activation at oil–water interface (interfacial activation). The specific activity of free and immobilized lipase was evaluated

in the batch reactor and in the biphasic membrane bioreactor (described in section 2.3.5, chapter 2) respectively. The specific activity of free lipase was 10 (± 0.98) U/mg, while the specific activity of lipase immobilized on NSG-PES-GA was 6 ± 0.6 U/mg. Therefore the retained specific activity of lipase immobilized on NSG-PES-GA was higher than lipase immobilized on PVDF-DAMP-GA membrane. This could be due to the lower amount of lipase immobilized on the NSG-PES-GA as a consequence of low reactive groups present on the modified PES membrane compared to the modified PVDF membrane.

3.5. Conclusions

In this study a non standard grade polyethersulfone membrane (NSG-PES) functionalized with a positively charged polyelectrolyte layer bringing free amino groups were used as support for immobilization of model biomolecules. This membrane was selected on the base of its hydrophilicity and reactive surface which is provided of sites for immobilization of biomolecules. The model biomolecules BSA and lipase were covalently immobilized on the membrane after activation of amino groups by glutaraldehyde reaction. The conditions for BSA and lipase immobilization were studied as well as the conditions for glutaraldehyde reaction. Afterword the specific activity of immobilized lipase was evaluated in a biphasic membrane reactor. The observed specific activity of lipase immobilized on NSG-PES-GA was about 60% of the activity of free lipase.

References

[1] B. Van der Bruggen. Chemical Modification of Polyethersulfone Nanofiltration Membranes: A Review Journal of Applied Polymer Science. 114 (2009) 630–642

[2] J. Pieracci, J.V. Crivello, G. Belfort, Photochemical modification of 10 kDa polyethersulfone ultrafiltration membranes for reduction of biofouling, J. Membr. Sci. 156 (1999) 223-240.

[3] S.W. Sun, Y.C. Lin, Y.C. Weng, M.J. Chen. Efficiency improvements on ninhydrin method for amino acid quantification. J. Food Compos. Anal. 19 (2006) 112–117.

[4] P. Grochulski, Y.G. Li, J.D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, M. Cygler, Insights into interfacial activation from an open structure of Candida rugosa lipase, J. Biol. Chem. 268 (1993) 12843–12847.

[5] J.R. Brown, P. Shockley, Serum albumin: structure and characterization of its ligand binding sites, in: P.C. Jost, O.H. Griffith (Eds.), Lipid–Protein Interactions, John Wiley, NewYork, 1982, pp. 25–68.

Chapter 4

Phosphotriesterase immobilization on polymeric membranes

4.1. Introduction

Organophosphates triesters (OPs) are a class of toxic compounds that have been employed in the agricultural industry as pesticides and insecticides; the warfare nerve agents (sarin, soman and VX) are also OPs [1]. The mechanism of action of these compounds is realized by the irreversible inhibition of the enzyme acetylcholinesterase, a key enzyme of the nervous system, resulting in accumulation of acetylcholine which interferes with muscular responses leading to death.

OPs poisoning may occur through: 1) consumption of liquids or foods contaminated with nerve agents, 2) contact with contaminated surfaces 3) terrorist acts or industrial accidents. Therefore, there is a strong necessity of different strategies-systems for OPs treatment and a growing public concern regarding the protection from these substances.

Owing to the high costs or environmental concerns of the methods used to destroy OPs (bleach treatment and incineration), alternative methods like enzymatic detoxification has become of great interest [2] also for the *in situ* monitoring and destroy of the OPs.

Bacterial phosphotriesterase (PTE) are appealing due to their broad substrate specificity and high catalytic rate [3], but their stability in solution is low [4]. It is very well known that immobilization of enzyme gives the possibility to improve the catalytic stability of the biocatalyst. Membranes are the optimal support for the PTE immobilization because they can mimic native microenvironment [5, 6].

In this work a single mutant of a Phosphotriesterase-like lactonase (PLL) from *Sulfolobus solfataricus*, a hyperthermophilic archaeal, has been immobilized on polymeric membranes and characterized in terms of catalytic activity and long term stability. The enzyme is a single mutant obtained by combining different approaches of protein engineering named *Sso*Pox W263F [7]. To our best knowledge, this is the first study reported on the

development of immobilized thermostable PLLs (*Sso*Pox W263F) and the characterization of the biocatalytic membrane system for organophosphates degradation. The exceptional intrinsic thermal stability of this enzyme is a property of valuable interest for applications in any detoxification system, in fact it is well known that a high thermal stability involves a high resistance to harsh condition (solvents, detergents, etc.) and long time stability. In order to produce systems for detoxification of OPs contained in water or air, the *Sso*Pox W263F was immobilized on membranes having different properties in terms of wettability. For this reason, the effect of membranes with various hydrophobic-hydrophilic properties on *Sso*Pox W263F immobilized activity and stability was investigated. Polyethersulfone (PES) and polyvinylidene fluoride (PVDF) were selected as hydrophobic and hydrophilic membranes, respectively. Membranes based on PES and PVDF are both widely used for micro and ultrafiltration because they exhibit high mechanical strength, good chemical resistance and thermal stability as well as excellent aging resistance. Furthermore, these materials are suitable for the construction of filters and masks.

The catalytic properties of free and immobilized *Sso*Pox W263F were evaluated by using a model organophosphate, the pesticide paraoxon, dissolved in water. Results showed that immobilized *Sso*Pox W263F retained catalytic activity and that biocatalytic membrane systems performance can be tuned on the basis of immobilized enzyme amount and hydrophilicity of membrane support.

4.2. Materials and methods

4.2.1 Chemicals

PTE enzyme was the mutant *Sso*Pox W263F from *Sulfolobus solfataricus* (more details are given in a following section). The pesticide ethyl paraoxon, 4-nitrophenol, hepes

sodium salt, trizma base, glutaraldehyde and 1,5-diamino-2-methylpentane were purchased from Sigma-Aldrich. BCA protein assay kit (Thermo Scientific) was used to evaluate protein solutions concentration [8].

4.2.2 Membrane synthesis and characterization

As support for *Sso*Pox W263F immobilization two different flat sheet microfiltration membranes were used: one hydrophilic and non supported membrane based on a non standard grade PES membrane functionalized with a positively charged polyelectrolyte layer (NSG-PES, GVS) and one hydrophobic membrane based on polyvinylidene fluoride (PVDF) supported by a polyester non-woven. The membranes were prepared by GVS Spa (Italy) by using a non-solvent-induced phase inversion (NIPS) process.

The NSG-PES membrane (GVS code M08A0020C) has a pore size equal to 0.2 µm, a thickness equal to 110 (± 10) µm and bubble point higher than 4.3 bar.

The PVDF membrane used in this study (GVS code M09G0020) was prepared by casting a PVDF solution on a non-woven polyester support. It is characterised by a nominal pore size equal to 0.2 µm, an air flow ranging from 2.10 to 5.40 l/min (at 1 bar, on a 1 cm^2 test sample, and T=25°C), a Water Break Through higher than 1.8 bar, a hydrorepellency grade higher than 9.5 according the DuPont Teflon Test standard, an oleorepellency grade higher than 3.5 according to the procedure described in AATCC118-2002, and a thickness ranging from 150 to 200 µm.

4.2.3. Membranes biofunctionalization and surface activation procedure

The NSG-PES membrane brings free amino groups which can be easily converted in reactive groups suitable for enzyme immobilization. The NSG-PES membrane cut into disk (Ø 47 mm) was treated with 5% v/v glutaraldehyde solution at 25°C to obtain a total

coupling of amino groups and generates reactive aldehyde pendant groups onto the membrane (NSG-PES-GA). After that the membrane was thoroughly washed with distilled water and stored at 4°C.

The PVDF membrane cut into disk (Ø 47 mm) was treated for 6 hours at 50°C with a 2 molar solution of 1,5-diamino-2-methylpentane (DAMP) in order to introduce onto the PVDF surface amino groups (PVDF-DAMP). Detailed description of the procedure has been already reported in chapter 2. The PVDF-DAMP membrane described above was then treated with 20 mL of 10% (v/v) glutaraldehyde solution at 25 °C for 2 hours so the amino groups were derivatized with glutaraldehyde (PVDF-DAMP-GA), after that the membrane was washed with distilled water and stored at 4°C until used.

4.2.4. Characterization of modified PVDF and NSG-PES membranes

FT-IR spectra were recorded using the Spectrum One System by Perkin-Elmer Instruments in the range of 4000–650 cm^{-1}. Colorimetric tests were performed using an UV spectrophotometer Perkin-Elmer Lambda EZ201. The wettability of the membranes was investigated by static water contact angle (SCA) measurement using a CAM 200 device (KSV Instruments, Ltd.). A drop of water (5 μL) was injected using a micro-syringe with automatic dispenser onto the membrane surface. Data for 4 drops on different locations were averaged to obtain the contact angle of each membrane sample. Samples for scanning electron microscopy (SEM) were analyzed using an EVO MA 10, Zeiss, Assing.

The mean pore diameters was measured by a capillary flow porometer (PMI, Porous Materials Inc. Ithaca, NY) using as wetting liquid 3 M™ Fluorinert™ Electronic Liquid FC-40.

Pure water permeation test on PVDF membranes were performed using a SteriltechTM HP4570 dead-end stirred cell having an active membrane area of 14.6 cm^2, pressurized by air. After about two hours of stabilization, permeate water was collected at regular time intervals in order to determine the flux (J) and Permeance (P_e) as reported in the following equations:

$$J_w = \frac{\mathrm{Q}}{\mathrm{A}} \quad (1)$$

$$P_e = \frac{\mathrm{Jw}}{\mathrm{TMP}} \quad (2)$$

Where J_w is the pure water flux (L $h^{-1}m^{-2}$), Q is the water flow rate permeated (L h^{-1}), A is the effective membrane area (m^2), and TMP (bar) is the trans-membrane pressure difference.

Pure water flux measurements on NSG-PES membranes were performed at a TMP within a range of 0.02-0.1 bar using a cross-flow filtration cells with 13.45 cm^2 active membrane area.

The water vapour permeation tests were performed by the "cup method" [9]. This method consists of a disk of membrane (0.003 m^2), sealed by a ring on a container containing 150 mL of ultrapure water. The space between the water level and the membrane was about 1 cm. The container was placed in a humidity-controlled chamber (humidity 30%, temperature 40°C), the atmosphere inside the chamber was ventilated. The transmembrane pressure was estimated from the difference of water vapour pressure between the cell (RH 100%) and the external chamber (RH 30%) at 40 °C. The flux of water vapour through the membrane was determined by the rate of weight decrease of the container. An experiment took about 30 h and the weight reduction was determined each hour.

4.2.5 SsoPox W263F production and purification

The pT7–7ssopox mutant constructs were transformed in E. coli BL21(DE3) cells to express the protein of interest, and all the procedures were carried out as reported previously [4, 7]. Briefly, wet frozen cells (40 g) were thawed and resuspended in 120 ml of buffer A (20 mM Hepes pH 8.5, 0.2 mM $CoCl_2$). Cells were broken by French pressure cell disruption (Aminco Co., Silver Spring, MD, USA), and cell debris was removed by centrifugation (80,000g, 20 min, 4 °C). After dilution with buffer A, Escherichia coli proteins were partially removed by incubating the crude extract at 60, 70, and 80 °C for 15 minutes under gentle stirring and with clarification, between each incubation, by centrifugation. Pellets were discarded. The enzyme solution obtained at 80°C was directly loaded onto a Q Sepharose Fast Flow FPLC column (Pharmacia) equilibrated with buffer A. After washing, a linear gradient of NaCl (0–0.5 M) was applied. The fractions with paraoxonase activity were pooled, concentrated and loaded on the High Load 16/60 Superdex 75 column (Pharmacia, Sweden) using run over an FPLC apparatus (Pharmacia, Sweden). The column was equilibrated and eluted with 20 mM Hepes buffer (pH 8.5) containing 0.2 mM $CoCl_2$ and 0.2 M NaCl. The semi purified fractions (about 30% pure) with paraoxonase activity were pooled and stored at 4°C or lyophilized and stored at -20°C.

4.2.6 SsoPox W263F immobilization

The modified PVDF or NSG-PES membranes were immersed in the enzyme (0.8 g L^{-1}) solution (9 mL, in 20 mM hepes buffer pH 8.5) at 25°C for 24 hours under gentle stirring. Rinsing with buffers at different pH (5.5-7-8.5) was followed, after that the membranes with immobilized *Sso*Pox W263F (NSG-PES-GA-PTE or PVDF-DAMP-GA-PTE) were

obtained. The rinsing at different pH is useful in order to remove eventual traces of enzyme not covalently or irreversibly linked on the surface (e.g. adsorbed by electrostatic interaction). Control test confirmed that no *Sso*Pox W263F traces were detected in the washing solution by BCA test kit (Thermo Scientific), indicating that the enzyme was irreversibly immobilized.

In order to evaluate the quantity of *Sso*Pox W263F immobilized on the membranes, protein concentration in the initial and final solution was measured by BCA test. The amount of immobilized protein was determinated by mass balance according to the equation 2.1. The immobilized mass was then normalized by the membrane area to estimate the immobilized enzyme amount related to membrane surface.

4.2.7 Phosphotriesterase activity

*Sso*Pox W263F was produced and stocked in lyophilized form; when activity tests were performed, it was suspended in buffer solution: 20 mM hepes buffer pH 8.5 and 0.2 mM $CoCl_2$. The enzyme concentration was 0.8 g L^{-1}. The pesticide paraoxon was used as substrate at concentration of 10^{-3} M. The activity was measured by monitoring the appearance of 4-nitrophenol at 405 nm, in 1-cm path-length cell of 3 ml with a spectrophotometer equipped with a thermo-jacketed cell holder at 25 °C. The molar absorption coefficient used for 4-nitrophenol, estimated at 25 °C and pH 8.5, was 19920 M^{-1} cm^{-1}. One unit (U) of enzyme activity is defined as the amount of enzyme which liberates 1 µmol of 4-nitrophenol per minute under assay conditions.

Immobilized *Sso*Pox W263F was assayed in a similar manner. A strip of *Sso*Pox W263F - loaded membrane (4 cm^2) was placed in a cell containing 3 mL of 10^{-3} M paraoxon in buffer. Cell was then placed into the spectrophotometer thermostated holder cell and the absorbance at 405 nm was recorded. Since here the enzyme is not suspended in solution,

but it is heterogenized on the membrane (which is placed along the cell wall in order to do not interfere with the light beam), the mass transfer will affect the 4-nitrophenol absorbance *vs* time response. Therefore, observed enzyme activity of immobilized enzyme will be underestimated.

4.2.8 Immunoelectron Microscopy

For indirect immunolabeling, biocatalytic membrane samples were embedded in Araldite (Fluka, Buchs, Switzerland) and placed in a 60 °C oven for 72 hours to polymerize. Ultrathin sections were prepared using a diamond knife and collected on Formvar carbon-coated nickel grids. Sections were then incubated with a rabbit monoclonal antibody in PBS 0.1% BSA at 4 °C overnight. The grids were then washed in three drops of PBS for a total of 15 min, transferred to 5 µL drops of secondary antibody conjugated to 10 nm gold particles (Goat-anti-Rabbit IgG, Aurion) for 1 h at room temperature. After immunolabeling, sections (80 nm) were washed with PBS and distilled water, dried, and examined with a Jeol JEM 1400 Plus electron microscope at 80 kV.

4.3. Results and discussion

In the following section results concerning membrane functionalization and characterization, enzyme immobilization, stability and catalytic performance as a function of enzyme amount and membrane type will be illustrated and discussed.

4.3.1. Activation and characterization of NSG-PES membrane

The amount of primary and secondary amino groups present on the NSG-PES membrane was quantified by ninhydrin method as already described in section 3.4.1, it was 0.1 ± 0.015 µmol/cm^2.

Afterwards, the enzyme was loaded to the functionalized membranes as described in previous sections. The amount of *Sso*Pox W263F immobilized on NSG-PES-GA was 5 ± 0.8 µg/cm^2. The FT-IR spectra of NSG-PES, NSG-PES-GA and NSG-PES-GA-PTE membranes are shown in Fig. 4.1a. The peaks at 1578 cm^{-1} and 1486 cm^{-1} are associated with the aromatic structure and are characteristic for PES, the band at 1661 cm^{-1} can be attributed to the N-H vibration in amino groups. A new peak at 1720 cm^{-1} appears for NSG-PES-GA membrane, it is due to the aldehyde carbonyl, the peak disappears after enzyme immobilization, indicating the reaction between aldehyde and the enzyme.

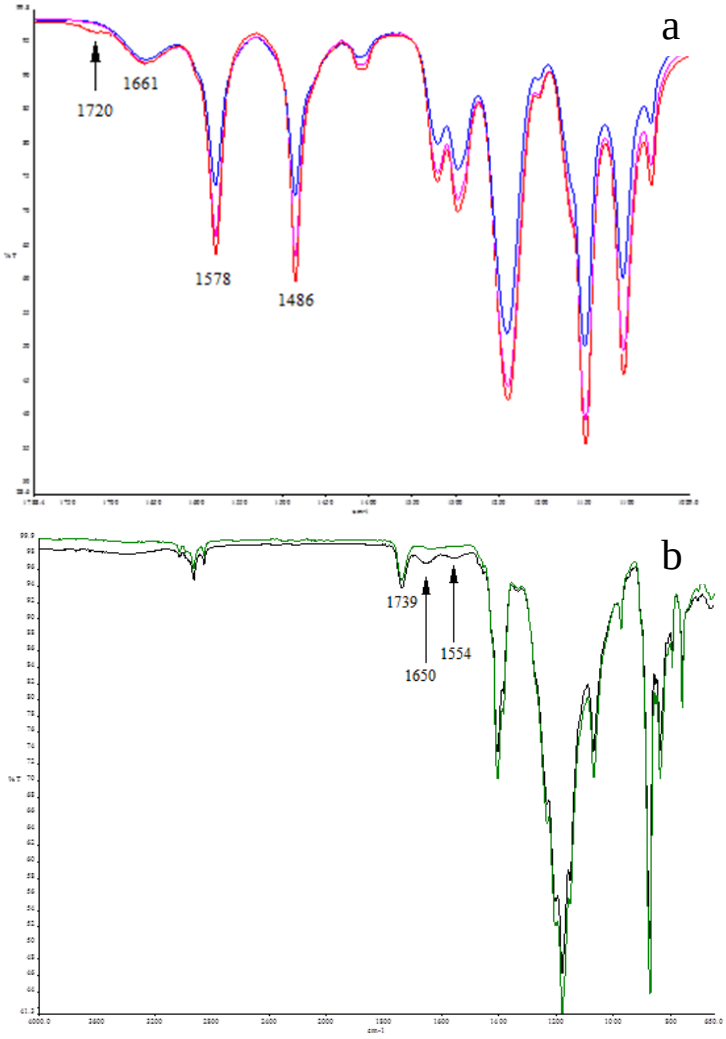


Fig. 4.1. FT-IR spectra of virgin and bio-functionalized membranes: a) NSG-PES membrane (blue line), NSG-PES-GA membrane (red line) and NSG-PES-GA-PTE membrane (fuchsia line), b) PVDF-DAMP-GA membrane (green line) and PVDF-DAMP-GA-PTE membrane (black line)

The water and vapour permeance and flux through the native NSG-PES and functionalized NSG-PES-GA-*Sso*Pox W263F membranes are illustrated in Table 4.1 together with pore size measurement before and after enzyme immobilization. As it is possible to note no evident changes in terms of pore size, membrane wettability, water permeance and vapour flux were noted after membrane biofunctionalization. This demonstrated that biofunctionalization process don't alter membrane properties.

Table 4.1: Properties of activated membranes used to immobilize *Sso*Pox W263F

MEMBRANE	PORE SIZE (μm)	CONTACT ANGLE (°)	VAPOUR WATER Flux ($L\ h^{-1}\ m^{-2}$)	LIQUID WATER PERMEANCE ($L\ h^{-1}\ m^{-2}\ bar^{-1}$)
NSG-PES	0.3 ± 0.1	33 ± 1	0.14[a] ± 0.01	3168 ± 883
NSG-PES-GA-PTE	0.3 ± 0.1	52 ± 2	0.15[a] ± 0.01	3100 ± 800
PVDF	0.21 ± 0.01	163 ± 9	0.14[a] ± 0.02	4.5 ± 0.4
PVDF-DAMP-GA-PTE	0.05 ± 0.01	136 ± 4	0.16[a] ± 0.02	29 ± 2

[a]: TMP 0.051 bar

4.3.2 Activation and characterization of PVDF membrane

The PVDF membrane was modified with DAMP (carbonate buffer 1 M pH 11). When the PVDF membrane was treated with the alkaline solution, an elimination reaction occurred, in particular fluorine and hydrogen were eliminated, and then DAMP was grafted onto the surface (PVDF-DAMP). The amount of grafted amino groups, quantified by ninhydrin

method, is 0.15 ±0.02 μmol/cm^2. Afterwards, the membranes were activated by reaction with 10% GA (v/v) for 2 hours at 25°C.

The immobilization of *Sso*Pox W263F was carried out on the glutaraldehyde-activated membranes. The amount of enzyme immobilized on PVDF-DAMP-GA was 35 ±1 μg/cm^2. FT-IR spectra of PVDF-DAMP-GA membrane and PVDF-DAMP-GA- *Sso*Pox W263F membrane are illustrated in Fig. 4.1b. The band at 1739 cm^{-1} is characteristic for PVDF, it is due to -CF=CH- bond present as defects into the structure of native PVDF, in addition the formation of these double bond is further promoted by the alkaline treatment during functionalization. The peaks at 1650 and 1554 cm^{-1} called "amide I" and "amide II" respectively, are characteristic of proteins [10] and point out the presence of *Sso*Pox W263F immobilized.

The liquid permeance and vapour flux through the native PVDF and functionalized PVDF-DAMP-GA- *Sso*Pox W263F membranes are illustrated in Table 4.1. As previously observed for NSG-PES any substantial change in terms vapour flux was observed. On the contrary, a slight decrease in contact angle and consequently an increase in water permeance was obtained. This was probably due to the reduction of the hydrophobic nature of PVDF due to hydrophilic species grafting (DAMP, enzyme) needed for membrane biofuctionalization.

Owing to the higher amount of reactive groups introduced on the PVDF membrane (as demonstrated by ninhydrin test) the quantity of *Sso*Pox W263F immobilized on PVDF-DAMP-GA was higher.

Moreover the higher hydrophobicity of the PVDF membrane compared to the NSG-PES membrane can play an important role on the amount of enzyme immobilized by adsorption. As reported in Table 4.1, the NSG-PES and PVDF membrane are very

different in terms of wettability. The NSG-PES membrane is hydrophilic whereas the PVDF membrane is a hydrophobic membrane and it is well known as the hydrophobic membrane is more susceptible to fouling of organic material in particular proteins due to the adsorption phenomena [11]. *Sso*Pox W263F is prone to be easily absorbed onto the PVDF-DAMP-GA membrane surface, promoting the interaction with the reactive aldehyde groups which involves irreversible immobilization. In addition the SEM micrographs (Fig. 4.2) show that the NSG-PES membrane has a porous structure and smooth surface while PVDF membrane has a porous structure and a rough surface. The higher surface roughness of the PVDF membrane in comparison with PES can favour the accumulation of *Sso*Pox W263F in the cavities [12]. In fact a pore size reduction was evident just in the case of PVDF (table 4.1).

However, part of these molecules may remain blocked and may not be reached by the hydrophilic substrate, reducing the specific activity.

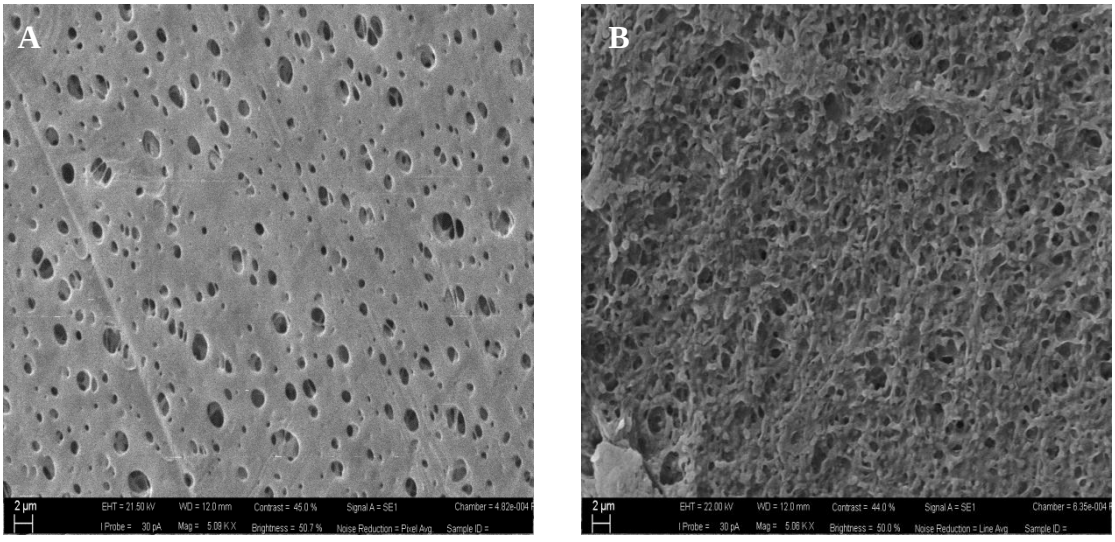
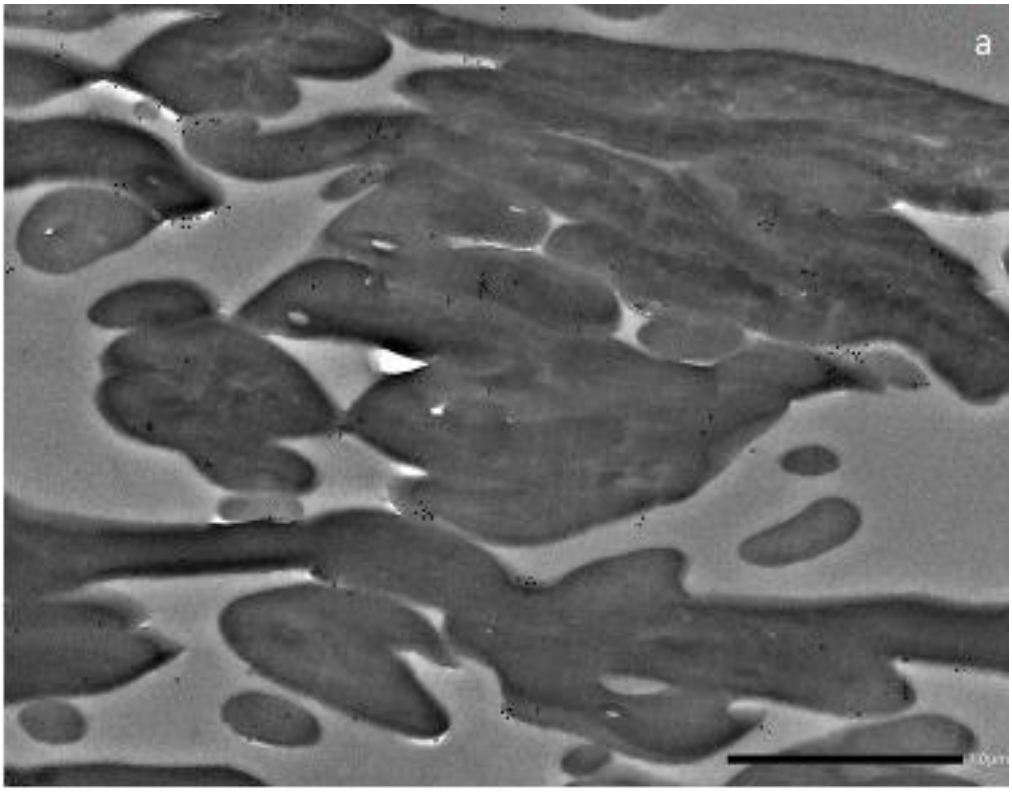


Fig. 4.2. SEM images of NSG-PES (A) and PVDF (B) membranes.

4.3.3. Immobilized SsoPox W263F enzyme in situ localization

In order to understand the *Sso*Pox W263F distribution within the two tested membranes, TEM analyses were carried out after hybridization of immobilized enzyme with a monoclonal antibody. The primary antibody was then coupled with a gold labelled secondary antibody, which permits the *in situ* localization of the immobilized *Sso*Pox W263F visible as black dots on the images (Fig. 4.3). Three zones of both biocatalytic PES and PVDF membranes cross section (top, middle and bottom) were analysed within the membrane cross-section and along the membrane length. Results demonstrated that the enzyme *Sso*Pox W263F was uniformly immobilized, without any preferential localization along the membrane thickness for both membranes. However it seems that a higher density of black dots is present on PVDF membranes, in agreement with quantitative evaluation of enzyme immobilized carried out by mass balance (Fig. 4.3a and Fig. 4.3b).


a

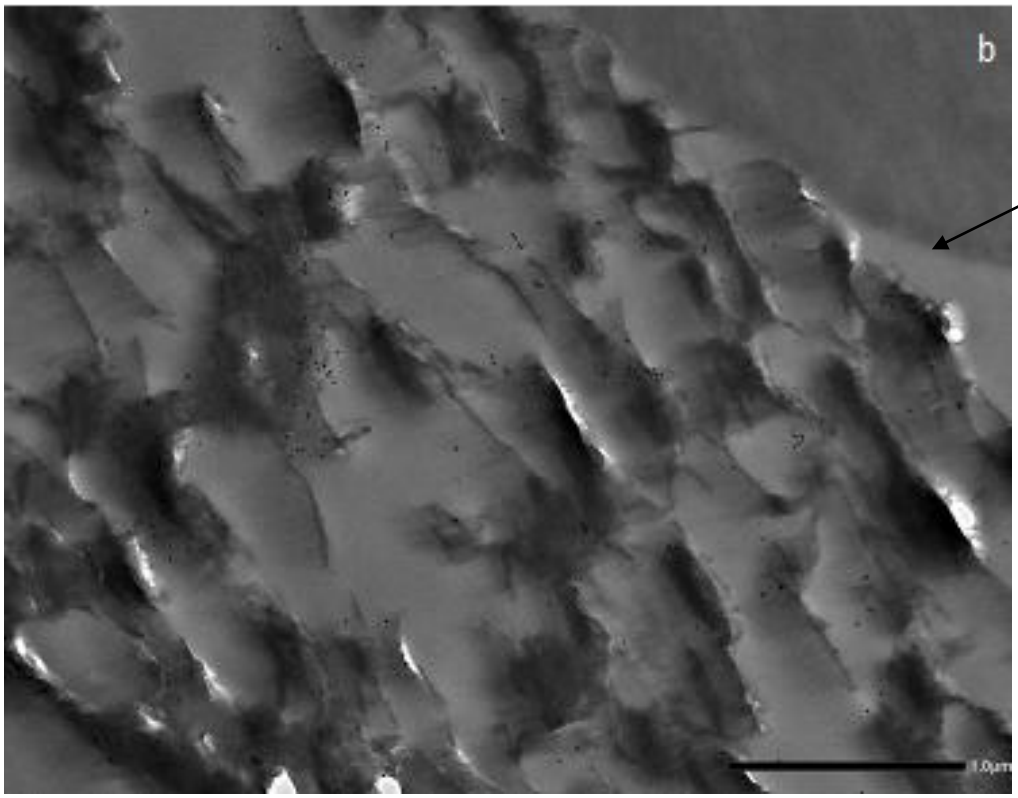

b
Membrane support

Fig. 4.3. Examples of cross section of biocatalytic membranes after immunolocalization of *Sso*Pox W263F observed by TEM: a) NSG-PES (middle part) b) PVDF (top part), section thickness 80 nm

In the image reported in Fig. 4.3b, about the PVDF biocatalytic membrane, it is possible distinguee the membrane and the membrane support. As it is possible to note, the enzyme is present just on the membrane and not on the membrane support. The absence of the biocatalyst on the PVDF membrane support was already demonstrated by IR analysis using lipase (see section 2.4.1).

4.3.4 Performance of free and immobilized SsoPox W263F

The specific activity of the free *Sso*Pox W263F freshly re-suspended in buffer from lyophilized lots, was 0.34 (± 0.04) Umg^{-1}. The stability over time of free enzyme was investigated for about one month; the *Sso*Pox W263F stock solution was stored at 4°C between activities tests carried out at 25 °C. The trend over time for free *Sso*Pox W263F is shown in Fig. 4.4. After initial reduction the specific activity is constant and it is about 0.24 (± 0.04) U mg^{-1}.

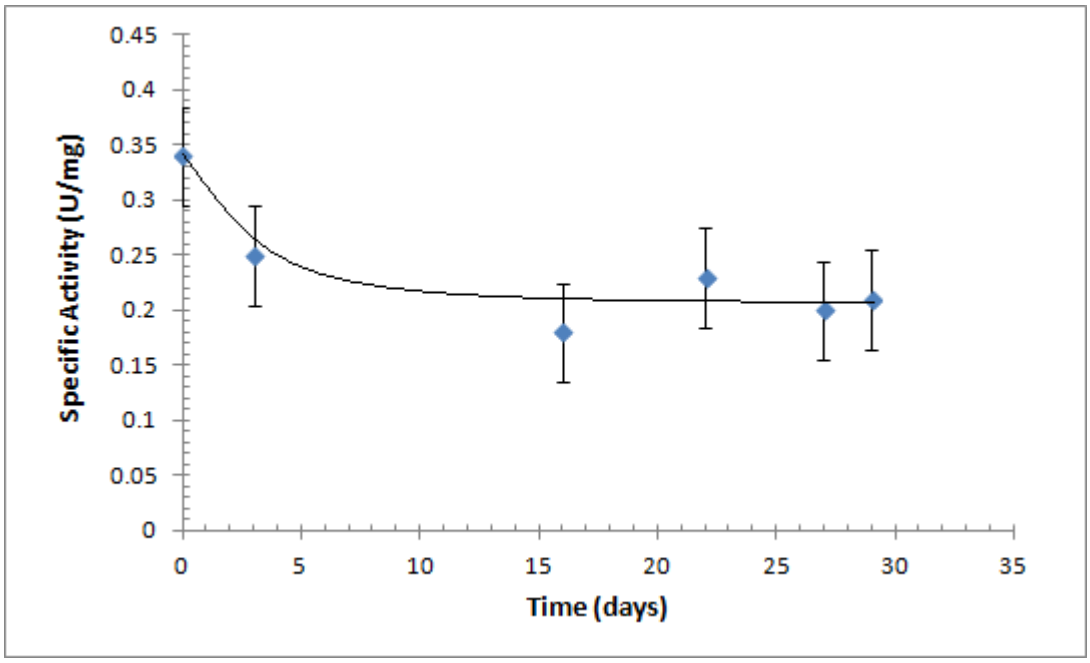


Fig. 4.4. Stability of free *Sso*Pox W263F as a function of time

The activity of *Sso*Pox W263F immobilized on both types of membranes (NSG-PES-GA and PVDF-DAMP-GA) was also evaluated at 25°C. Among them, the enzyme exhibited the highest activity per membrane area (8.25 ± 0.90 U/m^2) and specific activity (0.18 ± 0.02 U $mg^{-1}_{protein}$) on the NSG-PES-GA membrane, whereas for *Sso*Pox W263F immobilized on the activated PVDF-DAMP-GA membrane, activity per membrane area and specific activity were 4.2 ± 0.3 U/m^2 and 0.012 ± 0.001 U mg^{-1}, respectively.

The specific activity as a function of time is illustrated in Fig. 4.5. The stability over time of immobilized PTE was investigated for about five months. Between experiments, the *Sso*Pox W263F-loaded membranes were washed with hepes buffer pH 8.5 and stored at 4°C.

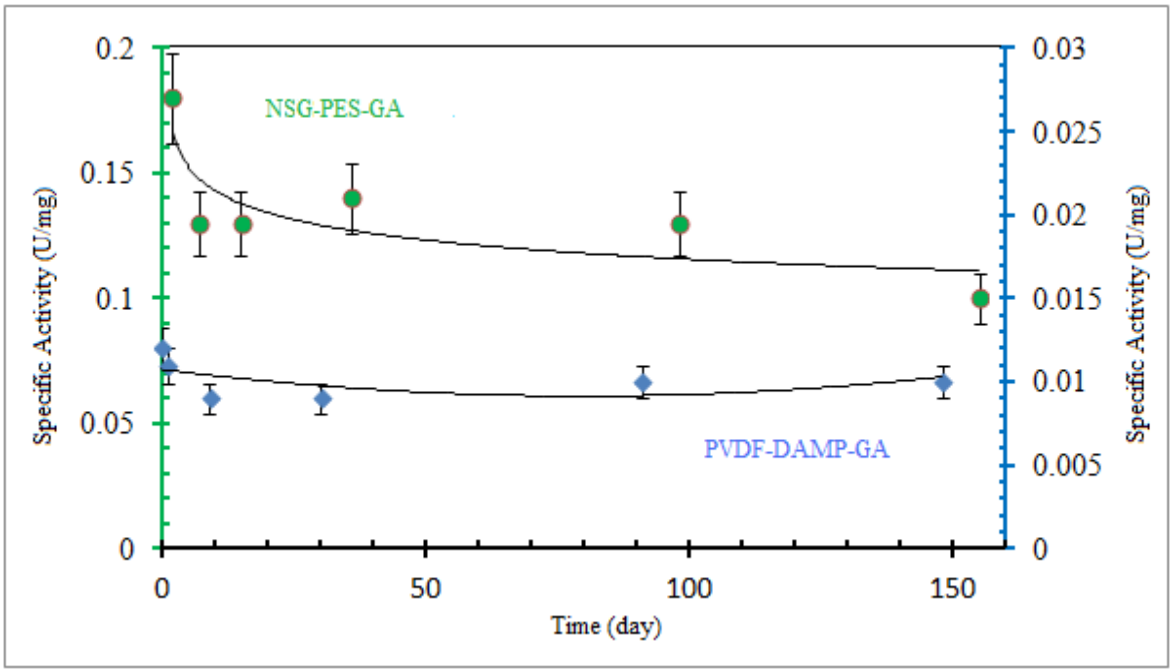


Fig. 4.5. Stability over time of *Sso*Pox W263F immobilized on modified NSG-PES-GA (green dots and y axis) and PVDF (blue dots and secondary y axis) membrane.

In good correlation with free *Sso*Pox W263F, also for immobilized *Sso*Pox W263F the specific activity decreases during few days from immobilization and then the catalytic systems becomes stable highlighting the possibility to recycle the biocatalyst in successive runs for example for water decontamination. It is important to mention that for enzyme immobilized on NSG-PES-GA membrane after one month, the reduction of specific activity (~20 %) respect to the initial one is lower if compared to the one of free enzyme (~ 30 %), whereas still less was the reduction (~10 %) for the enzyme immobilized on PVDF. This is anyway, the reflection of the respective lower initial specific activity.

It must be underlined that the biocatalytic membranes used to test the immobilized enzyme catalytic activity were the same samples (for each series of experiment), on the contrary for the free enzyme a new sample of enzyme, kept in buffer solution at 4 °C, was used at each

run. This perfectly reflects the advantages of immobilized system that permits biocatalyst re-use.

As before pointed out, a higher amount of *Sso*Pox W263F immobilized on PVDF compared to NSG-PES corresponded to a lower activity. This was probably due to both crowding phenomena (already observed using lipase, section 2.4.3) and mass transfer limitations for the case of PVDF base membrane. To prove this, catalytic activity was measured using different amount of immobilized biocatalyst. The different amount of immobilized enzyme was obtained by using different contact time between enzyme and functionalized PVDF-DAMP-GA membrane and correlating it with the corresponding specific activity.

As illustrated in Fig. 4.6, the amount of immobilized enzyme amount increases with contact time while specific activity decreases. In particular, in the range between 10 and 35 $\mu g/cm^2$ the specific activity as a function of immobilized enzyme decreases according to the following relationship:

$$y = 0.2037x^{-0.688}$$

where y is the specific activity (U/mg) and x is the amount of immobilized enzyme ($\mu g/cm^2$). Best biocatalytic membrane performance in terms of specific activity was obtained when the lowest amount of immobilized enzyme ($12 \mu g/cm^2$) was obtained.

However, if the catalytic activity was also considered, the better performance was obtained in the case in which the amount of immobilized enzyme was 19 $\mu g/cm^2$. In fact immobilizing 19 $\mu g/cm^2$ of enzyme, the catalytic activity was 5.9 U/m^2, while in the other case 4.2 U/m^2.

From the mass transfer point of view, considering the significantly different water contact angle of the two types of membranes (Tab. 4.1) it is reasonable to expect that they interact

in a different way with enzyme and substrate solution. In the case of the enzyme solution, from the TEM *in situ* analysis it was possible to see that the enzyme is present on top, middle and bottom of both membranes cross section, this means that the enzyme solution diffused into the pores of both membranes, probably due to the fact that long contact time between enzyme and membrane was used (24 h). In the case of the substrate solution the contact time of the biocatalytic membrane with substrate was few minutes (2-3 min), which are probably not enough to completely interact with PVDF membrane and saturate the entire immobilized enzyme.

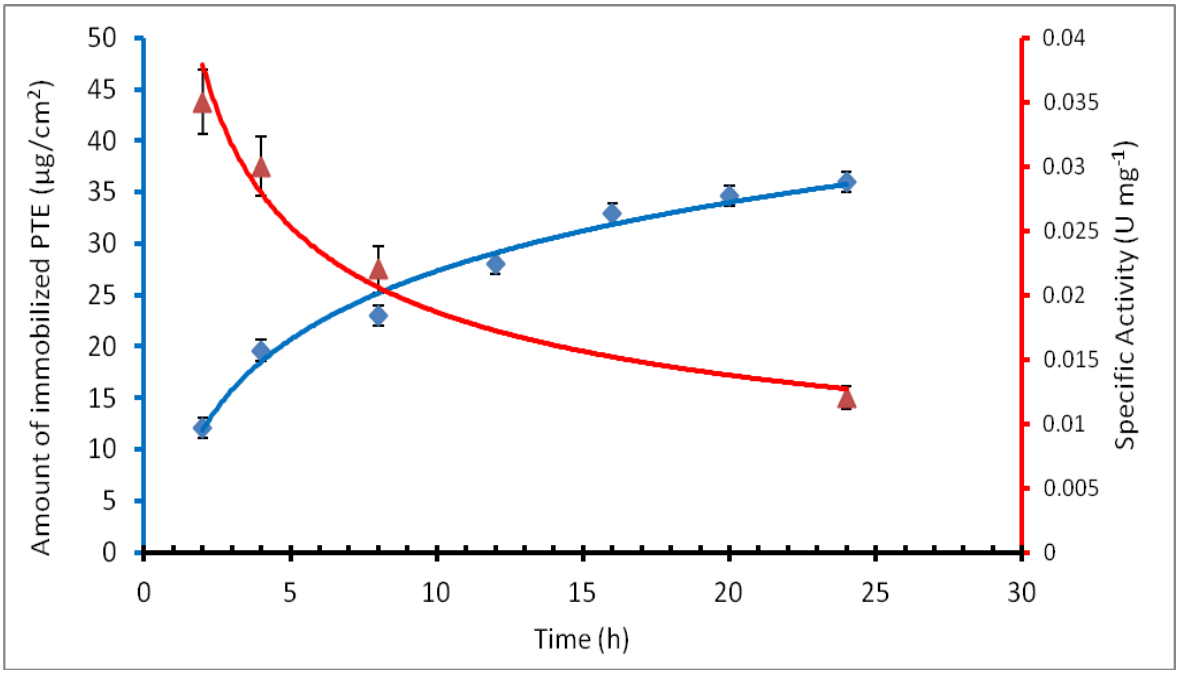


Fig. 4.6. Relationship between amounts of immobilized *Sso*Pox W263F and specific activity as a function of contact time between enzyme solution – PVDF-DAMP-GA membrane

4.4. Conclusions

In this work organophosphorus compounds (OPs) deactivation was studied immobilizing the single mutant *Sso*Pox W263F on polymeric membranes. Membranes with different

wettability, such as PES and PVDF membranes were tested with the final aim to treat either contaminated liquid or vapour streams.

In liquid phase, when the biocatalyst is immobilized on NSG-PES membrane the specific activity as well as the catalytic activity per membrane area were higher compared to the enzyme immobilized on PVDF. This was due to the high hydrophilicity of the membrane that makes it better wetted by the aqueous solution in which the substrate (paraoxon) is dissolved. PES membranes showed superior performance than PVDF membranes basing on both, enzyme activity and membrane permeability, in liquid phase decontamination.

References

[1] E. Ghanem, F. M. Raushel, Detoxification of organophosphate nerve agents by bacterial phosphotriesterase, Toxicology and Applied Pharmacology 207 (2005) 459 – 470

[2] K. E. Le Jeune and A. J. Russell, Covalent Binding of a Nerve Agent Hydrolyzing Enzyme Within Polyurethane Foams, Biotechnology and Bioengineering 51 (1996) 450-457

[3] A. A. Wang, W. Chen, A. Mulchandani, Detoxification of Organophosphate Nerve Agents by Immobilized Dual Functional Biocatalysts in a Cellulose Hollow Fiber Bioreactor, Biotechnology and Bioengineering 91 (2005) 379-386

[4] L. Merone, L. Mandrich, M. Rossi, G. Manco. A thermostable phosphotriesterase from the archaeon *Sulfolobus solfataricus* : cloning, overexpression and properties Extremophiles 9 (2005) 297–305

[5] E. Drioli, L. Giorno, Comprehensive Membrane Science and Engineering, 1, Newnes, 2010

[6] R. Mazzei, E. Drioli, L. Giorno, Enzyme membrane reactor with heterogenized β-glucosidase to obtain phytotherapic compound: Optimization study, Journal of Membrane Science 390 (2012) 121–129

[7] L. Merone, L. Mandrich, E. Porzio, M. Rossi, S. Müller, G. Reiter, F. Worek, G. Manco. Improving the promiscuous nerve agent hydrolase activity of a thermostable archaeal lactonase, Bioresource Technology 101 (2010) 9204-12

[8] P.K. Smith, et al., Measurement of protein using bicinchoninic acid. Anal Biochem 150 (1985) 76-85.

[9] S.J. Metz, W.J.C. van de Ven, J. Potreck, M.H.V. Mulder, M. Wessling, Transport of water vapor and inert gas mixtures through highly selective and highly permeable polymer membranes, Journal of Membrane Science 251 (2005) 29–41

[10] S. Belfer, R. Fainchtain, Y. Purinson, and O. Kedem, Surface characterization by FTIR-ATR spectroscopy of polyethersulfone membranes-unmodified, modified and protein fouled. Journal of Membrane Science 172 (2000) 113–124.

[11] F. Liu, N. Awanis Hashim, Y. Liu, M.R. Moghareh Abed, K. Li, Progress in the production and modification of PVDF membranes, Journal of Membrane Science 375 (2011) 1–27

[12] N. Lee, G. Amya, J. Crouè, H. Buisson, Identification and understanding of fouling in low-pressure membrane (MF/UF) filtration by natural organic matter (NOM), Water Research 38 (2004) 4511–4523

Chapter 5

Introduction on the basic concepts of inverse miniemulsion polymerization technique

5.1 Introduction

In order to improve the performances of enzymes immobilized on the hydrophobic PVDF membrane, a new method for enzymes immobilization was developed. The new strategy aims to immobilize the enzyme maintained in a hydrated microenvironment on the hydrophobic environment proper of PVDF membrane. To reach this aim polyacrylamide (pAAm) nanogels were selected as carrier for covalent enzyme immobilization. The pAAm nanogels were produced by inverse miniemulsion polymerization and then functionalized in order to covalent bind with both enzyme and modified PVDF membrane. Lipase was used as model enzyme. Basic concepts about inverse miniemulsion polymerization technique are described in the next section.

5.2 INVERSE MINIEMULSION POLYMERIZATION: BASIC PRINCIPLES

The miniemulsion technique is a useful tool for the preparation of high value nanomaterials. In particular, several routes (nanoprecipitation, phase separation, interfacial polymerization) can be used to produce nanoparticles with high encapsulation rates, and in the case of inverse miniemulsion, hydrophilic content. Nanoparticles produced by inverse miniemulsion polymerization are metastable colloids of diameter 50-500 nm composed by two immiscible fluids, one dispersed in the other, prepared by shearing a system containing unpolar continuous phase, water, and lypophobe (osmotic agent) in the presence of surfactants which provides colloidal stability [1]. The first general mechanism for any type of inverse emulsion polymerization was proposed in 1987 by Hunkeler et al. [2], it is shown in Fig. 4.1. The hydrophilic phase is dispersed in a hydrophobic continuous phase, the water droplets are stabilized with surfactants and hydrophilic osmotic agents are used to balance diffusion between the droplets [3].

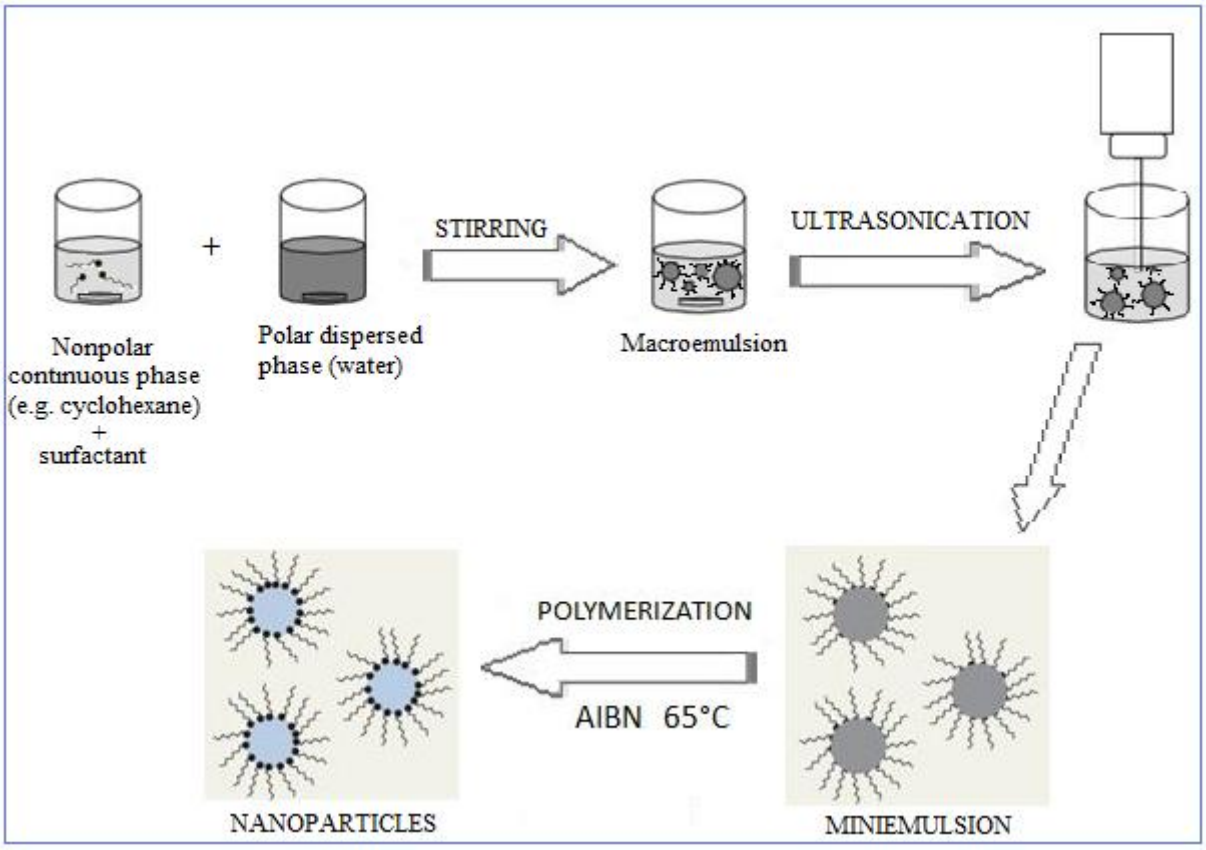


Fig. 5.1. General principle of inverse miniemulsion polymerization.

During the inverse miniemulsion polymerization each nanodroplet behaves like an independent nanoreactor. The main problem during the production of monomer-containing inverse miniemulsion is its stability before and during the polymerization of the monomer. Miniemulsions are usually kinetically stable, but under an ideal condition thermodynamically stable miniemulsions can also be prepared [4]. To achieve the stabilization of the nanodroplets, several requirements are needed.

The formation of small nanodroplets requires high-energy input to overcome the increasing interfacial energy as a consequence of droplets fission. Therefore, the overall interfacial area increases when the size of the droplets decreases, and the interfacial energy required *dE* is proportional to the variation of the interfacial area *dA*, following Equation (1):

$$dE = \gamma_{LL} dA \tag{1}$$

Here γ_{LL} is the surface tension between the two liquid phases ($N \cdot m^{-1}$). Usually the devices used for droplet fission are high-pressure homogenizers (high shear forces) or ultrasonication tips (cavitation pressure). The use of surfactant lowers the surface tension between continuous and disperse phase because the droplets requires to be stabilized to prevent fusion by collision. The concentration of surfactant is chosen such that after homogenization, the concentration in the continuous phase is below the critical micellar concentration (CMC) in order to avoid the micellar nucleation. The phenomenon of Ostwald ripening associated with the aging time is a manifestation of the thermodynamic instability. If the droplets do not all have the same size after homogenization, coalescence occurs over time: diffusion of monomer from the smaller droplets (with higher Laplace pressure) to the bigger droplets (thermodynamically more stable) progressively removes the smaller droplets and the average size of the droplets increases continuously. The driving force of the Ostwald ripening is the difference of Laplace pressures (Equation 2) between the smaller and bigger droplets.

$$P_L = \frac{2\,\gamma_{LL}}{r} \tag{2}$$

Here γ_{LL} is the surface tension between the two liquid phases ($N \cdot m^{-1}$) and *r* is the radius of the droplets (m). Higher is the solubility of the monomer in the continuous phase, more is the degree of Ostwald ripening because the monomer can diffuse faster to the bigger droplets. To counteract and minimize Ostwald ripening, an osmotic agent can be added to

the disperse phase. Incorporation of the osmotic agent (e.g. inorganic salts or sugars) into the system can retard the diffusional degradation of monomer saturated aqueous droplets and maintain the very small droplet size generated by ultrasonication. Osmotic agents such as sodium chloride (NaCl) lower the Gibbs free energy of the miniemulsion droplets, thereby decreasing the driving force for diffusion of the monomer into the continuous phase, they build up an osmotic pressure described by equation 3 what counteracts the Laplace pressure caused by the Interface Energy of the droplets.

$$\Pi_{osm} = c \cdot R \cdot T \tag{3}$$

Here c is the concentration of the osmotic agent ($mol \cdot L^{-1}$), R is the ideal gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$) and T is the temperature (K). Diffusion of monomer between the droplets can still occur but is compensated, under standard conditions and after ultra-homogenization, the osmotic pressure does not necessarily fully compensate the Laplace pressure. However the droplets are highly stable, thanks to the different stabilization mechanisms. Since nearly no surfactant is present as micelles, free radical polymerization of nanoparticles nucleated via entry of radical into monomer nanodroplets is possible. The inverse miniemulsion polymerization of water soluble monomers can be initiated by water-soluble and oil-soluble initiator as well as by radiation. The water-soluble initiator suppresses the homogeneous (organic phase) nucleation while the use of oil soluble one could result in an increase in this occurrence. The miniemulsion offers several advantages to more conventional bulk (or solution) processes, including higher polymer concentrations in the ultimate product.

The main interest in inverse miniemulsion is the possibility to prepare nanoparticles with a hydrophilic character. Therefore these materials are highly interesting for bio-applications after redispersion in water. In fact inverse-emulsion polymerization is a widely applied

technology for the preparation of high molecular weight water-soluble macromolecules [5], owing to the fact that high concentrations of monomers can be contained within the aqueous droplets, while maintaining a monomer/polymer latex. These polymers, which are generally based on acrylamide copolymerized with anionic or cationic monomers, can be used for several application such as compatibilizers, flocculants, separations, carriers etc. [6]. The polymerization of acrylamide by inverse water/oil miniemulsion offers several advantages. These include the high reaction rates, solids levels, polymer molecular weights, lower solution viscosities and higher heat removal rates [7].

5.3 NANOPARTICLES CHARACTERIZATION: LIGHT SCATTERING ANALYSIS

Light scattering was used to measure particle size and size distribution of the nanoparticles produced with the inverse miniemulsion polymerization methodology. Colloidal systems and polymer molecules in solution interact with visible light by scattering it in all directions. Dynamic light scattering (DLS) is a technique which measures Brownian motion of particles in suspension by illuminating the particles with a laser and analyzing the intensity fluctuations in scattered light. Brownian motions are correlated with the particle size: small particles move quickly and large particles move slowly. The fluctuation in scattering intensity depends on the speed of the Brownian motion and it is analyzed by a digital correlator which calculates a correlation function. The signal intensity detected fluctuates with time, because of the interferences within the scattered light, mainly due to the Brownian motion of the scattering particles. The mathematic treatment of the intensity I(t) with an autocorrelation function gives in first approximation an exponential decrease, with a characteristic time (or relaxation time). This relaxation time is inversely

proportional to the diffusion coefficient D of the particle or polymer chain (Equation (4)), where q is the norm of the scattering vector.

$$\frac{1}{\tau} = Dq^2 \tag{4}$$

The hydrodynamic radius R_H is obtained from the Stokes-Einstein Stokes-Einstein relationship:

$$R_H = \frac{k_B T}{6\pi\eta_S D} \tag{5}$$

Where K_B is the Boltzmann constant ($J \cdot K^{-1}$), T the temperature (K) and η_s the viscosity ($N \cdot s \cdot m^{-2}$) of the liquid.

Particle size distribution is expressed as polidispersion index (PDI), that is a dimensionless measure of the broadness of the size distribution calculated from the cumulant analysis [8]. This is in the range between 0 and 1 and monodispersed sample show a polydispersity below 0.1.

References

[1] I. Capek. On the Inverse Miniemulsion Copolymerization and Terpolymerization of Acrylamide, N;N0-methylenebis(acrylamide) and Methacrylic Acid *CEJC* 3 (2003) 291-304

[2] J. Hernández-Barajas, Hunkeler. Inverse-emulsion copolymerization of acrylamide and quaternary ammonium cationic monomers with block copolymeric surfactants: copolymer composition control using batch and semi-batch techniques. *DJ. Polymer* 38 (1997) 449

[3] K.Landfester, M. Willert, M. Antonietti, Preparation of polymer particles in nonaqueous direct and inverse miniemulsions**.** *Macromolecules* 33 (2000) 2370-2376

[4] I. Capek. On inverse miniemulsion polymerization of conventional water-soluble monomers. *Advances in Colloid and Interface Science* 156 (2010) 35–61

[5] RS Farinato, S-Y Huang. In: Farinato RS, Dubin P, editors. Fundamentals to practice. *New York: Wiley* (1999) 3–50.

[6] Buchholz FL. In: Elvers HSB, Schulz G, editors. Ullmann's encyclopedia of industrial chemistry. Weinheim, Germany: VCH (1992) 143–56.

[7] H. Ohde, C. M. Wai, J. M. Rodriguez The synthesis of polyacrylamide nanoparticles in supercritical carbon dioxide. *Colloid Polym Sci* 285 (2007) 475–478

[8] Barbara J. Frisken, Revisiting the method of cumulants for the analysis of dynamic light-scattering data. Applied Optics 40 (2001) 4087-4091

Chapter 6

Development of an immobilization method to keep enzymes in hydrated microenvironment on hydrophobic membranes

6.1. Introduction

Efficient immobilization techniques that preserve the activity of biomolecules have many potential applications, such as the development of bioreactors and biosensors [1]. The ideal immobilization method should: 1) Provide a stable biohybrid system; 2) Allow immobilizing desired amounts of biomolecules; 3) Employ mild conditions in order to avoid biomolecules denaturation; 3) allow optimal enzyme-substrate contact; 4) Minimize barriers to mass transport of substrates and products [2]. To date, the strategies for immobilizing biomolecules are adsorption or covalent attachment to a support, microencapsulation and entrapment within a membrane-film or gel [3]. Porous membranes are optimal support for enzymes immobilization because they allow process intensification combining a separation process with a chemical reaction [4]. In addition, porous membranes provide high specific surface area and minimize mass transfer limitations. Many materials of different chemical physical properties have been used as membrane materials. Because immobilisation of an enzyme entails the interaction of two species, the enzyme and the membrane, the surface properties of the membrane material are therefore important. The membrane material changes the microenvironments around the immobilized enzyme and can enhance or decrease the enzyme activity [5]. The material hydrophobicity for example can negative influence the enzyme activity either denaturing the enzyme or hindering the enzyme interaction with water-soluble substrates. However, the hydrophobicity is a valuable property for the membrane when it is employed in certain applications. Therefore, the aim of this work has been immobilize enzyme kept in hydrated microenvironment on hydrophobic membrane made of PVDF. In order to reach this aim hydrophilic nanogel has been used as carrier for enzyme immobilization on the PVDF

membrane. The PVDF membrane was first functionalized with amino groups and then activated with glutaraldehyde to allow covalent nanogels immobilization.

The nanogels were synthesized in the range 100-200 nm with a narrow particle size distribution using the inverse miniemulsion polymerization technique. In general nanogel and microgel particle formation can be achieved by different approaches including precipitation polymerization from aqueous or polar solutions [6-8] crosslinking of preformed polymers and oligomers in emulsions [9], or copolymerization of different water soluble monomers and cross-linkers in inverse (W/O) emulsion, microemulsion or miniemulsion systems [10,11]. Since nanogel preparation based on inverse miniemulsion polymerization enables the equal distribution of all components in each particle it is advantageous compared with other methods when homogenous gel properties and an equal distribution of all different functionalities is desirable. In addition particle sizes in the submicron size range, combined with a very narrow size distribution can be easily achieved by this method. The hydrophilic monomer used in this work has been acrylamide (AAm). The copolymerization of this monomer with *N,N'*-methylene-bis-acrylamide (MBAAm) in a stabilized inverse miniemulsion, yield chemically cross-linked strong hydrophilic polyacrylamide (PAAm) nanogels, which inherently swell in aqueous confinements. In this work the generated gel network was modified subsequently by partially degradation of amide groups to primary amine groups by Hofmann reaction [12,13]. The primary amine functionalities were used as binding sides to covalently bind a model biomolecule (the enzyme Lipase) via coupling reagents like glutaraldehyde (GA). The modified nanogels were employed in two different immobilization strategies. In the first strategy lipase was immobilized on the glutaraldehyde activated nanogels and then the lipase-loaded nanogels were immobilized on the GA-functionalized PVDF. In the second

strategy the PAAm nanogels after Hofmann degradation were immobilized on the GA-functionalized PVDF. Unreacted aldehyde groups were then quenched using ethanolamine and the lipase was immobilized on the nanogels-loaded membrane after activation of nanogels amino groups by GA. The catalytic efficiencies of lipases immobilized by these two strategies were evaluated using para-nitrophenyl palmitate (p-NPP) as substrate and compared with lipase immobilized on the PVDF membrane without nanogels. Results exhibited that lipase immobilized by means of nanogels, with both strategies, retained higher specific activity.

6.2. Materials and methods

6.2.1. Chemicals

All chemicals used in this work were commercially available and used without further purification unless otherwise stated. Used water was purified with a Milli-Q system from Satorius Stedim Biotech (Germany).

Acrylamide (AAm), the crosslinking agent *N,N'*-methylene-bis-acrylamide (MBAAm), the nonionic surfactant sorbitane monooleate (Span80), the non ionic surfactant polyehtylene glycol *tert*-octylphenyl ether (Triton - X100), Lipase from *Candida rugosa* (65 kDa), ninhydrin, ethanol, glutaraldehyde, 1,5-diamino-2-methylpentane, 4-nitrophenol, 4-nitrophenyl plamitate, sodium hypochlorite solution (NaOCl, available chlorine 10 – 15 %), 1,5-diamino-2-methylpentane were purchased from Sigma-Aldrich (Germany). The hydrophobic initiator 2,2'-azobis(2-methylpropionitrile) (AIBN) (Sigma-Aldrich, Germany) was recrystallized from methanol and vacuum dried over phosphorus pentoxide (P_2O_5) (Carl Roth GmbH, Germany).

Cyclohexane was supplied from Fisher Chemical (UK), while hydrogen phthalate (KHP), isopropyl alcohol (IPA), sodium chloride (NaCl), sodium hydroxide (NaOH) and hydrogen chloride (HCl) was received from Bernd Kraft GmbH (Germany).

Standard grade regenerated cellulose membrane tubing with nominal molecular weight cut-off (MWCO) of 12 - 14 kDa was used for dialysis. The membranes were pretreated by washing them six times in water for 15 min. Diafiltration was performed using a commercial polyethersulfone (PES) flat sheet ultrafiltration membranes with MWCO of 300 kDa kindly provided by Sartorius Stedim Biotech GmbH (Germany). Prior to use the membranes were cut into circular samples with diameter of 44 mm, washed in ethanol for 30 min and three times (30 min) in water to remove preservatives and soaked overnight in 0.01 M NaN_3 solution.

Flat sheet polyvinylidene fluoride (PVDF) membranes were used as support for biomolecules immobilization (see section 2.2.1. for detailed description).

6.2.2. PVDF membrane functionalization and characterization

The PVDF membrane cut into disk (Ø 54 mm) was soaked into 30 mL of 2 M 1,5-diamino-2-methylpentane (DAMP) solution in carbonate buffer pH 11 in order to introduce onto the PVDF surface amino groups (PVDF-DAMP) [14]. The PVDF-DAMP membrane was then treated with 20 mL of 10% (v/v) glutaraldehyde solution at 25 °C for 2 hours to couple the amino groups with glutaraldehyde (PVDF-DAMP-GA), after that the membrane was washed with water and stored at 4°C until used.

The wettability of the membranes was investigated by static water contact angle (SCA) measurement using a CAM 200 device (KSV Instruments, Ltd.). A drop of water (5 µL) was injected using a micro-syringe with automatic dispenser onto the membrane surface.

Data for 5 drops on different locations were averaged to obtain the contact angle of each membrane sample.

6.2.3. Synthesis of hydrophilic nanogels by inverse miniemulsion polymerization

The disperse phase was prepared by dissolving appropriate amounts of AAm and MBAAm in aqueous 0.5 M NaCl solution. Afterwards, the mixture was added to the continuous phase consisting of the nonionic surfactant Span80 in cyclohexane. After stirring the mixture at 1200 rpm for 1.5 h, the inverse miniemulsion was prepared by homogenizing the obtained pre-emulsion by ultrasonication for 2 min at 90 % intensity in a Bandelin (Germany) sonifier Sonoplus HD 3200 at 10 °C. Before addition of the oil soluble initiator (AIBN) in solid state, the miniemulsion was heated to 65 °C in an oil bath. This reaction was very quick and finished in several minutes. However, to ensure full conversion of the monomer, the reaction mixture was stirred at 300 rpm at 65 °C for 2 h. The typical composition of the dispersed and continuous phases and the used amount of initiator are summarized in Table 1:

Table 6.1: Typical recipe for inverse miniemulsion polymerization of acrylamide.

Component	Phase	Weight [g]	Remark
AAm[a]	Dispersed	1.2	20.8 % (w/w) [a)]
MBAAm[a]	Dispersed	0.04	4.76 % (w/w) [b)]
NaCl	Dispersed	0.094	2,85 % (w/w) [c)]
H_2O	Dispersed	3.2	4.8 % (v/v) [d)]
Span80	Continuous	1.2	27.3 % (w/w) [c)]
Cylcohexane	Continuous	50	95.2 % (v/v) [d)]
AIBN	Continuous	0.128	-

[a)]total polymeric solid content relative to water mass; [b)]relative to total polymeric solid content mass; [c)] relative to water mass; [d)] relative to total volume of the emulsion

After the polymerization, coagulates were removed by filtration and a sample of the resulting dispersions was investigated regard to the particle size distribution by means of dynamic light scattering. The main fraction of the dispersion was sedimented with aqueous 80 % (v/v) IPA mixture. The supernatant was removed and replaced by fresh aqueous 80 % (v/v) IPA mixture. Resuspension was carried out using a vortex. To remove the surfactant sufficiently, the suspensions were further washed five times, following the same procedure described above. The resulting surfactant free particles were than swollen over night in 30 mL of water at room temperature. To remove the osmotic agent NaCl and the solid content, which consisted of unreacted monomer and crosslinker, soluble (non crosslinked) polymers and oligomers, the aqueous dispersion was dialyzed in a large excess of ultrapure water under shaking for several days. The dialysis was monitored via conductivity and stopped when conductance of the washing water was lower than 1 µS. The purified dispersions were finally freeze-dried with an Alpha 1-4 freeze dryer (Christ, Germany) yielding the cross-linked polyacrylamide gel particles as deep white coloured fluffy powders with a yield of 70% (w/w).

6.2.4. Nanogel modification by Hofmann Reaction

In order to covalently bond lipase in the nanogel amino groups were created in the polymeric network applying Hofmann reaction according to literature procedures [12]. Prior to the modification the dried nanogels were redispersed in water by simply swelling at room temperature over night, followed by sonication in the ultrasonic bath (Trans Sonic Digital, Germany) using 100 % intensity for 30 min. After that the modification was performed in a thermostated jacketed reaction vessel by mixing the

resulting dispersion of PAAm nanogels (50 mL, 1 g L^{-1}) cooled at about 0 °C with 30 mL of a mixture consisting of 23 mM NaOCl and 10.7 M NaOH, cooled at -10 °C. The reactor was kept at -10 °C for 1.5 h in order to complete the first step of reaction (Fig. 6.1 a).

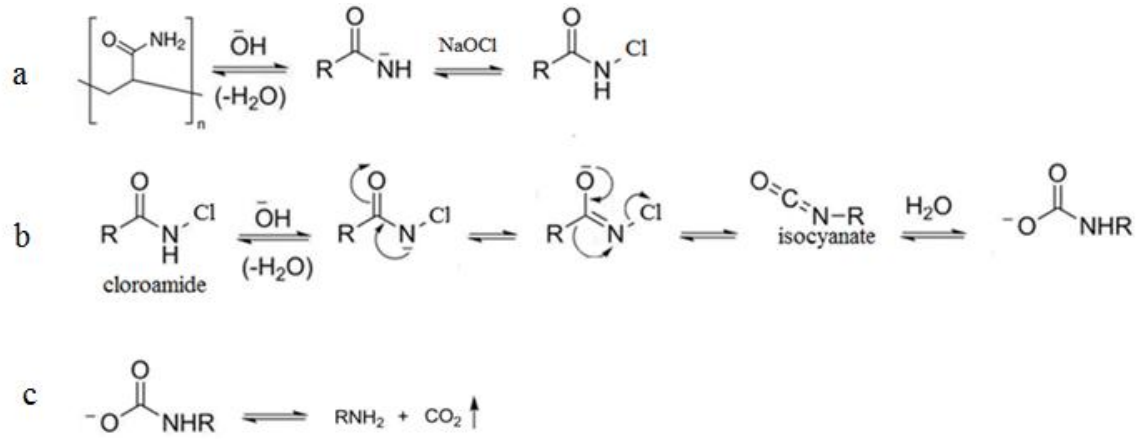


Fig. 6.1. Hofmann reaction of PAAm nanogels

Afterwards, 70 mL of 8.29 M NaOH was added and the reactor kept at -10 °C for 30 min, then the temperature was increased to 0 °C and reaction mixture was kept under stirring for 17 h to complete Hofmann reaction (Fig. 6.1 b-c). The mixture was then neutralized by dropwise adding 76 mL of HCl (conc. 37 %). The nanogels were purified by diafiltration in pressure driven dead-end setup with a stirred cell (Amicon 8050, Millipore, Germany), using pressure ranging from 0.4 to 0.5 bar and a volume of 2 L of ultrapure water. Finally water dispersions were freeze dried, yielding degraded nanogels as pale yellow powder with a yield of about 70 % (w/w).

6.2.5. Nanogel characterizations

Native and modified nanogels were characterized by dynamic light scattering (DLS) analysis using a StabiSizer PMX 200C (Microtrac, Germany) at fixed scattering angle of

180 ° (heterodyne) and laser light source with wavelength of 780 nm. The measurements were carried out at room temperature in diluted dispersions in the respective solvents. The results are the average from three separated measurements. For quality control measurements immediately performed after the polymerization the reaction mixture were diluted with cyclohexane down to 0.02 % (v/v) to avoid influence of the surfactant on the solution properties. Presented values are given as number weighted hydrodynamic mean diameters.

Liquid ^{1}H-NMR (300 MHz) spectra were measured with DRX200 (Bruker, Germany) using D_2O and $CDCl_3$. Quantitative measurements for conversion analysis of the inverse miniemulsion polymerization in D_2O were performed by adding KHP as internal standard. For quantification of the remained surfactant after the purification visible in $CDCl_3$ appropriated amounts of 1, 4-Dioxane were added as internal standard.

FT-IR spectra were recorded using Varian 3100 Excalibur Series (Varian, USA) in attenuated total reflectance (ATR) arrangement. The measurements were done with dry nanogel samples in range of 1000 - 4000 nm.

The nitrogen to carbon ratio of the samples was determined by elemental analysis of carbon, hydrogen, nitrogen and oxygen using the EURO EA Elemental Analyzer (EUROVector, Italy) under addition of vanadium oxide (V_2O_5). While absolute values were influenced by water the relative nitrogen to carbon ratio was used to determine the conversion of AAm during Hofmann degradation. Ninhydrin test was performed as qualitative test in order to detect free amino groups created by Hofmann degradation.

6.2.6. Glutaraldehyde activation of amino functionalized nanogels and Lipase immobilization

The amino functionalized nanogels where activated for enzyme immobilization by glutaraldehyde (GA) treatment. The nanogels (10 mg) were treated with 50 mL of 1% GA solution for two hours, in this way the amino groups were derivatized with GA by Schiff base reaction and reactive aldehyde groups introduced on the nanogels (GA-NANOGEL). The unreacted GA was removed by diafiltration.

The lipase from candida rugosa solution was prepared by dissolving 7 $g_{\text{raw powder}}$ / L in phosphate buffer (PBS) 50 mM pH 7. The suspension was stirred for 2 hours at room temperature and then centrifuged at 4500 rpm for 5 minutes. The supernatant was recovered and filtered by 300 kDa PES membrane, lipase concentration was finally measured by BCA test kit (0.5 ± 0.06 g L^{-1}).

The GA-nanogels dispersion in water was mixed with 50 mL of 0.5 mg mL^{-1} lipase solution in PBS pH 7 in order to load lipase on nanogels (lipase-GA-NANOGEL). The mixture was kept under stirring 16 h and because of presence of aggregates it was sonicated (10 minutes) and then centrifuged (1500 rpm, 5 minutes). The supernatant recovered and free lipase removed by diafiltration using a 300 kDa PES membrane. The diafiltration was stopped when lipase wasn't detected in permeate using BCA protein assay kit ((Thermo Scientific). Protein content of the obtained lipase-GA-NANOGEL dispersion (estimated by BCA) was 0.02 mg mL^{-1}.

6.2.7. Immobilization of lipase loaded nanogel on functionalized PVDF membrane

The PVDF-DAMP-GA membrane was immersed in the lipase-GA-nanogels dispersion (28 mL, 0.02 mg mL^{-1}) at 25°C for 24 hours under gentle stirring. Rinsing with water was followed, after that the membranes with immobilized lipase-loaded nanogels was obtained. In order to evaluate the quantity of lipase immobilized on the membranes, lipase concentration in the initial and final solution was measured. The amount of immobilized lipase was determinated by mass balance according to the equation 2.1. The immobilized mass was then normalized by the membrane area to estimate the immobilized enzyme amount related to membrane surface.

6.2.8. Immobilization of lipase on nanogels loaded PVDF membrane

The PVDF-DAMP-GA membrane was immersed in the amino functionalized nanogels dispersion (50 mL, 0.2 mg mL^{-1}). After 16 h of contact with the nanogels dispersion, the membrane was washed with water and treated 2 h with ethanolamine (20 mL, 0.1 M) in order to quench unreacted aldehyde groups, then the amino groups of the nanogels were activated by GA treatment (30 mL, GA 10% v/v). Afterwards, the membrane was washed with water and the lipase immobilized soaking the nanogels-loaded membrane in the lipase solution (0.5 mg mL^{-1}) for 24 hours. The amount of immobilized lipase was determinated by mass balance according to equation 2.1.

6.2.9. Direct lipase immobilization on PVDF-DAMP-GA membrane

Direct immobilization of lipase on the PVDF-DAMP-GA membrane was performed by soaking the membrane (Ø 54 mm) in a LCR solution (0.5 mg mL^{-1}, 30 mL phosphate buffer pH 7) for 24 hours at 25°C. Washings with the reaction buffer were then performed

in order to remove adsorbed lipase. The amount of immobilized lipase was determined by mass balance between lipase concentration into initial, final and washing solutions.

6.2.10. Lipase Activity measurements

The lipase activity was studied using the synthetic substrate p-nitrophenyl palmitate (p-NPP) [15] and measuring the release of p-nitrophenol (p-NP) spectrophotometrically at 405 nm. Unit of enzyme activity (U) was defined as the amount of enzyme which liberates 1 μmol p-NP per minute at pH 7.5 and temperature 30 °C. The specific activity was calculated using the following formula:

$$\text{Specific activity (U/mg)} = \frac{\Delta A / \text{min} \times V}{\varepsilon \times d \times E_w}$$

Where ΔA denotes the absorbance increment of the solution at 405 nm, V is the assay volume, ε is the molar extinction coefficient of p-NP (3909 $M^{-1}cm^{-1}$) estimated at pH 7.5 in PBS buffer containing triton x-100 (2 % v/v), d represents the cuvette path-length (1 cm) and E_w is the amount of free or immobilized enzyme.

In the case of free enzyme 25 μL of lipase solution 0.5 mg mL^{-1} were added to 2 mL of assay mixture consisting of 0.2 mL p-NPP 0.8 mM (dissolved in ethanol) and 1.8 mL PBS buffer (pH 7.5) containing triton x-100 (2 % v/v). In the case of immobilized lipase, the volume of assay mixture was increased to 10 mL and the lipase-loaded membrane (Ø 54 mm) was added to this mixture and incubated at 30 °C for 5 minutes, then the membrane was removed and the released p-NP measured by spectrophotometer.

6.3. Results and discussion

6.3.1. Activation and characterization of PVDF membrane

The PVDF membrane was modified with DAMP (carbonate buffer 1 M pH 11). When the PVDF membrane was treated with the alkaline solution, an elimination reaction occurred, in particular fluorine and hydrogen were eliminated, and then DAMP was grafted onto the membrane surface (PVDF-DAMP). The detailed mechanism of DAMP grafting and the characterization carried out are discussed in section 2.4.1. The PVDF-DAMP membrane was then treated with 20 mL of 10% (v/v) glutaraldehyde solution at 25 °C for 2 hours, in this way the amino groups were derivatized with glutaraldehyde (PVDF-GA).

The wettability of the membrane before and after the functionalization treatment was investigated by SCA measurements. The native PVDF membrane was strongly hydrophobic in fact the measured contact angle was 163° ± 9°. After the functionalization treatment the contact angle for the PVDF-GA membrane was 137° ± 5°.

6.3.2. Synthesis and Characterization of PAAm nanogels

Hydrophilic PAAm nanogels were successfully obtained via inverse miniemulsion polymerization. The addition of appropriate amounts of the nonionic low molecular weight surfactant Span 80 below its critical micelle concentration (CMC) in the continuous phase made sufficient stable the system. As described in literature the addition of NaCl as osmotic agent to the dispersed phase prevents diffusion between droplets and consequently the Ostwald ripening effect [8]. DLS measurements of PAAm in cyclohexane after the reaction indicating that the polymeric particles were generated in the desired size range around 130 nm ± 4 with narrow particle size distribution and polydispersity index (PDIs)

of 0.17 ± 0.02. After redispersion of the purified and freeze dried nanogels in water by swelling and application of ultrasound all samples show good colloidal stability but larger PDIs (0.27 ± 0.07) indicating the formation of agglomerates during the purification. In addition the mean particle size increased slightly up to 172 ± 8 nm in diameter, which can be explained by formation of dimer and oligomer particle assemblies and the swelling of the polymeric network without restrictions of the water droplet dimension in the heterophase system.

The network formation was monitored by conversion analysis via quantitative NMR experiments in D_2O. Since the conversion was estimated higher than 95 % (w/w) for all nanogel samples a proper network formation can be assumed, yielding gel particles with good structural integrity.

In order to ensure that the used surfactant has no influence on the targeted application of the nanogel particles, they were intensely washed to remove the Span80. Purification was confirmed by quantitative H-NMR analysis in $CDCl_3$ indicating that a surfactant amount lower than 1 % (w/w) of the total solid material remained in the hydrogel network, which was accepted as sufficient.

6.3.3. Preparation of nanogels with amino groups for enzyme immobilization

The Hofmann reaction was first discovered already at the end of the 19th century. However, only in the last decades it was intensively reinvestigated as suitable tool to convert amide functions into primary amine functions under elimination of a carbonyl group. It can be used to convert linear PAAm based polymers [16], as well as PAAm based microgel particles into copolymers with amine functionalities [17].

However, using this technique it has to be stated, that according to previous works some side reactions, such as hydrolysis of amide groups to carboxylic acids, formation of alkylacrylureas and alkylureas and the cleavage of the polymeric backbone may occur [18]. Achari et al. found that these reactions can be effectively prevented if the reaction temperature were low enough and a suitable molar ratio (α) of NaOCl to amide groups were used [12].

In this work the reaction was carried out at about α = 1 to avoid the simultaneous formation of carboxylic acids, what would have the potential to turn this material into an amphoteric ion exchanger with lower colloidal stability. In contrast to our expectations the nanogels obtained by Hoffman degradation showed prior to neutralization (pH = 14) good colloidal stability, but tends to form agglomerates around pH = 7. This is a first indication that the formation of carboxylic acid functions was not completely avoided leading to attractive interactions between positively charged amine groups and negatively charged carboxylic acid groups. After purification and freeze drying further analysis of the obtained nanogel was performed to investigate the formation of amines and side products.

After the reaction, FT-IR spectroscopy (Fig. 6.2) shows decreasing intensity for characteristic amide signals such as 1649 cm^{-1} (stretching vibration of amide carbonyl group), double peak at 1449 and 1414 cm^{-1} (stretching vibration of the amide carbon nitrogen bond) and the signals around 3189 cm^{-1} (stretching vibration of amide nitrogen hydrogen bond). In addition a new absorption peak can be detected at 761 cm^{-1}, it can be assigned to the nitrogen hydrogen wag typical for primary amines. The strong signal at 2918 cm^{-1} may be an indicator for hydrolysis products such as carboxylic acids, but no signal can be found around 1750 cm^{-1} what weakens this hypotheses.

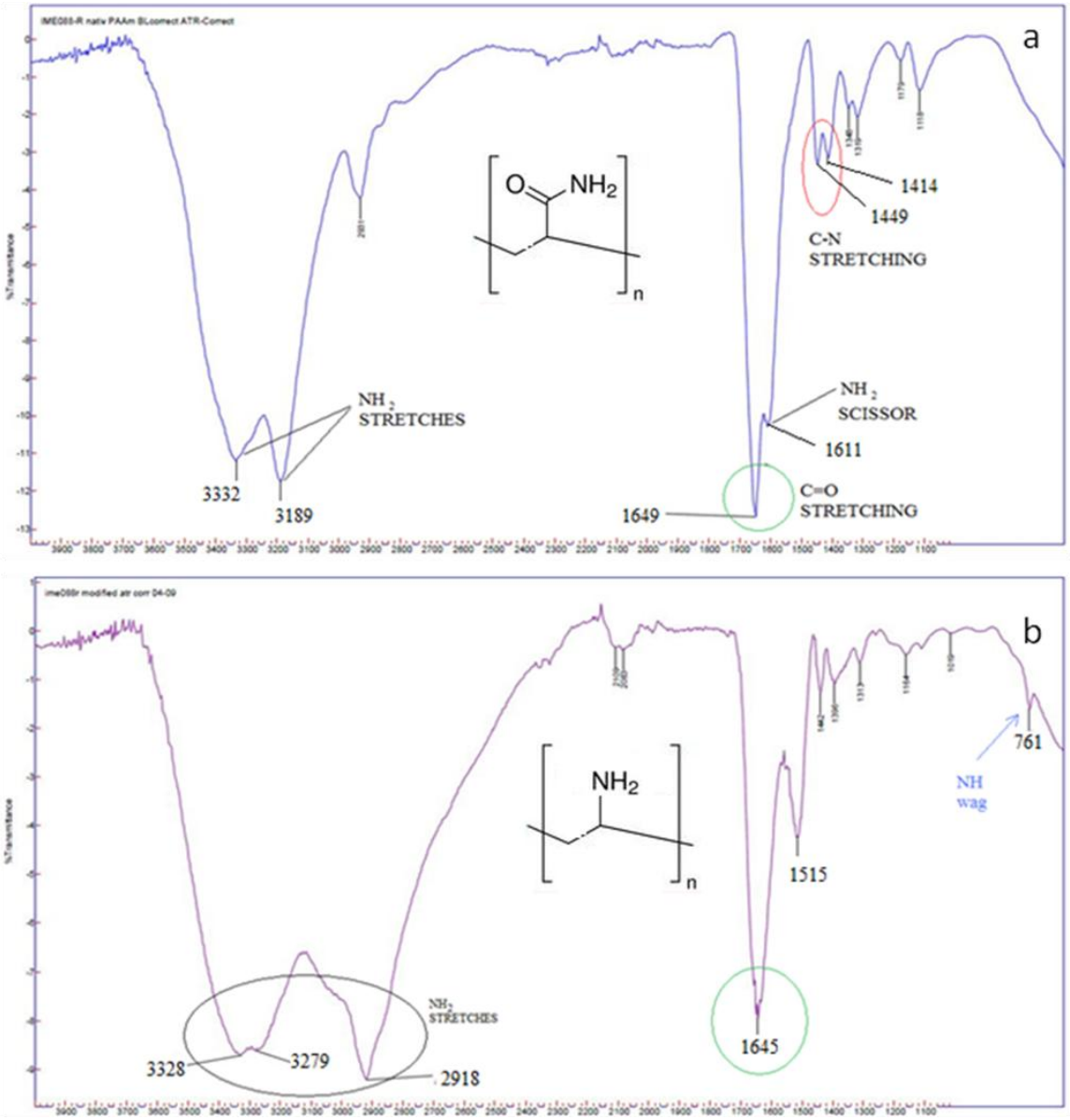
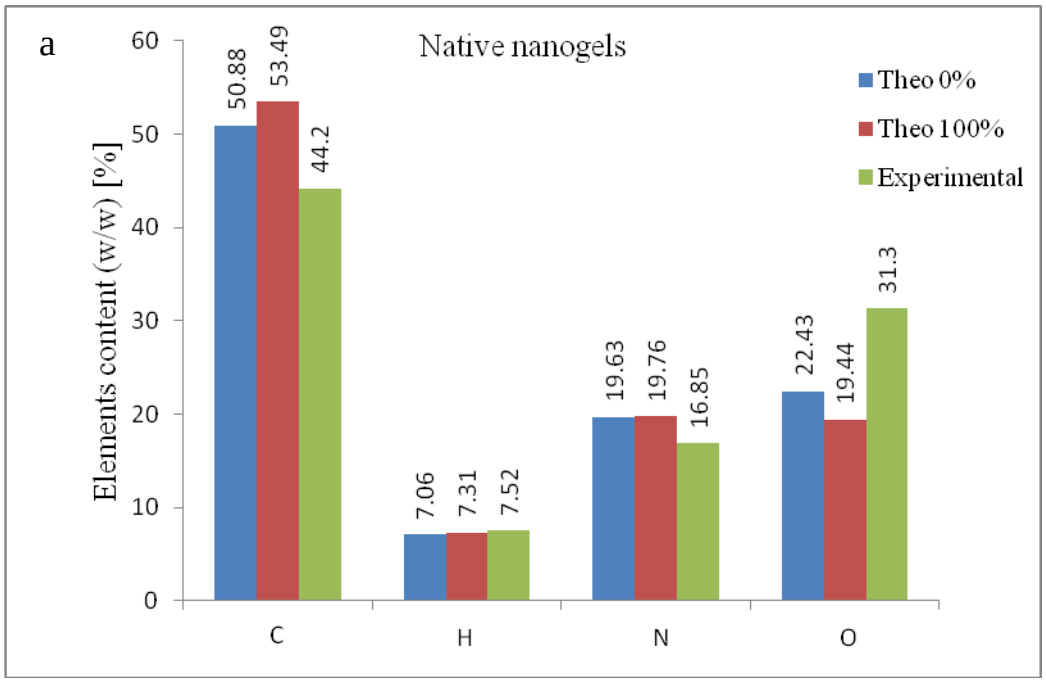


Fig. 6.2. FT IR spectra of native (a) and modified (b) PAAm nanogel

Additional a ninhydrin test was performed in order to detect primary and secondary amino groups. The ninhydrin reacts with free amino group under formation of a coloured complex in solution. In case of modified nanogels the solution changed colour indicating the presence of free amino groups.

The modification was also confirmed by measuring the C, H, N and O content of the native and modified samples via microanalysis. For interpretation of the analytical data, it was

important to calculate the theoretical C, H, N and O content of each sample by assuming a 100% yield of the hydrogel particle synthesis, using the amount of educts and initiator radicals (data presented in Fig. 6.3). Since for ideal inverse miniemulsion polymerizations the droplet nucleation mechanism is expected to be the dominant mechanism the 2-cyanoprop-2-yl radical formed by decomposition of azobisisobutyronitrile (AIBN) initiator is supposed to enter the droplets to initiate polymerization thus being incorporated in the hydrogel network. However, since the exact quantification of initiator incorporated in the network is not feasible, two unrealistic hypothetic cases have been considered. In the first case we consider that radicals are not incorporated in the network (Theoretical 0%), while in the second case, 100% of radicals are incorporated (Theoretical 100%). A realistic statement of the theoretic element contents can be achieved by using values between Theo 0% and Theo 100%. A proper value of the experimental elements contents should be comprised between Theo 0% and Theo 100%.



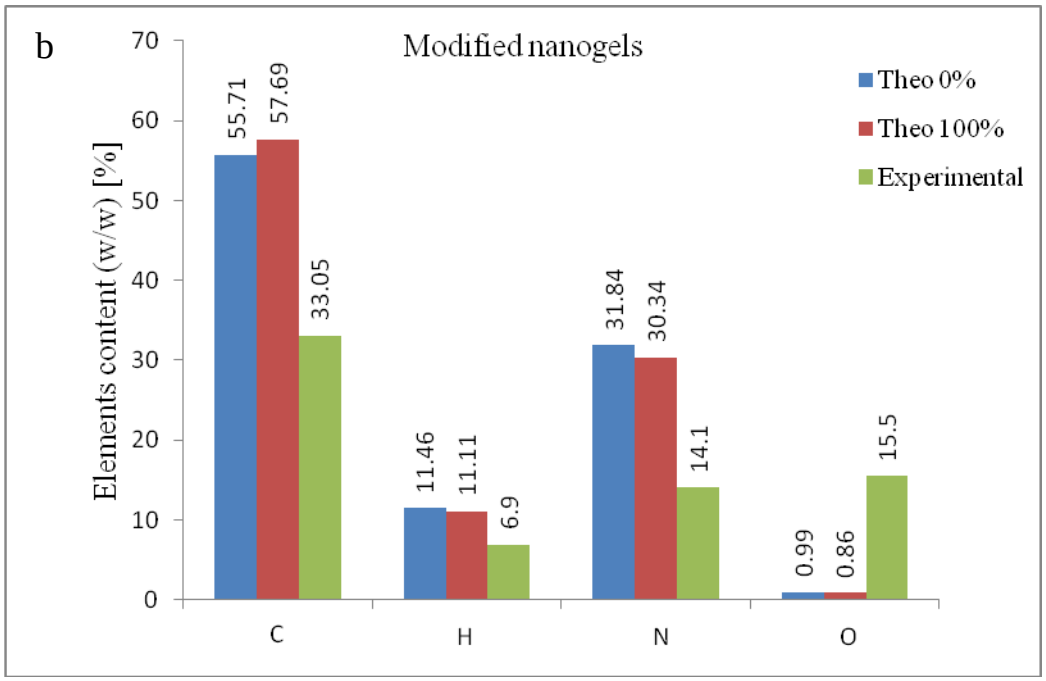


Fig. 6.3. C, H, N, O content of native (a) and modified (b) PAAm-nanogels related to the dry polymeric mass.

In Figure 6.3a, for native nanogels the experimental content of carbon and nitrogen is lower than the theory, while the hydrogen and oxygen content is higher than expected. These outcomes might be attributed to an incomplete drying process, in fact the nanogels are strongly hygroscopic, and therefore, a significant amount of water is still present in the sample. In this case water content increase the total mass, increasing the relative oxygen and hydrogen content, while lowering the carbon and nitrogen content pro rata.

However, if the carbon to nitrogen ratio is taken in consideration (see Fig. 6.4) it is evident that the experimental carbon nitrogen ratio (C/N) of the native PAAm-nanogels well fit to the theoretical values. When the modified nanogels are considered, a C/N value comprised between theoretical ratios for 0 and 100% conversions is expected. In fact, for the modified nanogels an experimental C/N value of 2.34 was found.

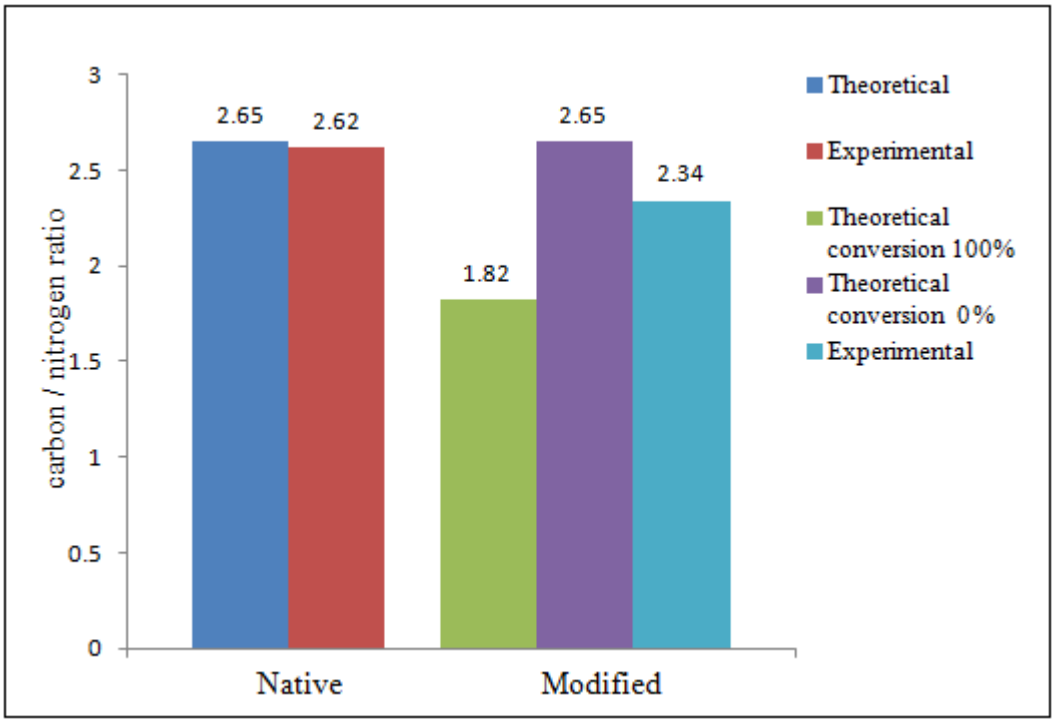


Fig. 6.4. Carbon to nitrogen ratio of native and modified nanogels

This result can be considered in order to estimate the conversion degree (% c) of the amide groups in amino groups. Using the equation 1 it was calculated to be 34%.

$$\% \, c = \frac{C/N_{(EXn)} - C/N_{(EXm)}}{C/N_{(Theo\ 0\%)} - C/N_{(Theo\ 100\%)}} \times 100 \qquad (1)$$

Where EXn is the experimental value for native nanogels, EXm is the experimental value for modified nanogels, Theo 0% is the theoretical value calculated for a 0% conversion and Theo 100% is the theoretical value calculated for a 100% conversion.

However, it has to be stated that this calculation neglected the possibility of side reactions such as the formation of carboxylic acid groups or urea derivates, which would increase or decrease the carbon to nitrogen ratio respectively.

6.3.4. Nanogels activation by GA treatment and Lipase loading

In order to covalent bind with lipase the nanogels were reacted with GA. Diafiltration was carried out to remove the unreacted GA. The presence of GA in the permeate was investigated by spectrophotometer, when GA wasn't detected in the permeate, diafiltration was stopped. The complete coupling of amino groups with GA was confirmed by the ninhydrin test that was negative after GA treatment.

Afterwards, lipase was immobilized on the GA-nanogels (Fig. 6.5). Aggregates appeared when the nanogels dispersion and the lipase solution were mixed. It was probably due to interactions of lipases molecule with different nanogels. The same lipase molecule reacting with aldehyde groups of different nanogels could promote the nanogels aggregation. However the macro aggregate were reduced by sonication indicating that lipase could promote also a reversible physical interaction between different nanogels particles. The remaining aggregate were removed by centrifugation and a clear supernatant (containing lipase loaded nanoparticles and free lipase) recovered. Free lipase was removed by diafiltration and the amount of lipase immobilized on the nanogels was 0.56 ± 0.1 mg.

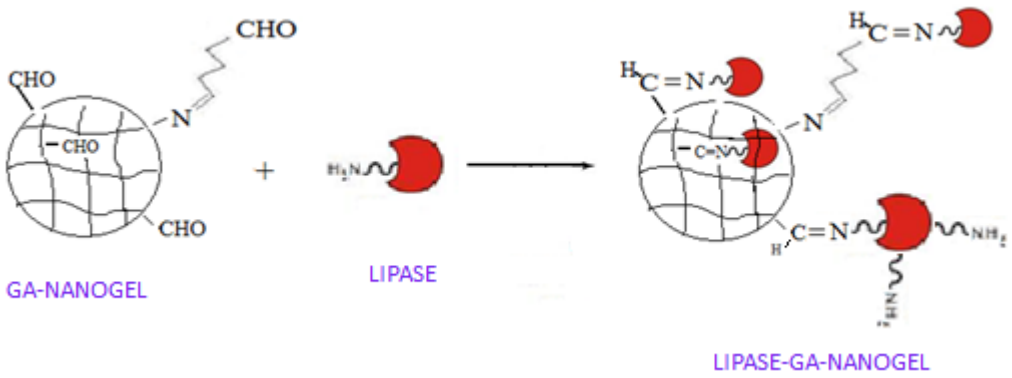


Fig. 6.5. Scheme of lipase immobilization on GA-functionalized nanogels

6.3.5. PVDF biofunctionalization by lipase-loaded nanogels

Immobilization of lipase-loaded nanogels on PVDF-DAMP-GA membrane was carried out by soaking the membrane in the nanogels dispersion. The immobilization protocol is illustrated schematically in Fig. 6.6. The aldehyde reactive groups of the membrane coupled with primary amino groups of lipase immobilized on the nanogels surface, and resulted in covalent conjugation of lipase-loaded nanogels onto the PVDF-DAMP-GA membrane (PVDF-GA-Lipase-GA-NANOGEL). The SCA measured for this membrane was 130° ± 7°. The amount of immobilized lipase was 26.2 ± 4.4 mg/m^2.

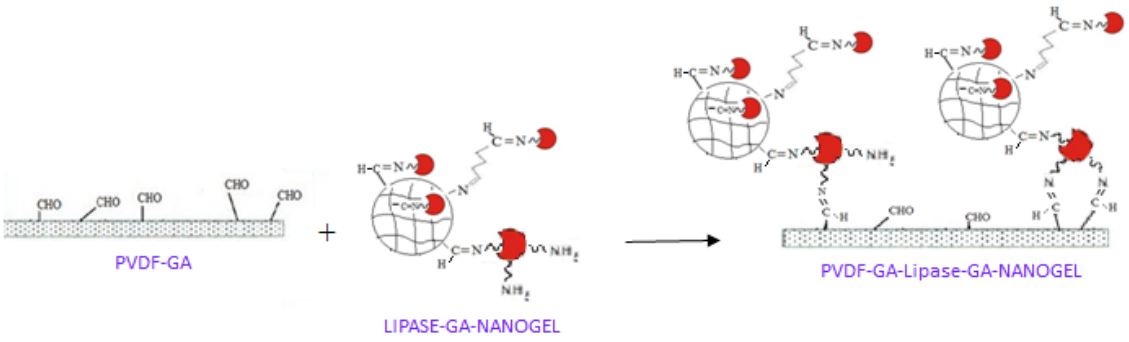


Fig. 6.6. Strategy used to immobilize lipase-loaded nanogels on functionalized PVDF membrane

6.3.6. Lipase attachment on nanogels-loaded PVDF membrane

An alternative strategy in order to immobilize enzyme using PAAm nanogels was developed. The immobilization protocol is showed in Fig. 6.7. In this protocol the amino groups introduced on the nanogel after Hoffman reaction were reacted with the aldehyde groups grafted on PVDF obtaining the nanogels-loaded membrane. The membrane was then washed and unreacted aldehyde groups were quenched by ethanolamine treatment [19]. Elimination of unreacted aldehyde groups is important to direct subsequent enzyme immobilization on the immobilized nanogels only. The amino groups of immobilized nanogels were then activated by GA treatment for enzyme immobilization. Afterwards,

lipase was immobilized on the membrane soaking the nanogels-loaded membrane in the lipase solution, in this way amino groups of lipase reacted with the aldehyde groups created on the immobilized nanogels obtaining the PVDF-GA-NANOGEL-Lipase membrane. The SCA measured for this membrane was 128° ± 4°. The amount of immobilized LCR was 743 ± 122 mg/m^2.

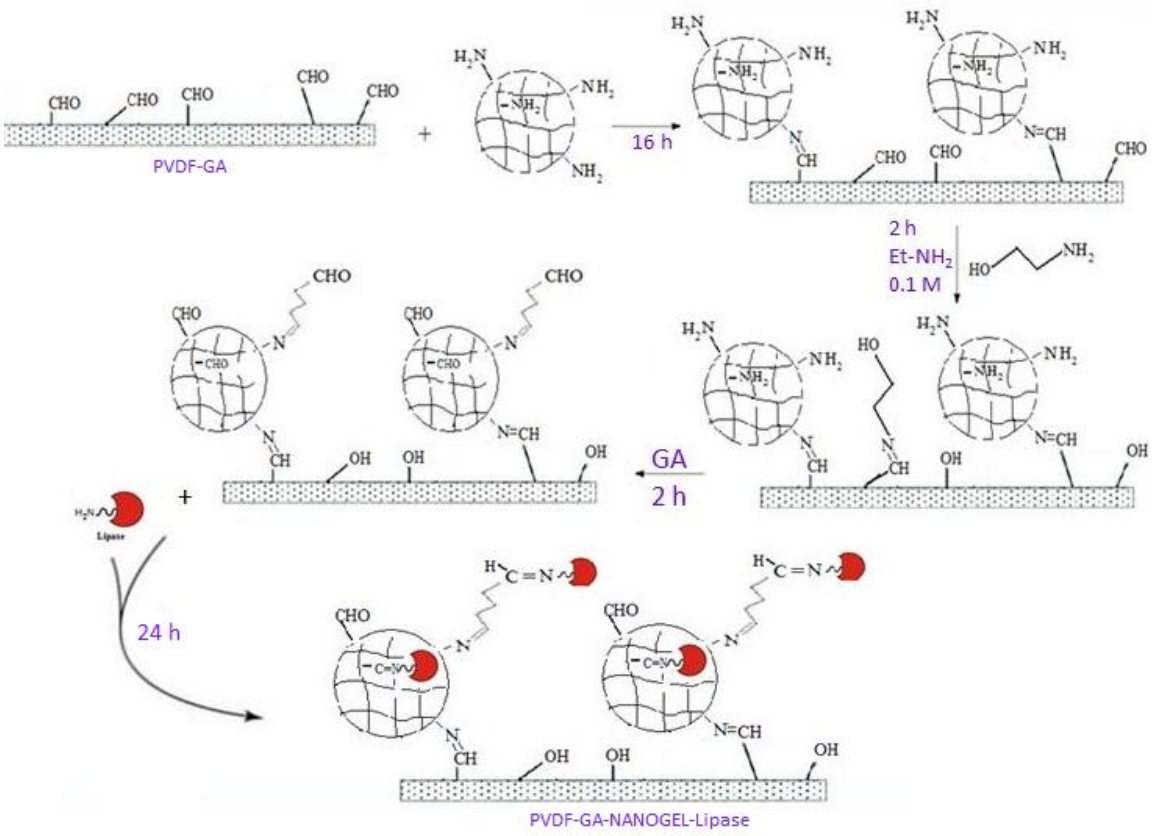


Fig. 6.7. Immobilization of LCR on a PVDF membrane previously loaded with nanogels

6.3.7. Lipase immobilization on PVDF-DAMP-GA membrane

The third type of enzyme immobilization was carried out exploiting aldehyde groups present on PVDF-GA membrane and primary amino groups present on lipase (PVDF-GA-Lipase). This easy methodology (illustrated in Fig. 6.8) takes place yielding a Schiff base

[20] and allows immobilizing lipase in mild conditions. The SCA measured for this membrane was 135° ± 3°. The amount of immobilized LCR was 976 ± 68 mg/m^2.

Fig. 6.8. Strategy used to perform direct lipase immobilization on PVDF-GA membrane

6.3.8. Catalytic performances of free and immobilized enzyme

The biocatalytic activity of free lipase was evaluated and compared with the activity of lipase immobilized by the three different strategies above reported. The specific activity of the free lipase in solution was determined to be 16.5 ± 1.7 U/mg. Among the immobilization protocols proposed, the enzyme exhibited the highest specific activity when it was first immobilized on the nanogels and then immobilized on the PVDF-GA membrane (PVDF-GA-Lipase-GA-NANOGEL). In this case the specific activity was 0.85 ± 0.1 U/mg with an activity per membrane area of 22.3 ± 3 U/m^2. However, when lipase was immobilized on the PVDF membrane previously loaded with the nanogels (PVDF-GA-NANOGEL-Lipase), its specific activity was only slightly lower (0.53 ± 0.11 U/mg) compared to PVDF-GA-Lipase-GA-NANOGEL membrane but the activity per membrane area was much higher (394 ± 76 U/m^2) due to the higher amount of immobilized lipase. Lipase immobilized without employ the nanogels (PVDF-GA-Lipase) exhibited the lowest specific activity (0.23 ± 0.3 U/mg) and activity per membrane area of 224.5 ± 26 U/m^2. Based on the contact angles results it is evident that the 3 strategies used for enzyme immobilization didn't affect significantly the PVDF membrane hydrophobicity, but the presence of nanogels which protect the enzyme from contact with the membrane and at the

same time ensures a better interaction of enzyme with water soluble substrates is able to improve the enzyme performance in both cases in which nanogels were applied. However should be considered that these observed specific activity values are highly influenced by mass transfer properties and therefore strongly underestimated. In fact in heterogeneous systems the overall reaction mechanism depends on the type of mass transfer of the reactants from the bulk solution to the enzyme and vice versa for the products [21].

6.4 Conclusions

An important challenge in enzyme immobilization strategies is the retention of biocatalytic activity of the bioconjugate. In this work we investigated the possibility of improve the performance of enzymes immobilized on hydrophobic membrane (PVDF) using colloidal polyacrilamide (PAAm) nanogels as carrier for enzyme immobilization. The proposed strategy allowed keeping the enzymes in a local hydrated microenvironment on the hydrophobic membrane without affect the membrane hydrophobicity. In order to promote a covalently interaction enzyme-nanogel or nanogel-membrane, amino functions have been imparted to the nanogels applying the Hofmann reaction; they were then activated for biomolecules immobilization by glutaraldehyde reaction. The model biomolecule lipase was immobilized on the PVDF membrane employing the PAAm nanogels in two different strategies. In the first one, lipase was immobilized on the nanogels and then the lipase loaded nanogels were immobilized on the functionalized PVDF membrane (PVDF-GA-Lipase-GA-NANOGEL). In the second one, the PAAm nanogels were immobilized on the functionalized PVDF membrane and then the lipase was immobilized on the nanogels-loaded membrane (PVDF-GA-NANOGEL-Lipase). Comparing the specific activity of lipase immobilized by means of nanogels, with both strategies, with the performance of

lipase immobilized without nanogels an improvement was noticed. We believe that the use of these strategies shows promise for extension to other enzyme systems, and this work is currently being pursued.

References

[1] S. Datta, L. R. Christena, Y. R. Sriramulu Rajaram, Enzyme immobilization: an overview on techniques and support materials. 3 Biotech 3 (2013) 1–9

[2] U. Hanefeld, L. Gardossi, E. Magner, Understanding enzyme immobilisation. Chem. Soc. Rev. 38 (2009) 453–468

[3] R. A. Sheldon, Enzyme Immobilization: The Quest for Optimum Performance. Adv. Synth. Catal. 349 (2007) 1289 – 1307

[4] P. Jochems, Y. Satyawali, L. Diels, W. Dejonghe, Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs), Green Chem. 13 (2011) 1609

[5] G.J. Chen, C.H. Kuo, C.I. Chen, C.C. Yu, C.J. Shieh and Y.C. Liu, Effect of membranes with various hydrophobic/hydrophilic properties on lipase immobilizer activity and stability, Journal of bioscience and Bioengineering 113 (2012) 166-172

[6] S. Nayak, L. A. Lyon, Soft Nanotechnology with Soft Nanoparticles. Angew. Chem. Int. Ed. Engl. 44 (2005) 7686-7708.

[7] A. Pich, W. Richtering, Microgels by precipitation polymerization: synthesis, characterization, and functionalization. Adv. Polym. Sci. 234 (2010) 1-37.

[8] A. Patanarut, E. H. Williams, E. Petricoin, L. A. Liotta, B. Bishop, Microspheres containing cibacron blue F3G-A and incorporated iron oxide nanoparticles as biomarker harvesting platforms Polymers 3 (2011) 1181-1198.

[10] K. Landfester, M. Willert, M. Antonietti, Preparation of polymer particles in non aqueous direct and inverse miniemulsions. Macromolecules 33 (2000) 2370-2376

[11] I. Capek. On inverse miniemulsion polymerization of conventional water-soluble

monomers. Advances in Colloidal and Interface Science 156 (2010) 36-61

[12] El Achari, X. Coqueret, A. Lablache-Combier, C. Loucheux, preparation of polyvinylamine from polyacrylamide: a reinvestigation of the Hofmann reaction. Makromol. Chem. 194 (1993) 1879-1891

[13] Y. Yamamoto, M.V. Sefton, Hofmann Degradation of Acrylamide Copolymer: Synthesis of Amine Functionalized Thermoplastic Hydrogel. Journal of Applied Polymer Science 61 (1996) 351-358

[14] G. Vitola, R. Mazzei, E. Fontananova, L. Giorno PVDF, Membrane biofunctionalization by chemical grafting, J. Membr. Sci. 476 (2015) 483-489

[15] N. Gupta, P. Rathi, R. Gupta, Simplified para-nitrophenyl palmitate assay for lipases and esterases. Analytical Biochemistry 311 (2002) 98–99

[16] N. Oz, A. Akar, Low Molecular Weight Polyacrylic Acid with Pendant Aminomethylene Phosphonic Acid Groups. Journal of Applied Polymer Science 78 (2000) 870–874

[17] J. Hong, P. J. Gong, D. Xu, L. Dong, S. Yao, Stabilization of α-chymotrypsin by covalent immobilization on amine-functionalized superparamagnetic nanogel. J. Biotechnol. 128 (2007) 597-605.

[18] Z. Hu, S. Zhang, J. Yang, Y. Chen, Some Properties of Aqueous-Solutions of Poly(vinylaminechloride). Journal of Applied Polymer Science 89 (2003) 3889–3893

[19] S.W. Park, J.W. Lee, S. I. Hong, S.W. Kim, Immobilization of Glutaryl-7-aminocephalosporanic Acid Acylase on Silica Gel and Enhancement of Its Stability Applied Biochemistry and Biotechnology 104 (2003) 185-198

[20] D.H. Lee, C.H. Park, J.M. Yeo, S.W. Kim, Lipase Immobilization on Silica Gel Using a Cross-linking Method, J. Ind. Eng. Chem. 5 (2006) 777-782.

[21] J. Zhang, G. Barbieri, F. Scura, L. Giorno, E. Drioli, Modeling of two separate phase enzyme membrane reactors for kinetic resolution of naproxen ester, Desalination 200 (2006) 514–515

OVERALL CONCLUSION

This thesis work was directed towards the development of biohybrid membranes able to decontaminate streams containing organophosphate pesticides. The work was focused on the study of functionalization methods for membranes based on polyvinylidene fluoride (PVDF) and polyethersulfone (PES) polymers. The functionalized membranes were used as support for biomolecules immobilization obtaining biofunctionalized membranes. The conditions for PVDF and PES biofunctionalization were investigated employing two model biomolecules, the protein bovine serum albumin (BSA) and the enzyme lipase from *candida rugosa* (LCR). Afterwards, the optimized conditions were used for biofunctionalization of PVDF and PES with phosphotriesterase (PTE).

For what concerns the PVDF membrane, reactive amino groups suitable for biomolecules immobilization were grafted on the PVDF surface using the bifunctional agent 1,5-diamino-2-methylpentane (DAMP) in alkaline conditions. The amino groups were then activated for biomolecules immobilization by glutaraldehyde treatment. The protein bovine serum albumin was immobilized on the functionalized membrane in order to evaluate the binding properties of the modified membranes. Afterwards, lipase has been immobilized on the PVDF membrane in order to develop a two separate phase biocatalytic membrane reactor in which the membrane works as both catalytic and separation unit. The performance of the biphasic enzymatic membrane reactor was evaluated based on the fatty acids extraction in the aqueous phase. The results obtained give a contribution to develop processes based on PVDF biocatalytic membrane, because by the proposed strategy the amount of biomolecules immobilized on the PVDF membrane can be predicted varying the degree of grafting and a suitable compromise can be found also considering the mechanical

properties of the membranes which are reduced in correlation with the increase of the degree of modification.

A phosphotriesterases enzyme from *Sulfolobus solfataricus* (a hyperthermophilic archaeal) has been immobilized on the functionalized PVDF membrane and on glutaraldehyde-activated PES membrane, with the aim to develop biocatalytic membrane systems able to perform organophosphate decontamination. For the first time a thermostable phosphotriesterase has been immobilized and characterized for hydrolysis of organophosphate pesticide (paraoxon). The impact of membranes with different wettability, such as PES and PVDF membranes was studied with the final aim to treat either contaminated liquid or vapour streams. Considering both retained phosphotriesterase activity and membrane permeability, PES membranes showed superior performance in comparison with PVDF membranes when the target was liquid phase decontamination. The better behaviour of phosphotriesterase immobilized on PES membrane was due to the high wettability of the PES that makes it better wetted by the aqueous solution in which the substrate (paraoxon) is dissolved keeping the enzyme in highly hydrophilic environment that is required to perform the hydrolysis reaction. On the other hand same results in terms of water vapour flux were obtained in preliminary studies performed on both biofunctionalized PES and PVDF membrane. This opens future positive perspectives in the use of both polymers for organophosphate gas degradation.

In order to improve the activity of enzymes immobilized on PVDF membrane, a strategy to immobilize the enzyme kept in hydrated state, within the hydrophobic membrane environment, was developed. Highly hydrophilic polyacrylamide (pAAm) hydrogel of colloidal dimension were synthesized using the inverse miniemulsion polymerization techniques. After dispersions in water the pAAm nanogels were characterized in terms of

hydrodynamic particle diameter and polydispersity index (PDI) by dynamic light scattering (DLS) measurements. Afterwards, the pAAm nanogels were functionalized to covalent bind enzymes, in particular amino groups were created into the polymer net by Hofmann reaction. The functionalized nanogels were then used as lipase carrier applying two different strategies. The first strategy was based on the production of biocatalytic nanogels immobilizing lipase on glutaraldehyde activated nanogels. The lipase-loaded nanogels were subsequently attached by covalent interaction on the glutaraldehyde-functionalized PVDF membrane. The second strategy was performed immobilizing the amino functionalized nanogels on the glutaraldehyde activated PVDF membrane to produce a nanogels-loaded membrane that was subsequently used to immobilize lipase. The performances of the lipase immobilized in presence or not of nanogels were evaluated using p-nitrophenyl palmitate as substrate. The results showed that an improvement in lipase activity was obtained in both cases in which nanogels were used. This open interesting perspectives for biohybrid systems in which the hydrophilic microenvironment is required to improve enzyme activity but the overall membrane hydrophobicity must be preserved.

APPENDIX

PUBBLICATIONS

- G. Vitola, R. Mazzei, E. Fontananova, L. Giorno PVDF membrane biofunctionalization by chemical grafting, J. Membr. Sci. 476 (2015) 483-489

- G. Vitola, R. Mazzei, E. Fontananova, E. Porzio, G. Manco, S. N. Gaeta, L. Giorno. Polymeric biocatalytic membranes with immobilized thermostable phosphotriesterase to detoxify organophosphate pesticides. (submitted to J. Membr. Sci.)
- G. Vitola, D. Buning, M. Ulbricht, R. Mazzei, L. Giorno. Development of a novel immobilization method for maintaining enzyme in hydrated microenvironment on hydrophobic membranes (in submission).

PROCEEDINGS

Oral presentations

- R. Mazzei, **G.Vitola,** E. Fontananova, C. Algieri, L. Donato, L. Giorno, Functionalized polymeric membrane for biomolecules immobilization, Early-stage and Experienced Researchers Seminars, December 18 -19, 2013, Rende (CS)

- **G.Vitola,** R. Mazzei, E. Fontananova, L. Giorno, Biomolecules immobilization on PVDF membranes functionalized with 1,5-diamino-2-methylpentane (DAMP), The 10^{th} international Congress on Membranes and Membrane Processes ICOM 2014, Suzhuo China, July 20-25, 2014.

- **G.Vitola,** R. Mazzei, E. Fontananova, E. Porzio, G. Manco, L. Giorno, Treatment of organophosphate pesticides using bio-catalytic membranes. Early-stage and Experienced Researchers Seminars, December 18 -19, 2014, Rende (CS).

- **G.Vitola,** D. Büning, M. Ulbricht, R. Mazzei, E. Fontananova, L. Giorno Development of a novel immobilization method to keep enzyme in hydrated microenvironment on hydrophobic membranes. Early-stage and Experienced Researchers Seminars, November 25 -26, 2015, Rende (CS).

Poster presentations

- **G.Vitola,** R. Mazzei, E. Fontananova, L. Giorno, PVDF membrane surface functionalization for biomolecules immobilization, EMS Summer School on "Membranes for Liquid Separation" , July 22-26, 2013, Essen (Germany)

- R. Mazzei, **G.Vitola,** E. Fontananova, C. Algieri, L. Donato, L. Giorno, Biomolecules immobilization on functionalized membrane, Early-stage and Experienced Researchers Seminars, December 18 -19, 2013, Rende (CS).

- **G.Vitola,** R. Mazzei, E. Fontananova, L. Giorno, Modification of polymeric membranes for biomolecules immobilization, XXXI EMS Summer School on "Innovative Membrane Systems", September 28 - October 3, 2014, Cetraro (CS).

- **G. Vitola**, R. Mazzei, E. Fontananova, E. Porzio, G. Manco, S. N. Gaeta, L. Giorno. Phosphotriesterase loaded membranes to detoxify organophosphate pesticides. Early-stage and Experienced Researchers Seminars, November 25 -26, 2015, Rende (CS).

EDUCATION AND TRAINING FROM 2012 TO 2015

- "Membrane course", January – March 2013, ITM-CNR, Rende (CS). Lecturers: Prof. E. Drioli, Dr. A. Figoli, Dr. Eng. Adele Brunetti, Dr. Eng. E. Curcio, Dr. Eng.

G. Di Profio, Dr. Eng. G. Barbieri, Dr. Lidietta Giorno, Dr. L. De Bartolo, Dr. S. Morelli, Dr. Alfredo Cassano, Dr. E. Fontananova

- "Course on Gas and Vapour Separation" a Course in the framework of the Erasmus Bilateral Agreement between UNICAL/ITM and ICT of Prague, 4-5 June, 2013, Rende (CS)
- EMS Summer School on "Membranes for Liquid Separation" in Essen (Germany) from the 22th to the 26th of July
- Symposium within the frame of the ITM-CNR - Hanyang University Joint International Laboratory on Membrane Technologies, October 4, 2013 , Rende (CS)
- "Membrane materials: Challenges and opportunities", Seminar series for young researchers, October 10, 2013, Rende (CS)
- "Polymers of intrinsic microporosity (PIMs): From idea to reality", Seminar series for young researchers, October 11, 2013, Rende (CS)
- "Graphene: Turning Nobel-prize winning science into practical technology", Seminar series for young researchers, October 14, 2013, Rende (CS)
- "Symposium on polymeric membranes for gas and vapour separations", October 15, 2013 Rende (CS)
- "Membrane operations and integrated membrane processes", ITM-CNR Seminar Days, October 30, 2013, Rende (CS)
- "Advances in membrane preparation and characterization", ITM-CNR Seminar Days, October 30, 2013, Rende (CS)
- "Membranes in Biotechnology and Biomedicine", ITM-CNR Seminar Days, October 31, 2013, Rende (CS)

- Early-stage and Experienced Researchers Seminars, December 18th - 19th, 2013, University of Calabria, Rende (CS)
- "Membrane processes course: phenomena at the phase interfaces" a Course in the framework of the Erasmus Bilateral Agreement between UNICAL/ITM and ICT of Prague, 10-12 June, 2014, Rende (CS)
- The 10th international Congress on Membranes and Membrane Processes ICOM 2014, Suzhuo China, July 20-25, 2014.
- XXXI EMS Summer School on "Innovative Membrane Systems", held in Cetraro (CS) from September 28 to October 3, 2014.
- Lecture: "Functionalized Separation Membranes", given by Prof. Dr. Mathias Ulbricht, July 7, 2015, University Duisburg-Essen
- Lecture: "Systematic Material Design and Development for Fuel Cells", given by Prof. Dr. Takeo Yamaguchi, July 7, 2015, University Duisburg-Essen