

# Synthesis of defined monodisperse microgel-protein conjugates and immobilization on Gold and ITO.

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Dedicated to those giants  
whose shoulders we stand on so that we can see further



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## **Abstract**

The goal of the study is to create a new method to preserve the activity of biocomponents. These active biocomponents will be further used to build up a photoelectrically driven enzyme system that combine bio-catalysis with electrochemistry with the final use as biosensor. In order to preserve the activity of proteins and enzymes, a novel way came up in the recent years: encapsulation in polymer microgels. In the first part of the thesis the encapsulation of enzymes and proteins will be exploited. The biocomponents that will be used are Glucose Oxidase and Bovine Serum Albumin. The encapsulation will be performed in two ways: with batch polymerization and with a pressure driven continuous synthesis plant (microfluidics device). The batch method gives a high rate of production with a low degree of monodispersity. The chosen polymer is Polyacrylamide. The activity after the syntheses will be characterized and for each synthesis a loss ratio will be defined. The characterization will contemplate also a morphological analysis. In order to reach a satisfactory loss ratio (good retaining of the enzymatic activity) the parameters of the synthesis will be engineered and the supplement of additives will be exploited. In order to reach a satisfactory degree of monodispersity, the microgel formation has been carried out by means of a microfluidics device. The parameters of the droplet formation will be presented.

A smaller topic that will be touched in the thesis is the immobilization of the microgels onto gold electrodes and Indium Tin Oxide (ITO) electrodes. Both physisorption and chemisorption will be treated.



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# Chapter 1

## Introduction

Nowadays, polymer microgels represent an interesting group of gels with numerous industrial and pharmaceutical applications. They undergo reversible volume change between collapsed and swollen states in response to an external stimuli. This change is due to any of the four fundamental interactions acting in these materials: Van der Waals, hydrophobic, electrostatic and hydrogen bonding [2]. Even if the interest in the scientific community has grown really fast and several studies have been performed in this topic, the knowledge about the structure of microgels is still limited because of the large number of parameters which influence their structure. Examples of these parameters are the type of solvent, the concentration of the polymer, the degree of crosslinking and ionization, the swelling and the concentration of added salt.

Although these synthesis drawbacks, microgels are one of the strongest candidates to revolutionize the contemporary world. The most interesting applications are in the biological and medical field. From drug releasing to encapsulation and protection of biological species, the applications are countless and they are growing day by day thanks to the efforts and fast progresses that science is doing in this field. The entrapment of drugs, enzymes and macromolecules inside microgel particles opens new possibilities also in the fabrication of new biosensors. In fact, enzymes and proteins can be used as sensors as long as they retain their activity. Therefore, chemical and physical stability of enzymes are essential requirements in enzyme sensors. In typical biosensors, enzymes are immobilized onto membranes or surfaces, being in contact with denaturing agents from the environment; they rapidly can lose their activity. By means of the encapsulation inside polymer microgels, enzymes can be well protected from inactivating agents such as solvents, mechanical stresses, changes in temperature, etc.

In view of their promising future, microgels and encapsulation have been studied in this thesis. The goal of the study is to synthesize well-defined microgels with biocomponent species entrapped inside. Another important goal is the achievement of the monodispersity, in order to not create imbalances in the enzyme/protein load inside the microgels. The chosen polymer to produce the microgels is Polyacrylamide (PAA) due to its low cost and ease of synthesis. The goal is to create a microgel template that can be filled with whatever the enzyme or protein and protect it. In order to perform the study, one protein and one enzyme have been chosen: respectively Bovine Serum Albumin (BSA) and Glucose Oxidase (GOD). BSA have been chosen since the low price and the possibility to calculate its concentration inside the microgels by means of Bradford assay or BCA assay. On the other hand, GOD have been selected since it is one of the most studied and important enzymes on the scientific panorama. In particular,

it is commonly used in biosensors for glucose, since its capacity to catalyze its oxidation reaction. Moreover, this oxidation reaction is simple to be performed and reproduced and the data survey can be easily carried out by means of a photometer. Among this encapsulation process, the goal is to preserve the activity of glucose oxidase during the synthesis of the microgels. In fact, immobilized enzymes usually experience denaturation due to the chemical process they have to undergo, i.e. the active centers of the enzyme can be inhibited by the interaction with the chemicals present in the synthesis. Another reason of denaturation originates from the change of the physical parameters of the environment. In fact during the synthesis of the microgels, changes in temperature or pressure of the system may occur. The synthesis of the microgels has been created and tailored in order to overcome the chemical drawbacks and retain the activity of the entrapped enzyme. In the study, the parameters that have been controlled are the crosslinking degree, the content of organic solvent, the content of surfactants and the load of GOD itself. In order to also control the physical changes, the supplement of additives have been tried.

In order to achieve a high degree of monodispersity of the microgels, a pressure-driven microfluidic setup will be built to carry out the synthesis also in this way. The study contemplates the droplet formation of the precursor of the microgels. A secondary goal that will be treated in the work is the immobilization of the microgels onto predetermined surfaces. This task has been thought in order to build up a new type of biosensors in which the biosensing species are durable and protected from any possible threat from the external environment. In fact, the substrates on which the attempts of immobilization will be carried out are Gold and Indium Tin Oxide. The characteristic of these two substrates is their high electrical conductivity, in order to transmit the received signal to the electronics that will display the result.

## Chapter 2

### State of the art

#### 2.1 Enzymes

Enzymes were discovered more than 5000 years ago when people started to store milk in the animal stomachs. Stomachs are full of enzymes called "rennet", able to turn milk into cheese. This is an appropriate example to describe how enzymes work: they speed up biological reactions. An enzyme is a macromolecular biological catalyst, it accelerates chemical reactions. At the beginning of the reaction, the molecule that is supposed to react (what in chemistry is named reagent) and catalyzed by the enzyme is called substrate. Then, at the end of the reaction, the substrate is transformed in another molecule (product) through the conversion by means of the enzyme.

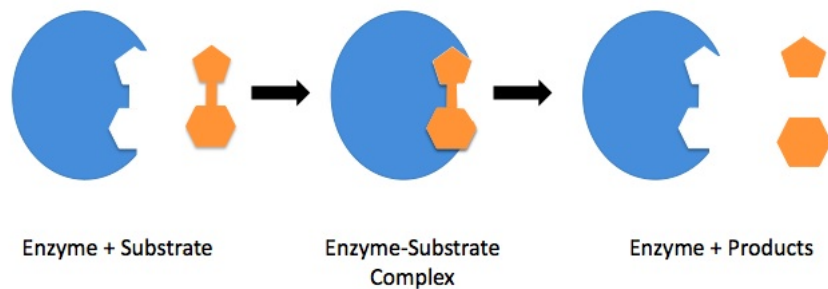


Figure 2.1: Enzyme catalyzed-reaction: formation of an intermediate complex before the obtaining of the products [3].

In Figure 2.1 the mechanism of an enzyme catalyzed-biological reaction is shown. The role of the enzyme is to provide to the substrate a spot (surface) to let the reaction happen. When the two components are in contact, the formation of an intermediate complex occurs. The substrate gets attached to the enzyme that has a specific structure; this step is selective. In fact, the structure of the enzyme can fit only in a similar substrate with a specific reading key. Some enzymes can convert their substrate with a rate magnitude  $10^6$  times higher. As schematized in Figure 2.2 the role of an enzyme is to decrease the activation energy of the reaction[4].

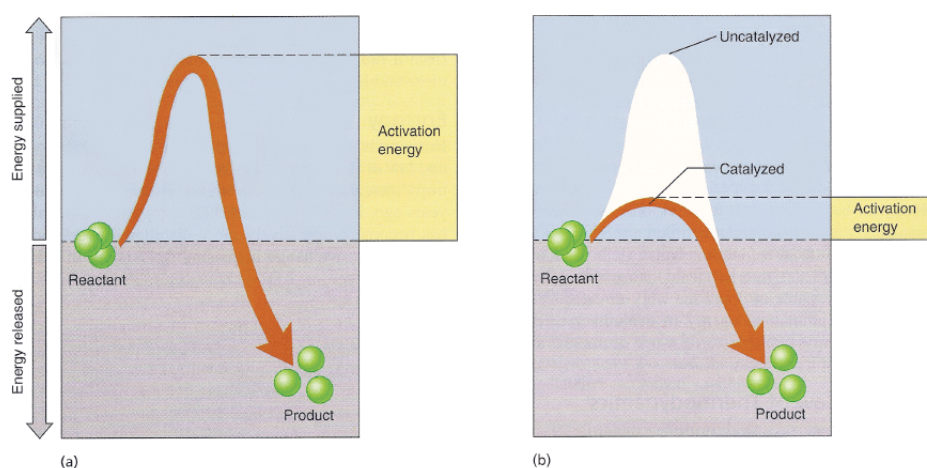


Figure 2.2: Lowering of the activation energy in a catalyzed reaction [5].

An extreme example of the lowering of the activation energy of an enzyme is given by 5'-phosphate decarboxylase. This enzyme shortens a reaction to milliseconds instead of millions of years [6]. From a chemical point of view, enzymes behave like any other catalyst. In fact, during a reaction they are not consumed and they do not alter the equilibrium of the reaction. The issue that made the entire scientific community spend a lot of time and funds on the discovery of new enzymes is that they are much more specific. Almost all the enzymes are proteins (some are RNA molecules). They have a three-dimensional structure that provides their unique specificity. An enzyme is characterized by an enzymatic activity, defined as the moles of substrate converted per unit time [7]. This activity might be lowered by some other molecules: inhibitors. For instance, drugs or poisons are inhibitors. On the other hand, some molecules can increase the activity of an enzyme: they are called activators. The activity is also affected by the environment in which they react. The most important parameters to control are temperature and pH [8].

### 2.1.1 History and etymology

The first scientist who discovered an enzyme was Anselme Payen (1833). The enzyme was diastase [9]. Successively, Louis Pasteur studied the fermentation of sugar to alcohol by yeast and concluded that the process was caused by a vital force that he called ferment [10]. This vital force was properly named by Wilhelm Kühne in 1877, who distinguished the word enzyme from the word ferment: the first refers to nonliving substances while the second refers to chemical activity produced by living organisms [11]. The definitive turning point was achieved by Eduard Buchner in 1897, who published the first paper about yeast extracts. At the university of Berlin, he discovered the cell-free fermentation of sucrose and he gained a Nobel prize in 1907 [12]. Buchner named his enzyme zymase. From that moment enzymes are usually denominated as the suffix *-ase* plus the substrate of the reaction they carry out (e.g., lactase is the enzyme that cleaves lactose) or to the type of reaction (e.g., glucose oxidase is the reaction of oxidation of glucose).

Further in the years, the scientific community tended to associate enzymes and proteins until Nobel laureates John H. Northrop and Wendell M. Stanley proved in 1930 that pure proteins can be enzymes, working on the digestive enzymes pepsin, trypsin and chymotrypsin [13]. The beginning of the field of structural biology and the research in order to understand how enzymes work at an atomic level started with the discovery of the crystallization of enzymes and the possibility to detect their structure by means of x-ray crystallography. This pivotal moment happened in 1965, when the group led by David C. Phillips solved the structure of lysozyme [14].

### 2.1.2 Structure and activity

Enzymes are generally globular proteins, acting alone or in larger complexes. It is a three-dimensional arrangement of atoms. The base concept of the structure of proteins is the same of the polymers: they are formed from sequences of monomer. Each monomer is called as an aminoacids residue, that refers to a sequence of aminoacids. The reaction that creates proteins is a condensation reaction: aminoacids lose one molecule of water per reaction in order to create a chain. The created bond between aminoacids is a peptide bond. A chain with less than 30 aminoacids is named as peptide; with more than 30 aminoacids it is named protein (polypeptides). Proteins are then folded into spatial conformations. The formation of this conformation is driven by non-covalent interactions between the chains such as hydrogen bonding, ionic interactions, Van der Waals forces and hydrophobic packing. The number of aminoacids present in the structure vary between tens to several thousands. As consequence, the physical size is not well determined. It is in the range of 1-100 nm, so proteins can be classified as nanostructures. While a protein is performing its biological function, it can undergo reversible or irreversible structural changes, as unfolding. This unfolding is also called denaturation: it means that the protein is losing activity. If irreversible denaturation happens not during the specific use of the enzyme, the molecule is not usable anymore; it has no more activity [15]. The structure of a protein/enzyme can be studied on four levels: primary structure, secondary structure, tertiary structure and quaternary structure. Those structure are:

- **Primary structure**

The linear sequence of aminoacids in a protein is the primary structure. The aminoacids are held together by covalent bonds, called peptide bonds. The linear aminoacids chain is named polypeptide. Depending on the nature of the extremities of the chain, they are called carboxyl terminus (called C-terminus) or the amino terminus (N-terminus). In Figure 2.3 a general primary structure is displayed.

In an aminoacid chain, the count of the aminoacids residues always starts with an non-bonded amino group . Every protein has a specific sequence of aminoacids residues that is extremely unique and specific to that protein: it defines the structure and the function of the protein itself. In the human body, there are more than 10,000 proteins which are composed of different arrangement of 20 types of aminoacids. In some proteins, post-formation

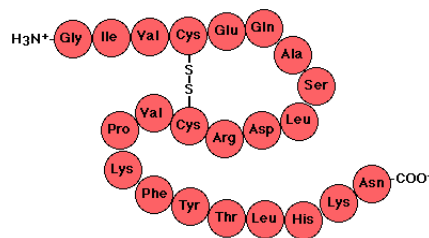


Figure 2.3: Protein primary structure: aminoacids chain [16].

modifications can occur. This is the case of disulfide bond formation, phosphorylations and glycosylations. This is usually still considered primary structure. An efficient example is insulin: it is formed of 51 aminoacids in 2 chains, one of 31 and the other of 20 [17].

- **Secondary structure**

On the secondary level, the structure refers to substructures on the polypeptide backbone chain. There are two possible substructures:  $\alpha$ -helix and  $\beta$ -sheet ( $\beta$ -strand) [18]. The two possible secondary structure are displayed in Figure 2.4.

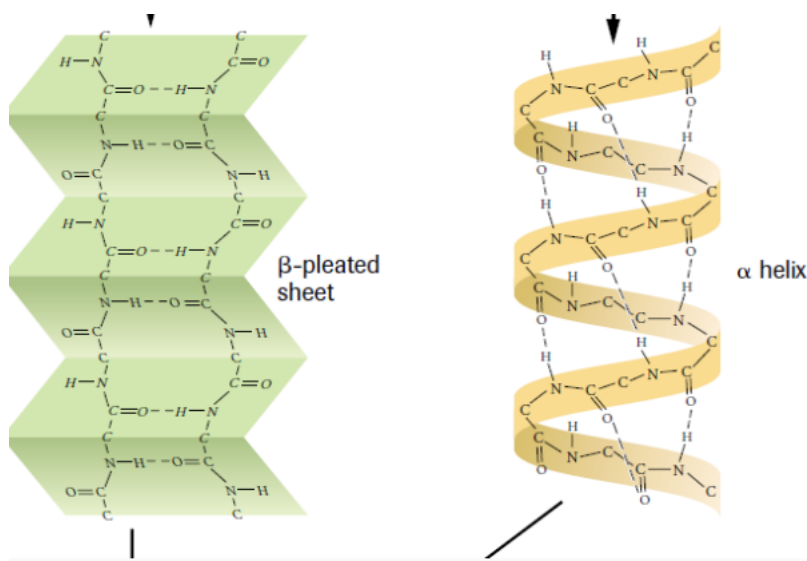


Figure 2.4: Protein secondary structure:  $\alpha$ -helix and  $\beta$ -sheet [19].

These structures are created due to hydrogen bond interactions between the main-chain peptide groups. When the structure is formed, all the hydrogen bonds in the backbone (donors and acceptors) on every chain are saturated.

- **Tertiary structure**

When the  $\alpha$ -helix and  $\beta$ -sheet are folded into a compact globular structure creating a three-dimensional structure of protein molecules, the structure belongs to the tertiary level of analysis. A visual clarifying example is given in Figure 2.5

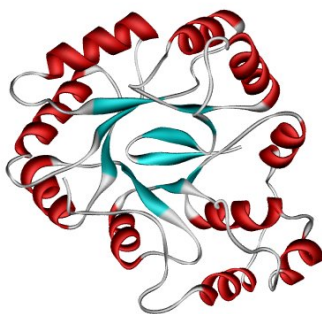


Figure 2.5: Protein tertiary structure: presence of lots of  $\alpha$ -helix (red) and some  $\beta$ -sheet (blue) and turn regions (grey)[20].

The folding is given by non-specific hydrophobic interactions. The structure is not stable: some protein domains need to be connected between them in order to stabilize the structure. This stabilization happens by means of specific tertiary interactions, mainly such as salt bridges and disulfide bonds. At this stage, a polypeptide is formed.

- **Quaternary structure**

The quaternary structure is the three-dimensional structure created by several sub-unit proteins and how they fit together (e.g. in Figure 2.6).

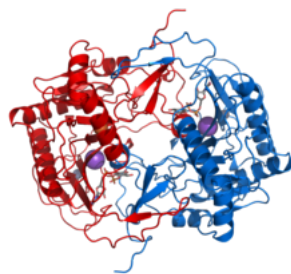


Figure 2.6: Protein quaternary structure: dimer composed by two polypeptides, red and blue [21].

A complex (or multimer) is composed by more than one polypeptide (sub-units) and it is stabilized again by non-covalent tertiary interactions, like disulfide bonds. Specifically, if the sub-units are two it would be called dimer; a trimer if it contains three sub-units; a tetramer with four, etc. If the sub-units are the same, a prefix *-homo* will be placed before. On the other hand, the prefix will be *-etero*. [17].

Another important characteristic of the enzyme is the **activity**. It is defined as the measure of the ability of an enzyme to catalyze a specific reaction [22]. Several factors affect the rate at which enzymatic reactions proceed. The most important are temperature and pH.

- **Temperature**

Alike most the chemical reactions, the rate of conversion of a enzyme catalyzed-reaction increase with an increase of the temperature, as Figure 2.7 shows. Variations of temperature of 1 or 2 degrees can introduce changes of 10-20%.

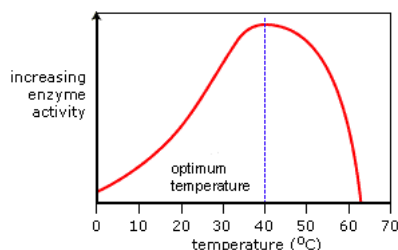


Figure 2.7: Trend of the activity with the temperature [23].

Before reaching the maximum, the heat energy causes more collisions between the enzyme and the substrate. Once the maximum level is reached, the enzyme activity abruptly decreases. This happens because most animal enzymes rapidly denature at temperature above 40°C by unfolding. Over a certain period of time, enzymes lose activity even at lower temperature. In fact, they should be stored a 5°C or below [24].

### • pH

pH also affects the activity of enzymes. The most suitable pH in which an enzyme is most active is named optimum pH. This optimum pH is not the same for every enzyme, for each enzyme there is also a region of pH optimal stability. As shown in Figure 2.8, extremely high or low pH values generally result in complete loss of activity for most enzymes.

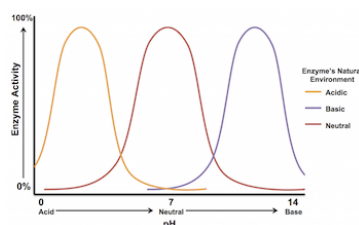


Figure 2.8: Trend of the activity with the pH [25].

### 2.1.3 Bovine Serum Albumin

Serum albumin is the most abundant protein in plasma, with a typical concentration of 5 g/100 ml. The full-length BSA precursor protein is 607 aminoacids. After the process, it reaches a length of 583 aminoacids. The molecular weight of the precursor is 69,324 kDa while the one of the processed BSA is 66,463 kDa. Albumin is composed by a low content of tryptophan and methionine and a high content of cystine and the charged amino acids, aspartic and glutamic acids, lysine, and arginine [26].

BSA is produced by means of the Cohn process, developed by Edwin J. Cohn. It consists in a series of purification steps of blood plasma, basing on the different

solubilities of the proteins. In fact, BSA solubility differs from the solubility of other plasma proteins tailoring pH, ethanol concentration and temperature. Albumin has the highest solubility and the lowest isoelectric point between all the major plasma proteins. Therefore, it is possible to precipitate and separate BSA during the Cohn process [27]. During World War 2, Bovine serum albumin was the most suitable candidate to replace blood plasma in order to grant a faster recovery for the soldiers. In the circulatory system, it contributes around 80% to colloid osmotic blood pressure [28]. Another function of albumin is the maintenance of blood pH, as discovered by Figge et al. in 1994 [29].

Though, the most important property of albumin is the ability to bind reversibly with an incredible variety of ligands. For instance, fatty acids are insoluble in plasma: thanks to the binding to albumin, they become soluble and can be transported in plasma. Another important function of albumin is the radical scavenging. In fact, by means of the ligand power of albumin, it is possible to sequester oxygen free radicals and inactivation of toxic lipophilic metabolites, e.g. bilirubin [30]. Again, very important is albumin carrier ability of nitric oxide (NO), implicated in numerous physiological processes as neurotransmission [31]. More information about the extraordinary ligand power of albumin can be found in the review of Fasano et al. [32]. Other than this, BSA has numerous biochemical applications: ELISAs (Enzyme-Linked Immunosorbent Assay), immunoblots and immunohistochemistry. Moreover, it is used as a nutrient component in culture of cells and microbes. Another very common use is the Bradford assay, to detect the concentration of proteins in solutions. BSA is so widely used because of its properties of increasing the signal in assays, its lack of effect in many biochemical reactions, and its low cost, since large quantities of it can be readily purified from bovine blood, a byproduct of the cattle industry.

#### 2.1.4 Glucose Oxidase

##### Properties

Glucose Oxidase (GOD) has been first discovered by Müller [33], who found this enzyme in *Aspergillus niger* and *Penicillium glaucum*. In 1928 he firstly found out the ability of GOD to catalyze the oxidation of glucose to gluconic acid in the presence of dissolved oxygen. The first purification to a moderate extent from *A. niger* was performed by Franke et al [34]. They classified GOD as a flavoprotein, since they found that the activity of different preparations was qualitatively related with the flavine content of the preparation. In 1965 Pazur et al [35] demonstrated that GOD is a glycoprotein, besides being a flavoprotein. DNA sequence of the GOX gene [36] as well as the structure [37] had subsequently been determined and published.

Glucose oxidase from *A. niger* is a dimer (80 kDa per monomer) with two molecules of flavin adenine dinucleotide (FAD). In Figure 2.9 the structure is shown: the two subunits are colored as dark and light blue, while the FAD coenzyme is depicted as pink. The role of FAD is to stabilize the 3-D structure and to act as electron carrier during catalysis.

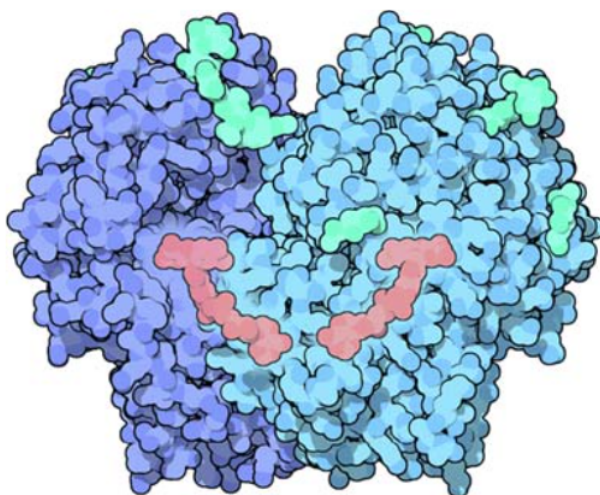


Figure 2.9: Structure of GOD: in dark and light blue the two dimers, in pink FAD coenzyme. [38].

The common commercial form of GOD is composed by about 20% amino sugars and 16-19% carbohydrates. The main properties of glucose oxidase from *A. niger* are listed in Table 2.1.

Table 2.1: Properties of glucose oxidase from *A. niger*. Data are taken from [1]

| Property                                            | Values                                             |
|-----------------------------------------------------|----------------------------------------------------|
| Molecular weight                                    | 150-186 kDa                                        |
| UV-VIS absorption                                   | 280 : 380 :450 (nm)                                |
| Extinction coefficient ( $M^{-1}cm^{-1}$ )          | 270,000 (at 280 nm)                                |
|                                                     | 25,180-28,200 (at 450 nm)                          |
|                                                     | 21,600 (at 452 nm)                                 |
| Specific activity (pH 5.6, 25–37°C)                 | 80–172 $\mu$ mol glucose/min/mg enzyme             |
| $K_m$ (Michaelis constant, with respect to glucose) | 198–248 mM (pH 5–7, 20–30°C, oxygen)               |
|                                                     | 110–120 mM (pH 5.6, 0–38°C, oxygen)                |
|                                                     | 50–74 mM (pH 5.5, 15–30°C, oxygen)                 |
|                                                     | 33 mM (pH 5.6, 25°C, oxygen)                       |
|                                                     | 41.8 mM (pH 6.86, 25°C, benzoquinone)              |
| Temperature range                                   | 20–50°C                                            |
| pH range                                            | 4–7                                                |
| Inhibitors                                          | $Ag^+$ , $Hg^{2+}$ and $Cu^{2+}$ ions ( $\mu$ mol) |
|                                                     | Arsenite                                           |
|                                                     | Arsenite                                           |
|                                                     | p-chloromercuribenzoate                            |
|                                                     | phenylmercuric acetate                             |
| Isoelectric point (pI)                              | 4.2                                                |

Glucose oxidase is extremely selective towards glucose. In fact, it catalyzes the oxidation of glucose (G) in gluconic acid (P) with formation of hydrogen peroxide  $H_2O_2$ . The overall reaction is shown in Figure 2.10.

A better definition for glucose oxidase could be an oxidoreductase that cat-

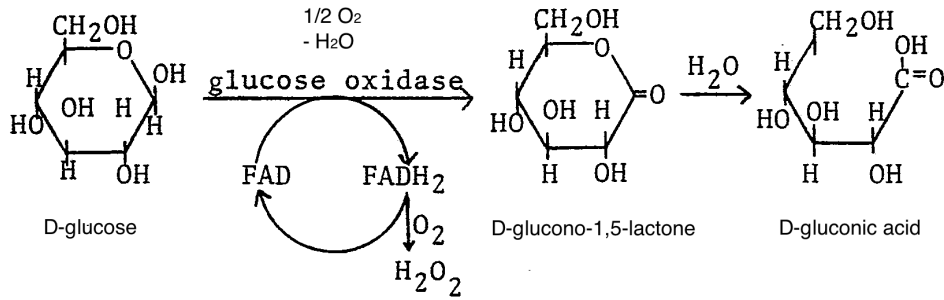
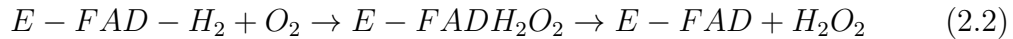
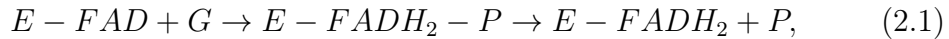


Figure 2.10: Reaction of oxidation of Glucose into Gluconic acid, catalyzed by glucose oxidase [39]

analyzes the reaction by which all the electrons taken from the substrate (glucose) are transferred to O<sub>2</sub> to form H<sub>2</sub>O<sub>2</sub>. The active center of the reaction is the FDA since the catalysis depends on the reduction-oxidation of its flavin group. In fact, the mechanism is based on the oscillation of FDA between its oxidized and reduced forms:



Glucose (G) reduces the FAD to  $FADH_2$  without the formation of intermediate free radicals and produces gluconic acid (P) as product. Subsequently, oxygen, the acceptor, oxidizes the  $FADH_2$  back to FAD and  $H_2O_2$  is released as product [40].

### Applications

The main application of glucose oxidase is biosensing. An estimation of the world's market value of biosensors has been carried out to be about 5 billion dollars, 85% of which depends on glucose biosensing. Therefore, the importance of GOD is highlighted: many glucose biosensors available in the market are based on immobilized glucose oxidase [41]. In medical environment, GOD is commonly used to assemble amperometric biosensors [42]. An amperometric sensor works thanks to a constant applied electric potential between the reference and the working cathode, promoting catalytic reaction, which drives the current flow that is proportional to the concentration of the target molecule. The amperometric glucose sensors can be divided in three generations according to their operating principle.

The base reaction is always the oxidation of glucose in gluconic acid, catalyzed by glucose oxidase. The differences between the three generations are given by the different mechanism of lecture and transport of the signal. For the first generation (Figure 2.11(a)) the rate of production of hydrogen peroxide is directly read by an appropriate electrode. In this design, the interference of other redox species such as oxygen, is a problem. In Figure 2.11(b) the functioning

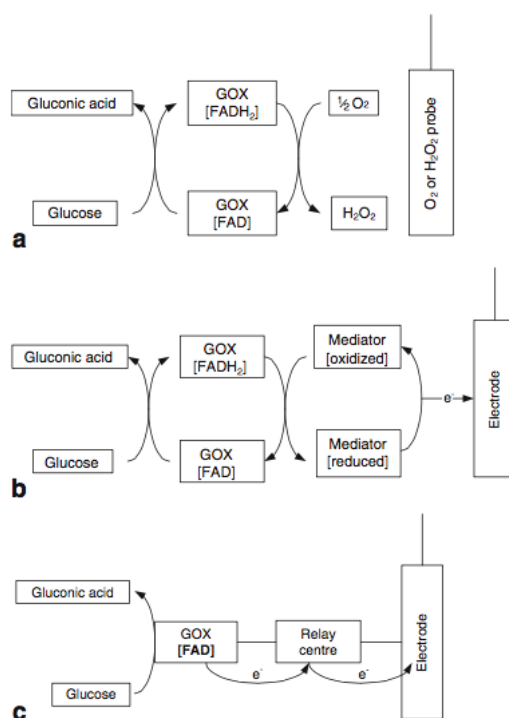


Figure 2.11: Operating principle for first (a), second (b) and third (c) generation of amperometric sensors [1].

of a second generation device is illustrated. In this case, a mediator is used to shuttle electrons back and forth between the enzyme and the electrode, limiting the contact with oxygen. Though, the mediator must be in proximity to the electrode. These kind of devices still suffer of a smaller extent of interference of redox species. In order to eliminate the interference, direct electron transfer was employed from the enzyme to the electrode in third generation (Figure 2.11(c)), via the use of a relay center. Recently, the direct immobilization of GOX onto carbon nanotubes [43] or nanofibers [44] has been studied in order to profit of the conductive properties of those materials. The use of GOD in biomedical environment is not limited on the traditional "fingerpricking" blood glucose measurement: continuous monitoring of glucose *in vivo* has been investigated by means of fluorescent based sensors [45][46]. The advantages of these biosensors are extreme sensitivity and non invasive techniques. These new biosensors allow a better representation of the glycemic history of the patient. In general, one of the problem with glucose oxidase is the intrinsic instability and the loss of activity with thermal changes, contact with chemicals (solvents), immobilization and storage.

## 2.2 Microgels

Structures with a network given by crosslinking, able to swell in an appropriate solvent, take the name of polymer microgels [47]. The importance of microgels is grown in the last decades: numerous theoretical studies of soft matter and

in applied fields have been published. This interest is born from the potential applications that have been thought. The facile synthesis and functionalization of microgels, their good colloid stability and control over particle size make them suitable subjects for product-oriented research. In fact, microgels find place in fields such as drug delivery [48, 49, 50], sensing [51, 52], the fabrication of photonic crystals [53, 54], template based synthesis of inorganic nanoparticles [55] and separation and purification technologies [56]. The most important characteristic of microgels that widen the array of applications is their stimuli-responsive nature. In particular, they undergo reversible volume phase transitions in response to environmental stimuli. Those studied stimuli are pH, temperature, ionic strength of the surrounding medium, quality of solvent and the action of an external magnetic field [57] (Figure 2.12).

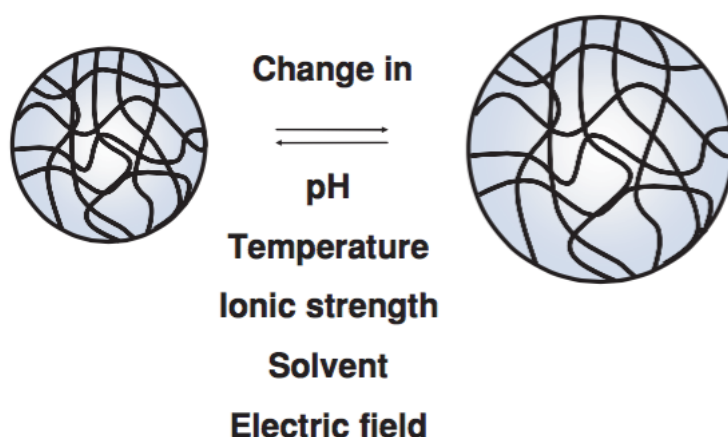


Figure 2.12: Scheme of the volume change in response to an external stimuli.

The swelling of microgels is due to the out-of-equilibrium situation of repulsive and attractive forces inside the particles. When ionic repulsion and osmotic forces go over attractive forces (hydrogen bonding, Van der Waals interaction, hydrophobic and specific interaction), swelling occurs [58].

### 2.2.1 Production of microgels

The main structural characteristic of microgel is the crosslinking. In fact, they are particles in which polymer molecules are crosslinked by chemical or physical bonds. Typically, the main two mechanisms of polymerization are free-radical polymerization or condensation polymerization. Many monomers have been synthesized in the shape of microgels. Between them it is possible to find styrene [59], methyl methacrylate [60], divinylbenzene [61], N-isopropylacrylamide [62], N-isopropylmethacrylamide [63] (NIPAM). NIPAM is the most studied polymer microgels for several applications. Its synthesis involves free radical precipitation polymerization with a crosslinking agent. The most used crosslinker is N,N-methylene-bisacrylamide (MBAM) [62]. Other synthesis methods are suspension emulsion polymerization, suspension polymerization and dispersion polymerization. For what concerns Emulsion Polymerization (EP), it is a radical polymerization that starts with the emulsification of a dispersed phase in a continuous

phase. The term emulsification refers to the formation of a stable dispersion of polymer microparticles, called emulsion or latex. The two phases are immiscible liquids, usually water and an organic solvent. To create the latex an emulsifier agent is added to the continuous phase in order to obtain the particles. This emulsifier agent is an amphiphilic molecule, composed by a hydrophilic head (water-loving, polar) and an hydrophobic tail (water-avoiding, apolar) [64]. Figure 2.13 shows a schematic representation of an amphiphilic molecule. Common amphiphilic substances are detergents, soaps and lipoproteins.

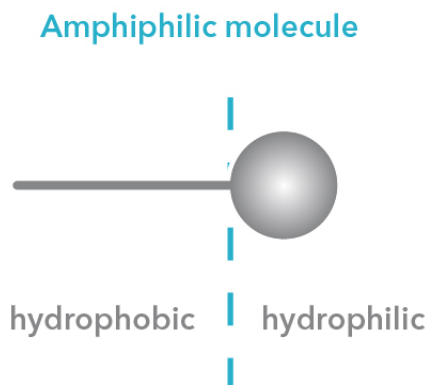


Figure 2.13: Schematic representation of an amphiphilic molecule. [65].

Mainly, there are two techniques of an emulsion polymerization:

- **Oil in water (O/W):** in this case the continuous phase is the aqueous phase while the dispersed phase is an organic solvent. This synthesis is usually used for non-water soluble monomers and with water soluble initiators.
- **Water in Oil (W/O):** in this case the continuous phase is the organic solvent while the dispersed phase is the aqueous medium. It is also called Inverse Emulsion Polymerization. Usually, this method is used for water soluble monomers.

Figure 2.14 represents an illustration of the mechanism of micelles formation of the two mechanisms. Figure 2.14 displays the two kinds of emulsions: (A) is W/O emulsion while (B) is a O/W emulsion. In Figure 2.14(A) the amphiphilic molecules ionize and dispose with the hydrophilic head towards the center in contact with the aqueous phase, in order to create the micelle. On the contrary, in Figure 2.14(B) the amphiphilic molecules dispose with the head outwards, where there is the aqueous phase [66].

### 2.2.2 Immobilization of enzymes in microgels

Enzymes are extremely important species in the current and future research, in many fields. Though, they present some drawbacks such as solubility, stability, recyclability by preserving or improving their catalytic properties. These issues play a key role for the use and applications of enzymes. In order to improve those

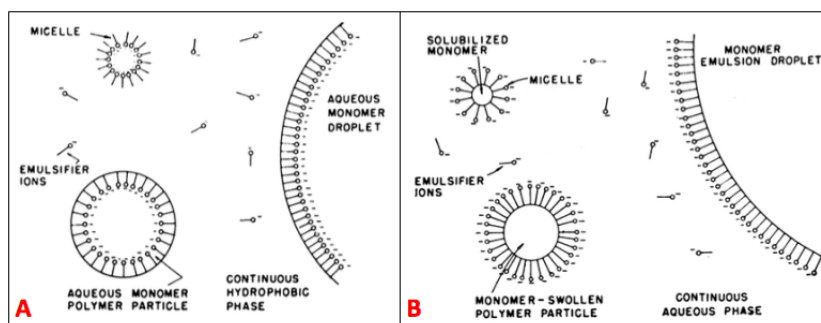


Figure 2.14: Schematic representation of (A) inverse W/O emulsion polymerization and (B) conventional O/W emulsion polymerization. [67].

properties, different immobilization techniques have been experimented. Mainly, immobilization can be performed in two ways: immobilization by coupling (physical adsorption or covalent bonding) or immobilization by encapsulation (enzyme is entrapped in a carrier matrix) [68]. The first technique is attractive since it does not influence the conformation of the enzymes and does not critically decrease the activity. Though, it presents a problem: the desorption process. Usually, during the desorption of enzymes a not negligible loss of activity occurs [69].

To graft the enzymes onto a substrate some functional groups (amino-, thiol- or carboxy-) in the enzyme structure are used for the covalent coupling. This leads to the reduction of the active centers with consequent loss of activity. In the case of entrapped enzyme inside a carrier matrix, the desorption process is easier. By means of changes of pH, ionic strength, temperature or mechanical stress the enzyme can leak out the carrier and be released. Different carriers like clays[70], porous glasses [71], active coal[72], porous silica particles [73, 74], cellulose fibers[75], nanoporous gold nanoparticles[76] have been used for the coupling of enzymes by adsorption or grafting. So far, the most suitable materials to encapsulate enzymes have been found in natural polymers (alginates, pectin or gelatin) and synthetic polymers (polyacrylamides (PAA), polyvinyl alcohol). Polyacrylamide (PAA) gels have been widely used for the matrix of electrophoresis and they have found applications as support for enzyme immobilization and drug encapsulation [77]. In addition, poly(acrylamide) gels in water have been used as models for investigation of molecular interactions in networks [78, 2]. Unfortunately, when enzymes are immobilized inside microgels, a huge loss of activity is observed. The scientific community is really interested in this new topic and it is putting a lot of efforts in the retaining of the activity of enzymes when encapsulated.

## 2.3 Microfluidics

At the beginning of the 80's, the research on microfluidic devices started. At Stanford University a gas chromatograph was studied [79] while at IBM the research focused on the developing of ink jet printer nozzles [80]. In the following years microfluidics technique has been modestly studied until 15 years ago,

when it became an "hot" research topic. Nowadays, microfluidic devices offer the ability to work with smaller reagent volumes, shorter reaction times, and the possibility of parallel operation. In addition to the traditional advantages that miniaturization brings, the most important benefit lies in the physics of the microscale. By understanding and managing phenomena at the microscale, experiments not possible on the macroscale can be carried out by means of microfluidics, allowing new ideas and functionality to emerge. Two devices are already considered working with microfluidics: gene chips and capillary electrophoresis [81]. Even though, gene chips are not technically microfluidic devices, but they still benefit of miniaturization. Before starting to work with microfluidics, one has to discover and understand the physics that dominate the microscale. Basically, microfluidics is the handling of fluids in structures of micrometer scale. Though, at the microscale several new forces different from those dominant in everyday life have to be taken in account. The effects that become important at the microscale are:

- **Reynolds number**

Every fluid has its Reynolds number (Re). It describes the flow regime: laminar or turbulent. A turbulent flow is chaotic and unpredictable, i.e. it is not possible to predict the position of a particle in the stream as a function of time. Re is calculated by means of the formula:

$$Re = \frac{\rho v Dh}{\mu} \quad (2.3)$$

where  $\rho$  is the fluid density,  $v$  is the characteristic velocity of the fluid,  $\mu$  is the fluid viscosity, and  $Dh$  is the hydraulic diameter (calculated from the cross section of the channel in which the fluid is flowing). Depending on the value of the Reynolds number the flow is laminar or turbulent. The threshold is 2300: when  $Re < 2300$  the flow is laminar. As soon as the number increases, turbulence starts to appear;  $Re > 2300$  is commonly considered as a turbulent flow. In microfluidic channels the Re transition might be different, according to literature. On the other hand, recent work states that the flows into the microchannels follow the theory [82].

- **Laminar flow.**

When the Reynolds number is lower than 2300 the regime of the flow is laminar. A laminar regime is that condition in which it is possible to predict the position of a particle in the stream as a function of time. In microchannels, the flows are almost always laminar due to the dimensions [83]. Consequence of that is that two or more flows in contact with each other do not mix except by diffusion (Figure 2.15. This effect has been used in research to select particle by size [84]).

- **Diffusion.**

Diffusion occurs when a concentrated group of particles in a volume spread out over time by Brownian motion. At the end, the final average concentration of particles in the volume is constant. In one dimension, diffusion can be represented by the equation:

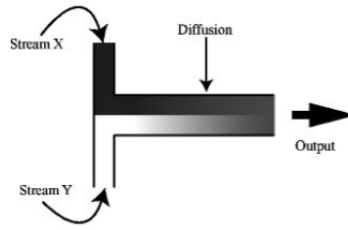


Figure 2.15: Two laminar streams flowing in contact will not mix except by diffusion. As the time of contact between two streams increases, the amount of diffusion between the two streams increases.

$$d^2 = 2Dt \quad (2.4)$$

where  $d$  is the distance a particle moves in a time  $t$ , and  $D$  is the diffusion coefficient of the particle. Considering the square power on the distance, diffusion is one of the most important parameter at the microscale. Since diffusion times at the microscale are short, complex profiles of concentration gradients can be built inside the microchannels [85].

- **Fluidic resistance.**

In microchannels, the fluidic resistance is governed by a set of equation whose solutions are known [83]. The flow rate is given by

$$Q = \frac{\Delta P}{R} \quad (2.5)$$

where  $Q$  is the flow rate inside the microchannels,  $P$  the pressure drop across the channel and  $R$  the channel resistance. The resistance in a circular tube (the most common geometry, e.g. vessels) is given by:

$$R = \frac{8\mu L}{\pi r^4} \quad (2.6)$$

where  $\mu$  is fluid viscosity,  $L$  is the channel length, and  $r$  is the channel radius. Other channel geometries and resistances can be found in References [86] and [83].

- **Surface to volume ratio.**

Surface area is another factor that becomes important at the microscale. When scaling down from the macro- to the microscale, an increase in the surface area to volume (SAV) ratio by orders of magnitude is not uncommon. A very large SAV ratio makes capillary electrophoresis more efficient in microchannels by removing excess heat more rapidly [87].

- **Surface tension.**

Another significant force at the microscale is the surface tension. Surface tension results from the cohesion between liquid molecules at the liquid/gas interface. A parameter that explain how much tension a surface contains

is the surface free energy. When microchannels are used, the liquid will travel throughout the capillary by means of the capillary forces alone. The path the water will do is directly related to the water's surface free energy and inversely related to the radius of the capillary. The pressure generated by a liquid surface with perpendicular radii of curvature  $R_1$  and  $R_2$  can be calculated with the Young-LaPlace equation:

$$\Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right), \quad (2.7)$$

where  $\gamma$  is the surface free energy of the liquid. If the surface is spherical, and  $R_1 = R_2$ , then the equation reduces to

$$\Delta P = \frac{2\gamma}{R} \quad (2.8)$$

and allows the calculation of the pressure contained within a spherical drop of liquid.

The typical production methods of microgels produce particles with insufficient control over their dimensions. Polydispersity of microgels can be a disadvantage in the optimization of drug uptake and release profiles or in the controlled encapsulation of biological species in the microgel interior. Over the past five years, microfluidic synthesis and assembly have offered a facile approach to the continuous production of solid micrometre-size polymer particles with unprecedented control over their sizes, shapes, and morphologies [88, 89]. Microfluidic generation of microgels is a two steps process: the emulsification of the solutions of monomers and on-chip (gelation in the droplets during their residence in microchannels) or off-chip gelation of the precursor droplets. The dimensions, the morphology and the shape are controlled by the corresponding properties of the precursor droplets. An excellent control over the microgels dimensions is provided by microfluidic synthesis, higher than any other batch synthesis (emulsion, suspension or precipitation polymerization). The dimension is predetermined in the stage of the emulsification. Several other morphologies are available with microfluidic synthesis by means of the control of the hydrodynamic and thermodynamic parameters. Structures alike are core-shell or Janus morphologies [90, 91]. Variation in the composition of precursor droplets (and hence, the corresponding microgel particles) can be achieved by mixing liquid reagents in the concentration ratio predetermined by the ratio of their flow rates [92]. In addition, parallel microfluidic reactors can enable high-throughput screening of the compositions of microgel properties and the properties of the encapsulated species. The most important part of the process is the generation of the droplets inside the microchip. Four different geometries of droplet generator are the most common; they are displayed in Figure 2.16.

In a flow-focusing microchip (Figure 2.16(a)) an aqueous phase and a oil phase (labeled as A and B) are let flow respectively to the central and side channels. The aqueous phase is focused in the narrow orifice by the shear forces imposed by the oil phase. A periodic break-up of the thread of the disperse phase produces droplets with desired constant size [94]. Varying the ratio of flow

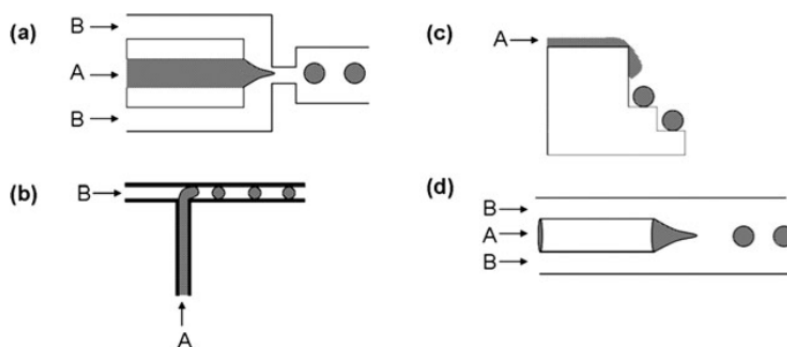


Figure 2.16: Schematic drawings of various types of microfluidic droplet generators: (a) flow-focusing, (b) T-junction, (c) terrace-like, and (d) and co-flowing stream geometries. The aqueous and oil phases are labeled as A and B, respectively [93].

rates the size of the droplets can be tailored, so that with increasing flow rate ratio, the diameter of droplets decreases. In Figure 2.16(b) a T-junction device is shown. As the aqueous phase flows into the junction, the oil phase forms a thin film layer between the dispersed phase and the walls of the device, causing an increase in pressure which "squeezes" the other flow to form a droplet [95]. The size of generated plugs (droplets spanning the entire width of the channel) decreases with increasing ratio of flow rates of the continuous-to-droplet phases. In contrast, the size of spherical droplets is not directly related to the ratio of flow rates of the liquids. An alternative droplet generator is a terrace-like design, shown in Figure 2.16(c). At the end of the terrace, a stream of the disperse phase flows into a well and breaks into disc-like droplets which rapidly transform into spherical droplets due to the action of interfacial tension. The droplets are forced towards the exit of the device by a cross-flowing continuous phase. In this method, the diameter of the droplets is controlled by the dimensions of the terrace channel and the ratio of the viscosities of the disperse phase and continuous phases [96]. A microfluidic droplet generator can also be designed as two concentric capillaries, in which the two phases are flowing (Figure 2.16(d)). The operating principle is the same as the flow-focusing chip.

The encapsulation of biological species has been widely studied. The specie has to be dissolved in the aqueous phase in order to be present inside the microgel at the end of the droplet formation. Important requirements for bio-encapsulation are:

1. Reagents used in microfluidic encapsulation of bioactive species have to be biocompatible.
2. The microfluidic device and the supplying tubing have to be sterilized before and after encapsulation.
3. Following on-chip or off-chip gelation, microgel particles have to be mechanically strong to bear their successive use or processing.

As well as the bio-specie has been entrapped in microgels synthesized with one of the traditional methods, it can be immobilized also with microfluidics. It is

possible to fill the microgels with protein, enzymes, cells, peptides, etc. The most common off-chip gelation is the chemical gelation. When the polymer droplets are collected, they need to polymerize and crosslink in order to become mechanically stable. This route has been exploited for the preparation of microgels by using photoinitiated or redox-initiated polymerization of N-isopropylacrylamide [97], dextran-hydroxymethyl methacrylate [98] and acrylamide [90]. In this study, the setup of the pressure driven microfluidic machine and droplet formation are presented.

## Chapter 3

### Experimental processes and methods

This chapter explains all the experimental processes and methods that have been used during the work for this thesis. In addition, the specifications of the utilized devices will be given.

#### 3.1 Batch synthesis

In order to synthesize the microgels several attempts have been carried out. The polymerization have been performed through Inverse Emulsion Polymerization. At a first stage, Bovine Serum Albumin (BSA), purchased from Roth has been used as the protein immobilized inside the microgel in order to set a well defined protocol of the synthesis. At a later time, Glucose Oxidase (GOD) has been encapsulated in the microgel in order to perform the activity tests. Referring to Rubio-Retama et al. [99] some first attempts of synthesis have been carried out. The used technique is named Concentrated Inverse Emulsion Polymerization (CIEM) since the amount of continuous phase is kept very low. In order to compose the aqueous phase, the following materials have been dissolved in Phosphate Buffer solution (PB) (0,1 M, pH= 7,2):

- The monomer: Acrylamide (AA), purchased from Sigma-Aldrich
- The crosslinker: N,N'-Methylenebisacrylamide (MBAM), purchased from Sigma-Aldrich
- The initiator: Ammonium Persulfate (APS),  $(NH_4)_2S_2O_8$ , purchased from Sigma-Aldrich
- The enzyme: Glucose Oxidase (GOD), purchased from Roth

The content of MBAM defines the crosslinking degree  $\eta$  of the polymerization. It is defined as:

$$\eta = \frac{MBAM(mg)}{MBAM(mg) + AA(mg)} \quad (3.1)$$

In Table 3.1 a list of the amounts of added chemicals for each synthesis is given.

Table 3.1: Immobilization of Glucose Oxidase through Concentrated Emulsion Polymerization

| Label | PB [ml] | AA [mg] | MBAM [mg] | APS [mg] | GOD [mg] |
|-------|---------|---------|-----------|----------|----------|
| E1    | 5       | 1250    | 41,3      | 25       | 250      |
| E2    | 5       | 1250    | 41,3      | 25       | 10       |
| E3    | 5       | 1250    | 41,3      | 25       | -        |

In order to compose the oil phase, 250  $\mu\text{l}$  of the surfactant SPAN80, purchased from Sigma-Aldrich, (SP80) has been dissolved in 750  $\mu\text{l}$  of the organic solvent Dodecane ( $\text{CH}_3(\text{CH}_2)_{10}\text{CH}_3$ ). The oil phase has been degassed with Nitrogen for 30 minutes. Subsequently, the aqueous phase has been added to the oil phase. The mixing is carried out by dropwise addition of the aqueous phase by means of a Pasteur pipette in order not to break the hydrophilic/hydrophobic equilibrium. The reaction vessel is degassed for 30 minutes. In order to catalyze the reaction, 63  $\mu\text{l}$  of N,N,N',N' tetramethylethylenediamine (TEMED) are added to the emulsion (TEMED is purchased from Roth). The reaction has been let react for 1 hour. The consecutive purifying process entailed the following steps:

- Centrifuge (10 min, 10.000 rpm). Removal of the organic supernatant.
- Addition of acetone, sonication (20 min) and centrifuge (10 min, 10.000 rpm), removal of the supernatant.
- Addition of water, sonication (20 min), centrifuge (10 min, 10.000 rpm), and removal of the supernatant.
- Again, addition of water, sonication (20 min), centrifuge (10 min, 10.000 rpm), and removal of the supernatant.
- Lyophilization: the machine worked with vacuum of 0,05 mbar, temperature of the ice condenser  $-60^\circ\text{C}$  and temperature of the shelf  $-20^\circ\text{C}$ .

This purification process has been used as protocol for all the following syntheses in this dissertation.

### 3.1.1 Encapsulation of Bovine Serum Albumin

Other two attempts with the CIEM have been performed. In these syntheses, the immobilized biocomponent was BSA. The composition of the oil phase was the same of the previous attempts with GOD. The recipes are shown in Table 3.2.

Table 3.2: Immobilization of Bovine Serum Albumin through Concentrated Emulsion Polymerization

| Label | PBS [ml] | AA [mg] | MBAM [mg] | APS [mg] | BSA [mg] |
|-------|----------|---------|-----------|----------|----------|
| E4    | 5        | 1250    | 41,3      | 25       | 10       |
| E5    | 5        | 1250    | 105,7     | 25       | 10       |

The reaction was initiated with 63  $\mu\text{l}$  of TEMED. Also in this case, the post-reaction process followed the purification protocol.

Afterwards, in order to visually check if the protein was inside the microgels or not, the bare BSA was stained with Fluoresceinisothiocyanate (FITC) and characterized by means of fluorescence microscopy. The protocol of the chemical coupling BSA-FITC was performed through EDC-NHS coupling as follows [100]:

1. Equilibration of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) to room temperature.
2. Preparation of 60 ml of solution 1mg/ml BSA in Phosphate Buffer.
3. Preparation of solution 1mg/ml FITC in Phosphate Buffer.
4. Addition of 0,4mg of EDC and 0,6 of NHS,
5. Reaction 15 minutes protected from light.
6. Reaction vessel FITC solution with 60 ml of BSA solution in Phosphate Buffer (1mg/ml). The molar ratio BSA:FITC is 1:3.
7. Reaction overnight protected from night.
8. Purification with Size Exclusion Chromatography (SEC), with a machine Sephadex G-25.
9. Lyophilization.

In Figure 3.1 the chemistry of the coupling is illustrated. The carboxylic acid is FITC while the amine is the protein.

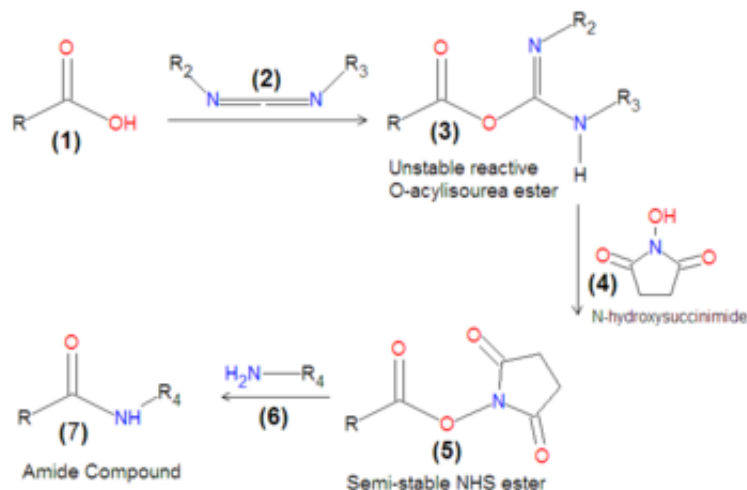


Figure 3.1: Reaction mechanism of NHS-EDC coupling [101].

Using the coupled BSA, more CIEM have been carried out. The oil phase is always composed by 250  $\mu\text{l}$  of the surfactant SPAN80 and 750  $\mu\text{l}$  of the organic solvent Dodecane. The crosslinking degree  $\eta$  is the changing parameter, as Table 3.3 shows:

Table 3.3: Immobilization of FITC coupled-Bovine Serum Albumin through Concentrated Emulsion Polymerization

| Label | PB [ml] | AA [mg] | $\eta$ | MBAM [mg] | APS [mg] | FITC-BSA [mg] |
|-------|---------|---------|--------|-----------|----------|---------------|
| E6.1  | 5       | 1250    | 3%     | 38,66     | 25       | 10            |
| E6.2  | 5       | 1250    | 6%     | 79,79     | 25       | 10            |
| E6.3  | 5       | 1250    | 9%     | 123,63    | 25       | 10            |
| E6.4  | 5       | 1250    | 12%    | 170,45    | 25       | 10            |
| E6.5  | 5       | 1250    | 20%    | 312,5     | 25       | 10            |

Combining the previous recipe with the one from Schachschal et al [102], a successful protocol has been determined. This protocol is no more based on the concept of Concentrated Emulsion Polymerization; the amount of organic solvent is increased, while the other components are set basing on the two papers that we took in account. The final protocol of the recipe is:

**1. Preparation of the Oil Phase:**

- SPAN 80 (SP80),  $V=500 \mu\text{l}$
- TWEEN 20 (TW20),  $V=100 \mu\text{l}$
- n-Heptane (nH),  $V=25 \text{ ml}$

**2. Preparation of the Aqueous Phase:**

- Phosphate Buffer solution,  $V=3 \text{ ml}$
- APS,  $m=108 \text{ mg}$
- AA,  $m=600 \text{ mg}$
- MBAM,  $m=38,3 \text{ mg}$  ( $\eta=6$ )
- FITC Coupled-BSA,  $m=10 \text{ mg}$

**3. Degassing of the oil phase:** The oil phase has to be degassed for 30 minutes in Nitrogen atmosphere.

**4. Mixing of the phases:** By means of a Pasteur pipette the aqueous phase is added to the reactor in which the oil phase is contained. The addition is dropwise, in order not to brake the hydrophilic/hydrophobic equilibrium. Thanks to the high stirring, an emulsion is formed.

**5. Degassing of the emulsion:** The emulsion is degassed in Nitrogen atmosphere for 30 minutes.

**6. Initiation of the polymerization:** The polymerization is initiated through the addition of TEMED ( $V=166 \mu\text{l}$ ).

**7. Purification and Lyophilization:** The post-reaction process follows the protocol explained before.

Again, in order to study the concentration of the biocomponent inside the microgels the crosslinking degree has been varied. In Table 3.4 the crosslinking degree and the equivalent amount of crosslinker is reported. All the other chemicals of the recipe are kept constant.

Table 3.4: Changing of the crosslinking degree  $\eta$  and the relative amount of crosslinker in the aqueous phase.

| Label    | Crosslinking degree $\eta$ | MBAM [mg] |
|----------|----------------------------|-----------|
| HOBL-3%  | 3%                         | 18,6      |
| HOBL-6%  | 6%                         | 38,3      |
| HOBL-9%  | 9%                         | 59,3      |
| HOBL-12% | 12%                        | 81,8      |

### 3.1.2 Encapsulation of Glucose Oxidase

The same protocol for the synthesis of the microgel has been used for the encapsulation of GOD. In order to perform the study, different parameters of the synthesis have been tailored. The protocol recipe is composed as in Table 3.5 with the name of standard. Several parameters has been tailored in the synthesis in order to retain the activity of GOD. In details, the parameters are:

- The crosslinking degree
- The content of organic solvent (nH)
- The content and type of surfactants
- The content of Glucose Oxidase

The detailed recipes of the polymerizations are reported in Table 3.5. All the synthesis are made 600 mg of Acrylamide, 108 mg of APS and 166  $\mu\text{l}$  of TEMED dissolved in 3 ml of Phosphate Buffer. Only the chemicals that change are reported in the table.

Table 3.5: synthesis of GOD filled-microgels

| Label                                        | SP80 [ $\mu$ l] | TW20 [ $\mu$ l] | nH [ml] | MBAM [mg]            | GOD [mg] |
|----------------------------------------------|-----------------|-----------------|---------|----------------------|----------|
| <b>STANDARD</b>                              | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| <b>Crosslinking degree <math>\eta</math></b> |                 |                 |         |                      |          |
| HOG-3%                                       | 500             | 100             | 25      | 18,6 ( $\eta=3\%$ )  | 10       |
| HOG-6%                                       | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| HOG-9%                                       | 500             | 100             | 25      | 59,3 ( $\eta=9\%$ )  | 10       |
| HOG-12%                                      | 500             | 100             | 25      | 81,8 ( $\eta=12\%$ ) | 10       |
| <b>Content of n-Heptane</b>                  |                 |                 |         |                      |          |
| nH-5                                         | 500             | 100             | 5       | 38,3 ( $\eta=6\%$ )  | 10       |
| nH-10                                        | 500             | 100             | 10      | 38,3 ( $\eta=6\%$ )  | 10       |
| nH-15                                        | 500             | 100             | 15      | 38,3 ( $\eta=6\%$ )  | 10       |
| nH-25                                        | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| <b>Content of Surfactant</b>                 |                 |                 |         |                      |          |
| 2X                                           | 1000            | 200             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| 3X                                           | 1500            | 300             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| 4X                                           | 2000            | 400             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| 6X                                           | 3000            | 600             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| <b>Content of GOD</b>                        |                 |                 |         |                      |          |
| GOD-1                                        | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 1        |
| GOD-5                                        | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 5        |
| GOD-10                                       | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 10       |
| GOD-15                                       | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 15       |
| GOD-20                                       | 500             | 100             | 25      | 38,3 ( $\eta=6\%$ )  | 20       |

As done for BSA, also GOD has been conjugated with FITC in order to obtain fluorescence labeling. Successively, an imaging analysis with the fluorescent microscope has been carried out in order to check if the GOD was entrapped in the microgel. The staining protocol is the same mentioned in paragraph 4.1.1, with GOD instead of BSA. The synthesis of the microgels followed the standard recipe. Another study has been performed in order to obtain better results: additives have been added to the synthesis. These additives are Vinylimidazole (VIm), Glycerol (GLY) and Trehalose (TREA). Vinylimidazole has been studied as an activity retainer of Laccase in microgel encapsulation. In fact, VIm has been put in the aqueous phase in order to counterbalance the electrostatic interaction between the polymer shell and the enzyme during and after the polymerization. It showed positive results in reference [102]. Moreover, Glycerol is a well known protein stabilizer. In fact, thanks to mechanisms such as excluded volume effect [103] and the solvophobic effect [104]. Basically, the solute particles tend to cluster as the attractive interaction between solvent particles is strengthened. AS a consequence, the protein unfold and denature less likely. Moreover, Trehalose has been proved to be a thermal stabilizer for GOD, protecting the enzyme from the unfolding given by an increase of the temperature [105][106]. The syntheses listed in Table 3.6 show the amounts of additives added to the standard protocol of the synthesis. The additives were dissolved in the aqueous phase.

Table 3.6: Concentrations of additives supplied to the standard recipe of the synthesis.

| Label   | Additive       | Amount |
|---------|----------------|--------|
| VIm     | Vinylimidazole | 5%mol  |
| Glyc100 | Glycerol       | 100mM  |
| Trea100 | Trehalose      | 100mM  |

## 3.2 Calculation of concentration

A lot of attempts to calculate the concentration of the Enzyme/protein inside the microgel have been done. The used techniques are: Bradford assay, BCA assay, Fluorescence assay, Elemental analysis. In this subchapter, all the protocols will be clarified. All the measurements but the elemental analysis are done with a multimode microplate readers Tecan Infinite®200 PRO (Figure 3.2).



Figure 3.2: Image of Infinite®200 PRO, machine used for the measurements[107].

### 3.2.1 Bradford Assay

The Bradford assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. In this case, Bradford assay is meant to calculate the concentration of protein inside the microgels in solution. The measurements have been performed with the microplate reader, with triplicates and using the same serial dilution of the activity assay. At first, a calibration curve has been defined: it has been built with 6 solutions of BSA in PBS with concentrations 0, 20, 40, 60, 80, 100  $\mu\text{g}/\text{ml}$ . The used dye is Coomassie brilliant blue G-250 provided as Roti®-Quant solution. The dye has been diluted in water with a ratio 2:5.5 (dye:water). Each well of the microplate was filled with 25  $\mu\text{l}$  of BSA solution and 100  $\mu\text{l}$  of the dye solution. The two components were let react for 5 minutes light protected. After that, the microplate was inserted into the machine and the program was run. The adsorbance was measured at 595 nm [108]. For what concern all the measurement of the microgels, the specifics are in Table 3.7

Table 3.7: Protocol for the Bradford assay.

| Volume[ $\mu$ l] | Component                          | Final amount  |
|------------------|------------------------------------|---------------|
| 25               | Microgel in PBS (5 mg/ml)          | 0.125 $\mu$ g |
| 100              | Roti®-Quant in Water (ratio 5.5:2) | -             |
| Tot=125          |                                    |               |

### 3.2.2 BCA assay

The Bicinchoninic Acid (BCA) assay was performed completely following the walkthrough given by the instructions of BCA Protein Assay Reagent Kit, provided by ThermoFisher scientific. The BCA assay is used to calculate the concentration of a protein in a solution. The concentration is shown by a color change of the sample solution from green to purple in proportion to protein concentration, which can then be measured using colorimetric techniques. The stock BCA solution contains the following ingredients:

- Bicinchoninic acid
- Sodium carbonate
- Sodium bicarbonate
- Sodium tartrate
- Copper(II) sulfate pentahydrate

First, the peptide bonds in protein reduce  $Cu^{2+}$  ions from the copper(II) sulfate to  $Cu^+$ . The amount of  $Cu^{2+}$  reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each  $Cu^+$  ion, forming a purple-colored complex that strongly absorbs light at a wavelength of 562 nm. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions of known concentration. It is showed in Figure 3.3. The whole protocol can be found in reference [109].

First a calibration curve has been built with the concentrations 2000, 1500, 1000, 750, 500, 250, 125, 25, 0  $\mu$ l/ml. Then the BCA assay has been carried out with the standard procedure. The procedure implies the use of the microplate reader at wavelength 562 nm. The assay has been done with serial dilution as aforementioned and with triplicates per each sample. In Table 3.8 the specifics are listed.

Table 3.8: Protocol for the BCA assay.

| Volume[ $\mu$ l] | Component                           | Final amount  |
|------------------|-------------------------------------|---------------|
| 25               | Microgels in PBS (5 mg/ml)          | 0.125 $\mu$ g |
| 200              | BCA working reagent (as in the kit) | -             |
| Tot=225          |                                     |               |



Figure 3.3: BCA Protein Assay Reagent Kit [109].

### 3.2.3 Fluorescence assay

Another attempt to calculate the concentration of the protein inside the microgels has been carried out with the Fluorescence assay. In order to obtain the precise value of concentration of BSA in the mixture BSA-FITC a Bradford assay has been performed. Thus, the stained BSA was used to build a calibration curve: 6 calibration solutions have been prepared with the concentration of 0, 20, 40, 60, 80, 100  $\mu\text{g}/\text{ml}$  (the  $\mu\text{g}$  refers to the real mass of BSA and not to the mass of the whole stained BSA). Once that the calibration was done, the real measurements have been performed. Using a black microplate in order to retain the fluorescence of the protein, the fluorescent microgel solutions (in PBS) were put into the wells in triplicates and with serial dilution. Then the microplate reader was run in fluorescence top reading mode, at excitation wavelength 488 nm and emission wavelength 525 nm. In Table 3.9 it is possible to read the specifics of the assay.

Table 3.9: Protocol for the Fluorescence assay.

| Volume[ $\mu\text{l}$ ] | Component                 | Final amount      |
|-------------------------|---------------------------|-------------------|
| 100                     | Microgel in PBS (5 mg/ml) | 0.5 $\mu\text{g}$ |
| Tot=100                 |                           |                   |

### 3.2.4 Elemental analysis

Elemental analysis (EA) is a process where a sample is analyzed for its elemental composition. The result is both qualitative, detecting what element are present in the sample, and quantitative, detecting the respective amounts of elements. EA refers to CHN analysis, i.e. the determination of the mass fraction of carbon, hydrogen and nitrogen. In modern machines, it is also possible to determine heteroatoms X like halogens or sulfur. Usually this technique is widely used to ascertain the purity or structure of synthesized materials. In the case of encapsulation of BSA or GOD, EA has been used to detect the content of sulfur in the sample, in order to detect the amount of protein/enzyme entrapped in the

microgels. The EA is carried out by combustion: in a chamber with excess of oxygen and various traps, a sample is burned. The products are carbon dioxide ( $\text{CO}_2$ ), water and nitric oxide ( $\text{NO}$ ). By means of the masses of these products, the composition of the original sample can be calculated. Figure 3.4 shows a common machine for EA.



Figure 3.4: Common Elemental Analysis set up [110].

### 3.3 Activity test

The activity test has been performed for first on pure GOD. Then, all the samples with GOD inside have been tested in order to compare the activity with the bare enzyme in order to define the loss ratio. The activity test is based on the reaction of Glucose with Oxygen, catalyzed by GOD (see paragraph 3.1.2). The components for the activity test are:

- **Glucose:** it is the main substrate of the reaction. It converts in D-glucono- $\beta$ -lactone (GDL) with production of  $\text{H}_2\text{O}_2$  (GOD catalyzed).
- **GOD or GOD filled-microgels:** in both the cases the material is dissolved in Phosphate Buffer Saline (PBS). The presence of GOD catalyzes the reaction of glucose.
- **Horseradish Peroxidase (HRP):** it is used to amplify the weak signal and make it detectable in the spectrophotometric machine. The generated  $\text{H}_2\text{O}_2$  activates the HRP, which oxidizes a light adsorbing dye molecule that can be detected by photometric based spectroscopic measurements. The overall reaction is shown in Figure 3.5
- **2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS):** it is the dye used as substrate with HRP. Its use allows the reaction kinetics to be followed by photometric technique. Thanks to its oxidation, it is possible to quantify the content of  $\text{H}_2\text{O}_2$ , so the rate of conversion of the GOD reaction (i.e. the retaining of activity of GOD).

In Table 3.11 the quantities of the protocol of the activity tests are reported.

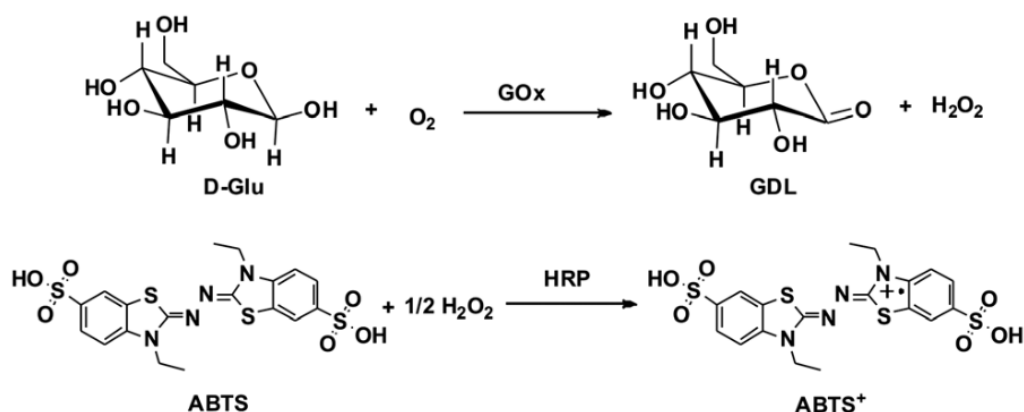


Figure 3.5: Reactions of the activity assay: oxidation of glucose and oxidation of the dye ABTS [111].

Table 3.10: Protocol for the activity test on pure GOD.

| Volume[ $\mu$ l] | Component                | Final amount |
|------------------|--------------------------|--------------|
| 50               | Pure GOD (1 mg/ml)       | 0.05 $\mu$ g |
| 50               | ABTS in PBS (0,25 mg/ml) | 1.25 $\mu$ g |
| 50               | HRP in PBS (0,5 U/ml)    | 0.125 U      |
| 50               | Glucose in PBS (10 mM)   | 90 $\mu$ g   |
| Tot=200          |                          |              |

Table 3.11: Protocol for the activity test on microgels.

| Volume[ $\mu$ l] | Component                 | Final amount |
|------------------|---------------------------|--------------|
| 50               | Microgel in PBS (5 mg/ml) | 0.25 $\mu$ g |
| 50               | ABTS in PBS (0,25 mg/ml)  | 1.25 $\mu$ g |
| 50               | HRP in PBS (0,5 U/ml)     | 0.125 U      |
| 50               | Glucose in PBS (10 mM)    | 90 $\mu$ g   |
| Tot=200          |                           |              |

Every sample has been measured in triplicates and with a serial dilution in a 96 wells microplate . The dilution factors per each well of a column (every column is a replicate) are 0, 2, 4, 8, 16, 32, 64. In each replicate, the last well of the column was filled with water instead of microgels as negative control. Measurements with empty microgels have been performed in order to estimate the interaction of Polyacrylamide with the components of the assay. After mixing the materials, the microplate was immediately put into the machine and the program was started. The measured characteristic is the adsorbance at a wavelength of 420 nm. The assay lasted 10 minutes and the adsorbance was measured in intervals of 20 seconds. The calculation of the activity were performed using the Beer-Lambert law:

$$Activity(U/ml) = \frac{m \times V_{tot} \times f}{\epsilon \times V_{test} \times L} \quad (3.2)$$

where  $m$  is the slope of the plotted adsorbance resulted from the measurements,  $V_{tot}$  is the total volume of liquid in the well,  $d_f$  is the dilution factor of the well,  $\epsilon$  is the extinction coefficient with the value of 36,8 ml/ $\mu$ molcm,  $V_{test}$  is the volume of microgel solution in the well and  $L$  is the light path (i.e. path length through the solution) with value 0,57 cm. The result in U/ml was converted in U/mg, rationalizing with 5 mg/ml, concentration of microgels in the solution put in the well with dilution factor 0. In order to define the loss ratio, a rationalization with the amount of enzyme put into reaction vessel  $m_{GOD}$  and with the production rate of the reaction  $m_{Microgel}$ . The following formula has been used:

$$LossRatio = \frac{(A_{GOD} \times m_{GOD}) - (A_{Microgel} \times m_{Microgel})}{(A_{GOD} \times m_{GOD})} \times 100 \quad (3.3)$$

$A_{GOD}$  is the activity of the bare enzyme GOD and  $A_{Microgel}$  is the measured activity of the microgels.

### 3.4 Setup of the pressure driven microfluidics synthesis plant

Another possibility to synthesize the microgels and to obtain a high degree of monodispersity has been exploited: microfluidics. A setup for a pressure driven continuous synthesis plant has been designed. Usually, in a microfluidics setup the fluids are injected from two syringe pumps. In our case, in order to reach a more precise control on the pressure (range of mbars), it has been necessary to switch from syringe pumps to a pressure control setup given by a continuous stream of nitrogen. Figure 3.6 shows scheme of the components.

When the blocking valve (1) is released, the stream of Nitrogen goes through a separator that splits and brings the nitrogen flows towards the pressure controller (2). In these pressure controller the flows of nitrogen is put under pressure. The pressure can be tailored to mbar changes thanks to a control knob. After, the nitrogen under pressure goes into the pressure vessels (3), where the reaction flasks with the oil phase and the aqueous phase are placed. The values of the applied pressure can be read on the pressure gauge (4), connected to the pots. Thanks to the applied pressure, the liquid can flow through the outlet tubes and reach the microfluidics chip (5). In Figure 3.7 the components are shown.

All the components have been connected with suitable high pressure tubes. The tightness of all the junction has been tested with a solution of soap in water.

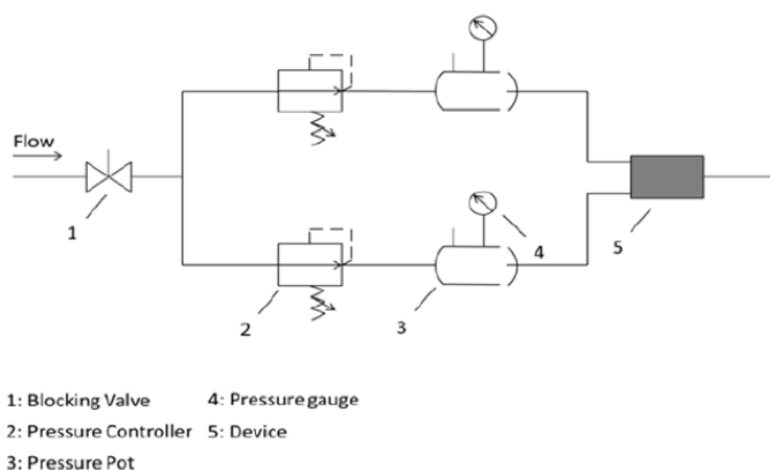


Figure 3.6: Schema of the pressure driven continuous microfluidics setup.

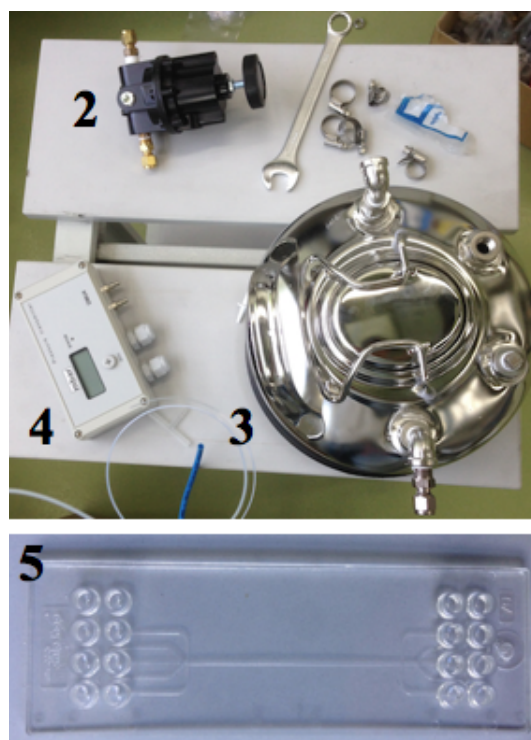


Figure 3.7: Component of the Microfluidics device: (2) pressure controller, (3) pressure pot, (4) pressure gauge, (5) microchip.

### 3.5 Immobilization

Another small task that the thesis will treat is the attachment of the microgels onto the electrodes. The chosen materials for the electrodes are gold (Au) and Indium Tin Oxide (ITO). The attempts of both physical and chemical attachment have been investigated. For the chemical immobilization of the surfaces, a reduction of the amide groups of the microgels have been tried. Before every physical or chemical treatment, a cleaning protocol has been applied to the

wafers:

1. Sonication of the wafers in Acetone for 5 minutes;
2. Again, sonication of the wafers in Acetone for 5 minutes;
3. Sonication of the wafers in Isopropanol for 5 minutes;
4. Again, sonication of the wafers in Isopropanol for 5 minutes;
5. Sonication of the wafers in water for 5 minutes;
6. Again, sonication of the wafers in water for 5 minutes;
7. Snowjet cleaning with solid  $CO_2$  ice crystals as a jet medium.

This cleaning process has been applied to every used wafer. After the cleaning, all the wafer were processed with a plasma oven in order to increase the reactivity and hydrophilicity. The process was carried out in Oxygen atmosphere for 5 minutes. Finally, the electrodes were ready to be functionalised for the immobilization. The goal of the immobilization is to obtain a robust, uniform, monolithic thin film of microgel onto the electrode (Self Assembly Monolayer, SAM).

### 3.5.1 Reduction of the amide groups

A certain amount of microgels were treated with reductive chemicals in order to reduce the amide group of Polyacrylamide to an amine group. The process for the reduction is:

- Dissolution of 20 mg of microgels in 5 ml of Tetrahydrofuran (THF);
- Addition of 0,5 mol (3,8 mg) of  $LiAlH_4$ , the reductive agent;
- Reaction time under stirring: 60 minutes;
- Separation of the microgels in solution with centrifuge (10.000 rpm, 20 min);
- Deactivation of  $LiAlH_4$  solution with dropwise addition of water followed by 15% sodium hydroxide and water ( $n$  grams of  $LiAlH_4$  require  $n$  ml. of water,  $n$  ml. sodium hydroxide, and  $3n$  ml. of water, added in succession).

The protocol is inspired by reference [112].

### 3.5.2 Immobilization on Gold

**Physisorption.** Firstly, the immobilization on Gold has been attempted via physisorption. Physisorption (or physical adsorption) is adsorption in which the forces involved are intermolecular forces (van der Waals forces) and which do not involve a significant change in the electronic orbital patterns of the species involved.

- The first process that has been tried is the **"Paint on"** protocol [113]. An aliquot of given microgel solution was centrifuged for 30 min at 10000 rpm in order to pack the microgels and create a compact pellet. A protocol cleaned wafer (1cm x 1cm) of Gold was put on a hot plate at 30°C. Thus, 40  $\mu$ l of the concentrated microgels were deposited onto the wafer. By means of the micropipette tip the microgel solution was spread along the entire wafer surface, paying attention to not scratch the Au surface. The spreading continued as long as the microgel layer started to dry and an increase of the viscosity was noticed. Finally, the microgels were let dry completely by increasing the temperature to 35°C. The whole procedure is schematized in Figure 3.8

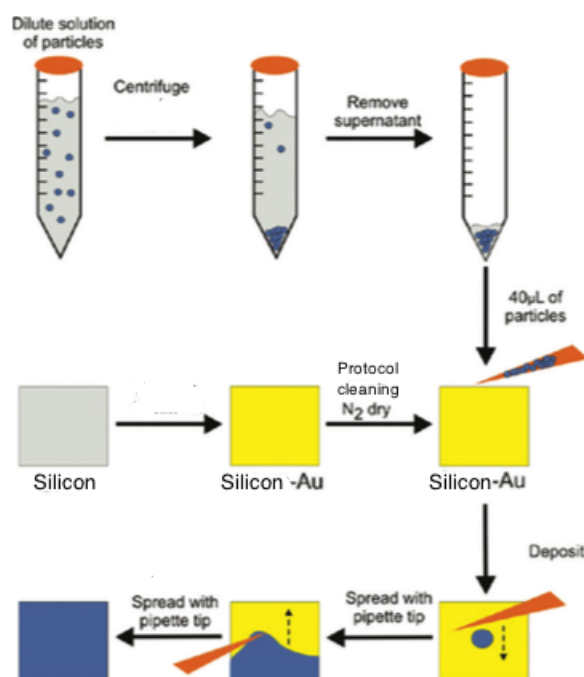


Figure 3.8: Schematic description of the "Paint on" protocol.

The excess of microgels was removed rinsing the wafer with copious water. In order to characterize the stability and the robustness of the attachment of the microgels, the treated wafer was rinsed first with deionized water, then with acetone.

- Another attempt of attachment via physisorption has been carried out through **"Spin Coating"** [114]. The layers of microgel has been prepared with a standard spincoater. A solution of about 200 ml of microgel has been dispensed on the wafer. Then, the spincoater was started with a spinning rate of 1000 rpm for 5 min. The liquid evaporated. Again, the stability and the robustness of the attachment of the microgels was characterized rinsing the wafer first with deionized water, then with acetone.

**Chemisorption.** Chemisorption (or chemical adsorption) is adsorption in which the forces involved are valence forces of the same kind as those operating

in the formation of chemical compounds.

- The procedure for the chemical attachment of the microgels on the Au wafers was inspired by the studied interaction Gold-Thiol [115] [116]. The chosen molecule to bind the microgel was Lipoic Acid (LA)(purchased by Sigma-Aldrich). As Figure 3.9 shows, LA is a organic molecule with a Thiol group on one extremity of the chain and with a carboxyl group on the other. Following the same staining procedure of the proteins (NHS-EDC coupling), it has been tried to immobilize the microgel on the Au electrode.

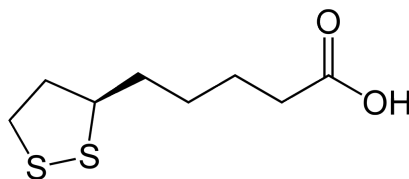


Figure 3.9: Molecule of Lipoic acid [117].

The whole protocol has been carried out as follows:

1. Preparation of the LA solutions in ethanol and water (1:1). The concentrations are 1 mM, 10 mM, 50 mM, 100 mM;
2. Reaction with the wafer in a microplate 12 wells for 1 hour. The wafer were then washed with ethanol and water to remove the unreacted LA;
3. Reaction with the EDC-NHS coupling protocol (in this case the LA substitute the protein);
4. Reaction in vessel with microgel solution (5 mg/ml) with mixing. Reaction time: 3h;
5. Vortex with PBST (PBS + Tween20 2% w) and washing with ethanolamine to remove the unreacted microgels.

In order to characterize the stability and the robustness of the bonds, the treated wafers were rinsed first with deionized water, then with acetone.

### 3.5.3 Immobilization on Indium Tin Oxide

In the case of ITO, the immobilization have been performed only via chemisorption. Recent studies confirmed the formation of a chemical bond between ITO and ethoxysilane groups [118] [119] [120]. The chosen molecule to functionalize the surface is 3-(Triethoxysilyl)propyl isocyanate (IPTES).

As shown in Figure 3.10, the Si atom in the middle is surrounded by three ethoxysilane groups and one cyanate group. While the ethoxysilane will react with the surface of ITO, the cyanate will hydrolyse and the amine groups of the microgels will substitute. The chemical procedure has been carried out on clean wafer in two steps:

1. Reaction in a microplate 12 wells between the wafers and a sufficient amount of IPTES solution composed as follow: 25 ml of ethanol, 2,1 ml of Ammonium hydroxide ( $NH_4OH$ ) and 200  $\mu$ l of IPTES. The ITO wafers were let

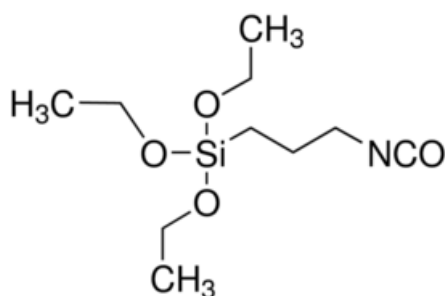


Figure 3.10: Molecule of IPTES [121].

react overnight at room temperature. They were then washed with toluene ethanol to remove the byproducts;

2. Reaction in vessel with microgel solution (5 mg/ml) with mixing. Reaction time: 3h;
3. Vortex with PBST and washing with ethanolamine to remove the unreacted microgels.

In Figure 3.11, the reaction is shown.

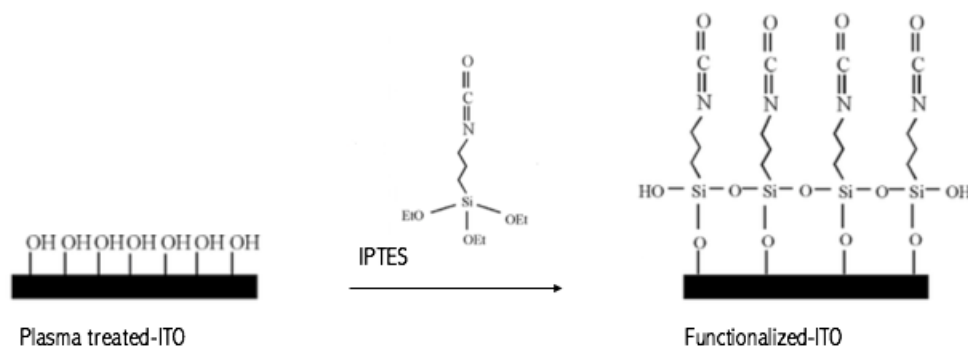


Figure 3.11: Formation of the isocyanate-silane monolayer by IPTES on the ITO substrate [118].

In order to characterize the stability and the robustness of the bonds, the treated wafers were rinsed first with deionized water, then with acetone.

## Chapter 4

### Results and discussion

This chapter will show the results of the experiments. The first section will concern the syntheses of the microgels. The results of the path from the concentrated emulsion polymerization to the protocol recipe will be analyzed. The second section is about the debate regarding the calculation of the concentration of the protein inside the microgels. All the results of the different techniques will be compared and discussed. Moreover, the most important task will be explained in the third section: the retaining of the activity of encapsulated GOD. This section will show the results with the changes of the parameters of the recipe as well as the supplement of additives. At last, the fourth section will illustrate the results of the attempts of immobilization of the microgels onto the electrodes (Au and ITO).

#### 4.1 Batch synthesis

At the beginning, the syntheses were carried out with the technique of Concentrated Inverse Emulsion Polymerization (CIEM) with encapsulation of Glucose Oxidase, following the recipe suggested by Retama et al. [99]. The first samples were:

- E1, with encapsulation of GOD (250 mg)
- E2, with encapsulation of GOD (10 mg)
- E3, empty microgels

The sample E1 was synthesized with 250 mg of GOD, strictly following the recipe suggested in the paper. Considering the not irrelevant price of GOD, it has been decided to reduce the content of GOD in the synthesis to 10 mg (sample E2). In addition, a blank sample with empty microgels has been synthesized in order to compare the result and the hypothetical interactions and effects of GOD on the synthesis. The results of the syntheses can be seen in Figure 4.1.

All the images taken with the optical microscope show that the microgels are formed. In fact, in Figure 4.1 can be clearly seen the presence of a lot of spheres, i.e. the microgels produce by the syntheses. Another issue that can be noticed in the picture, is that the microgels tend to stay closer to each other and form agglomerates.

Since the result of the first syntheses were not as promising as expected due to the agglomeration, a decision has been taken: switching from GOD to BSA as immobilized protein. This change has been done in order to contain the initial costs, at least at the first steps of the defining of a protocol recipe. BSA was

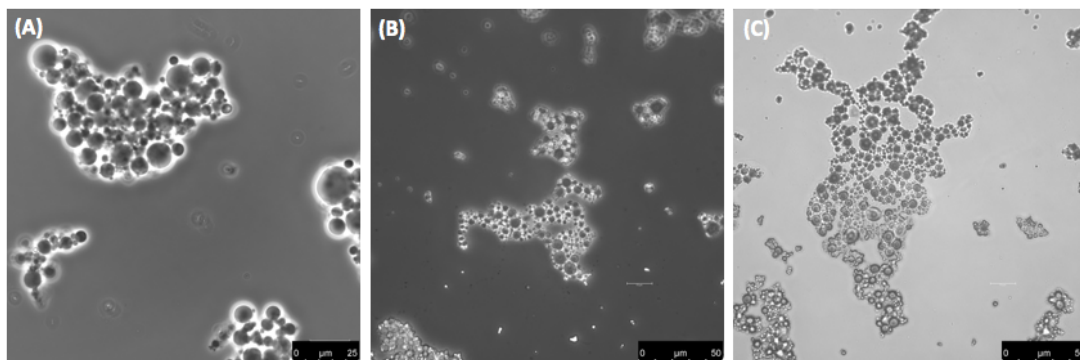


Figure 4.1: Optic images of the samples E1 (A), E2 (B) and E3 (C), results of the syntheses with concentrated emulsion polymerization.

chosen has biocomponent to encapsulate since it is cheap, easily to stain and make fluorescent. Another good point of using BSA was the eventual possibility to directly calculate the concentration of entrapped protein inside the microgel by means of Bradford assay. In order to compare with the previous syntheses, other two tries have been done with concentrated emulsion polymerization (E4, E5). The difference between the two was the crosslinking ratio: E4 was 3,2% while E5 was 7%. The crosslinking has been computed to 7% taking in account the different molecular weight and size of the two proteins. The result is shown in Figure 4.2.

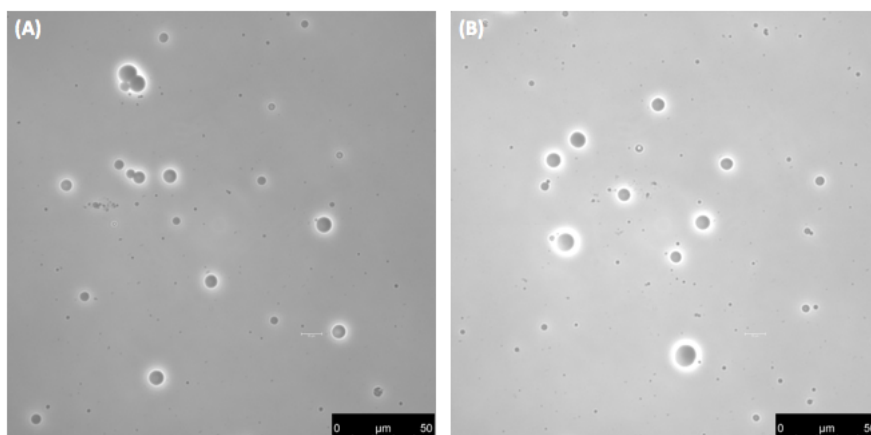


Figure 4.2: Optic images of the samples E4 (A), E5 (B), results of the syntheses with concentrated emulsion polymerization with entrapped BSA. The difference is the crosslinking degree  $\eta$ : 3,2% in (A) and 7% in (B)

These syntheses were definitely more promising than the previous one: the agglomerates disappeared. Likely, the change of protein nor the changing of  $\eta$  did not affect the result. The agglomeration did not occur anymore due to a thorough and intense stirring during the emulsion formation and during the synthesis. The stirring became a key point for all the following attempts. In these new tries another aspect started to gain attention: the size and the monodispersity. The calculated average size is 3,52  $\mu\text{m}$ . In Figure 4.3 it is possible to see the size

distribution of the microgels.

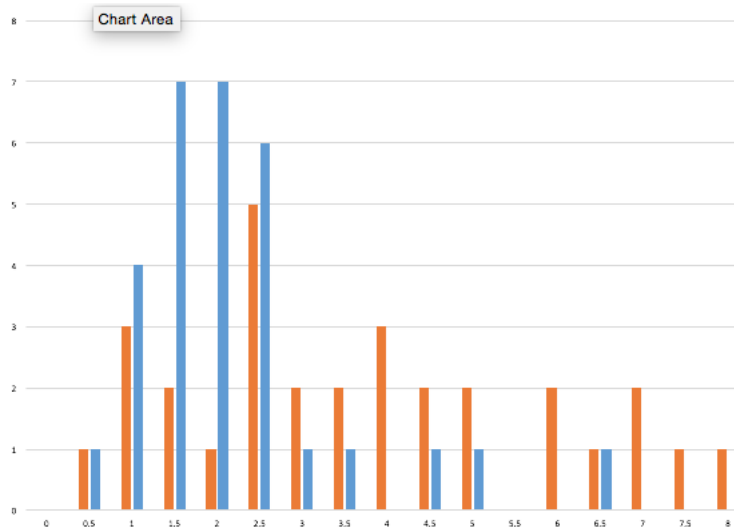


Figure 4.3: Size distribution of the samples E3 (orange), E4 (blue)

The size distribution is not uniform in both the samples. Moreover, the graph shows the high degree of polydispersity of the microgels.

At that stage, it has been possible to characterize the morphology of the microgels but there were no indications about the encapsulation: it was not possible to say if the protein was inside the microgel or not. In order to overcome this setback, BSA was coupled with FITC, a fluorescent dye. After that, we could check if the results of the immobilization in the microgel by means of the fluorescent microscope. Thus, new syntheses have been performed with the stained-BSA:

- E6.1, crosslinking  $\eta = 3\%$
- E6.2, crosslinking  $\eta = 6\%$
- E6.3, crosslinking  $\eta = 9\%$
- E6.4, crosslinking  $\eta = 12\%$
- E6.5, crosslinking  $\eta = 20\%$

In Figure 4.4 an image of the microgels is shown. It is an overlay of two pictures: one has been taken in Bright Field mode while the other in Fluorescence mode.

The fluorescence was present in all the samples. Therefore, the protein has been well encapsulated in the microgels. Visually, the intensity of the fluorescence does not substantially change in the samples with  $\eta$  equal to 3%, 6% and 9% and 12 % (Figure 4.4(A)(B)(C)(D)). It decreases with a really high crosslinking of 20% (Figure 4.4(E)). This might be due to the small porosity of the microgels that prevents the penetration of the protein. Comparing the pictures, it can be noticed that the microgels look very different. The agglomeration came back in the cases of (A), (B) and (C) of Figure 4.4. The size distribution is completely random and incoherent.

Considering the performed attempts, it has been decided that the concentrated

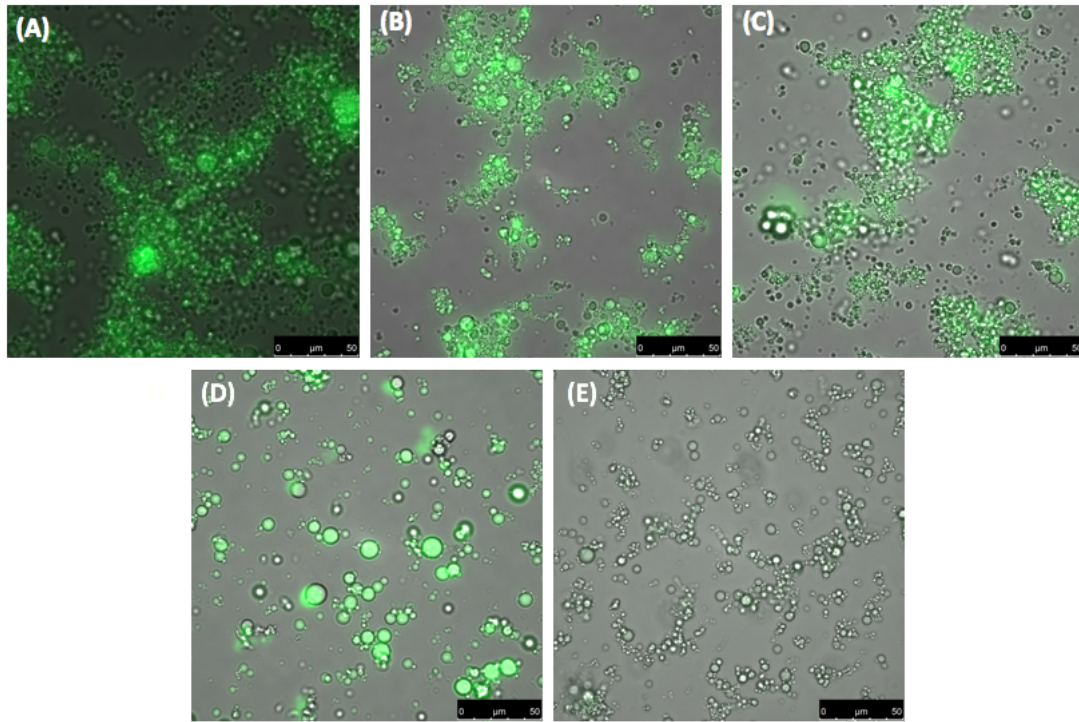


Figure 4.4: Overlay images of bright field and fluorescence mode of samples E6. The crosslinking changes: 3% (A), 6% (B), 9% (C), 12% (D), 20% (E).

emulsion polymerization is not an adequate recipe to be promoted as protocol synthesis. The results were not constant and reproducible; the synthesis is not reliable. Getting idea from the the concentrated emulsion and merging it with the recipe suggested by [102], a new promising synthesis have been tried (specifications are in paragraph 3.1.1). The syntheses have been performed with stained-BSA again. The produced samples are named HOBL-3%, HOBL-6%, HOBL-9%, HOBL-12%, where the percentage is the value of  $\eta$ .

The result was definitely as good as expected. The microgels in Figure 4.5 (A)(B)(C) are perfectly round, they do not agglomerate and they are uniformly and massively fluorescent. Though, in picture (C), the surface roughness increases. Another particularity is (D) ( $\eta = 12\%$ ) in which the microgels lose their circular shape to become irregular. Increasing too much the crosslinking degree some surface interactions appear on the microgels during the synthesis, producing instabilities. An important issue of these batch of synthesis regards the size. Figure 4.6 shows the size distribution of samples HOBL-3%, HOBL-6%, HOBL-9%. HOBL-12% has not been taken in account since the microgels are not round.

The average size has increased: it respectively is  $111,20\ \mu\text{m}$ ,  $76\ \mu\text{m}$  and  $56,91\ \mu\text{m}$  per HOBL-3%, HOBL-6%, HOBL-9%. A decrease in the average size is noticed with the increase of the crosslinking degree. Unfortunately, the microgels are still largely polydisperse since the size range varies between  $20$  and  $150\ \mu\text{m}$ . Nonetheless, this recipe is suitable to be promoted as protocol synthesis for the study. The chosen crosslinking degree for the protocol is 6%, since the microgels

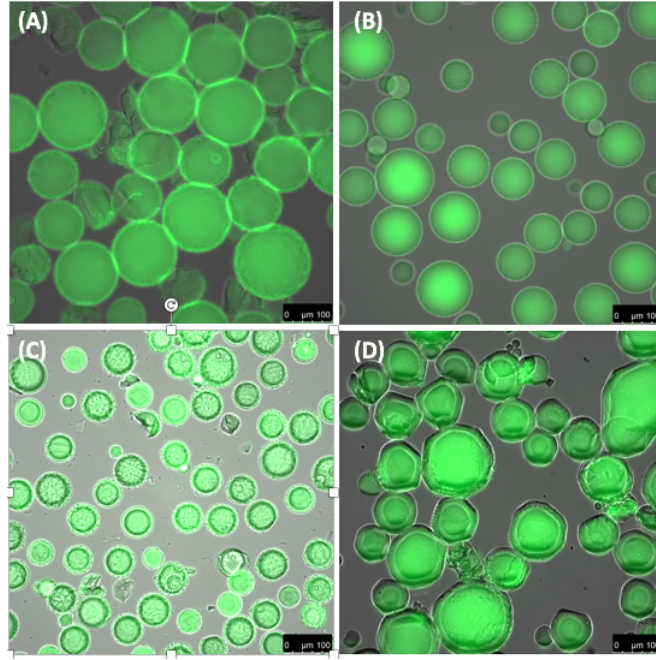


Figure 4.5: Overlay of images in bright field and fluorescence mode. The crosslinking  $\eta$  is in (A) 3%, in (B) 6%, in (C) 9% and in (D) 12%.

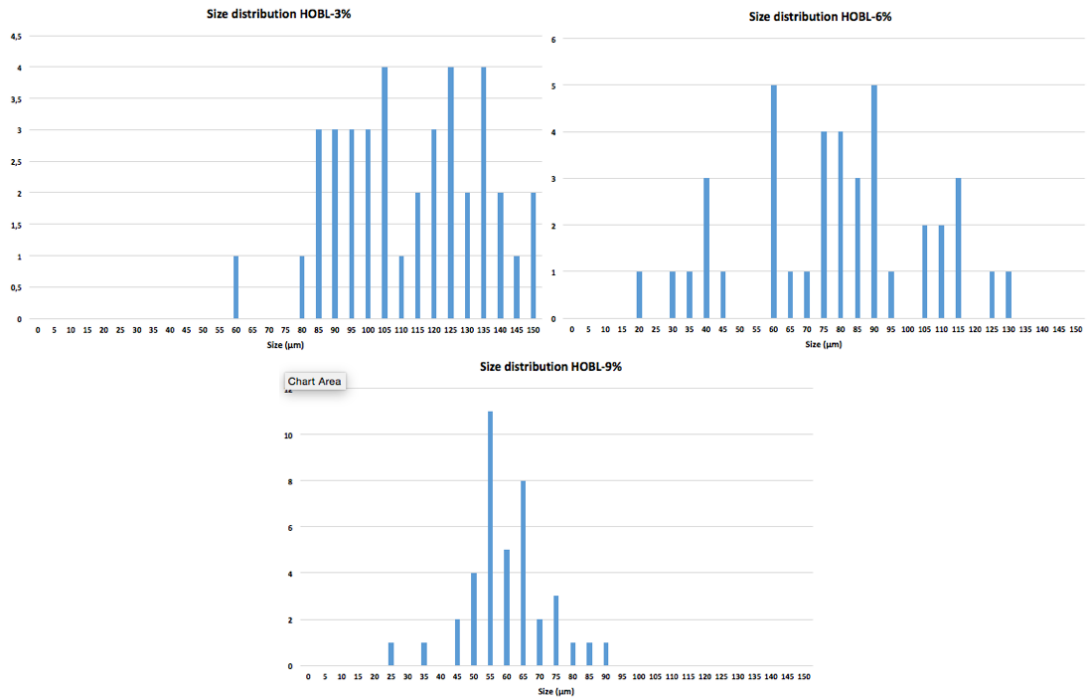


Figure 4.6: Size distribution of the samples E3 (orange), E4 (blue)

are the best looking.

To confirm the good results obtained with the new protocol, the encapsulation of GOD was tried. The synthesis was performed with the standard recipe,  $\eta =$

6%. Figure 4.7 shows the result.

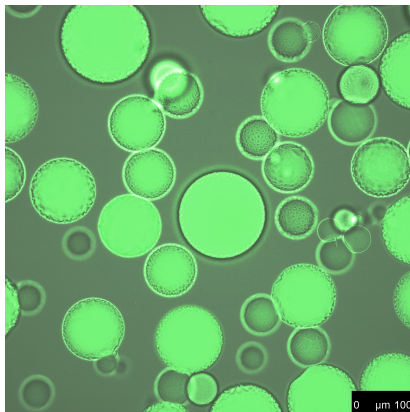


Figure 4.7: Microgels with stained-GOD encapsulated, protocol synthesis.

The microgels are well round-shaped and the fluorescence is present inside the microgels. The encapsulation succeed also in case of GOD. The tailoring of the synthesis was the following step. Other four synthesis have been performed with not stained GOD in order to study the effect

## 4.2 Calculation of concentration

The first characterization was to calculate how much biocomponent was encapsulated in the microgels. In order to detect that, different techniques have been used:

- Bradford assay
- BCA assay
- Fluorescence assay
- Elemental analysis (EA). With the EA, the goal was to detect the quantity of Sulfur in the samples. Thus, knowing the content of Sulfur in the protein and in the synthesis, it has been calculated the amount of protein encapsulated. The test has been performed only on HOG-6%, as a negative control.

The tests were performed on the samples synthesized with the standard recipe and with stained-BSA encapsulated (HOB-3%, HOB-6%, HOB-9%, HOB-12%). All the tests have also been carried out on empty microgels, in order to subtract the obtained values from the full microgels. In Table 4.1 are indicated the amount of protein detected and the loss ratio related to the initial amount of the recipe and to the production rate of the synthesis. The initial amount of stained-BSA put in the synthesis is 10 mg, while the mass of the bare BSA is 2,47 mg (computed by means of Bradford assay on the bare stained.BSA).

Table 4.1: Additives put in the protocol synthesis.

| HOB $\eta$ | Bradford [mg] | BCA [mg]  | Fluorescence [mg] | EA [mg] |
|------------|---------------|-----------|-------------------|---------|
| 3%         | 2,17          | 38,52     | 1,16              | -       |
| 6%         | 3,50          | 39,10     | 0,72              | 112     |
| 9%         | 3,41          | 39,31     | 1,11              | -       |
| 12%        | 5,1           | 40,55     | 0,87              | -       |
|            |               |           |                   |         |
| 3%         | 9,6%          | -1461,5 % | 51,7%             | -       |
| 6%         | -45,5%        | -1485,3%  | 70,1%             | -       |
| 9%         | -41,8%        | -1493,7%  | 53,6%             | -       |
| 12%        | -111,9%       | -1543,9%  | 63,9%             | -       |

The values listed in Table 4.1 for what concern Bradford and BCA assay are not coherent and pointless. The values of detected concentration of protein inside the microgels is higher than the amount of protein put in the synthesis. Consequently, the loss ratio is negative. The loss ratios actually express a gain of mass of the protein. The values are not relevant from a physic point. An explanation of these results might be given by the scattering. In fact, the emitted light could have scattered inside the wells, affecting the value of the detected adsorbance. A proof of this explanation can be the precipitation of the microgels in the well. This visual proof is giving by the activity test (explained later in paragraph 4.3). In fact, during the activity assay, the microgels turned blue. After some time, it has been noticed that the blue was present only in the precipitated microgels in the well. It was possible to notice this effect, thanks to the big size of the microgel (tens of  $\mu\text{m}$ ). On the other hand, the values given by the Fluorescence assay were reasonable. The amount of protein inside the microgels was less than the amount put in the recipe and the loss ratio expressed an actual loss. Following these results, from 50% to 60% of the initial amount of protein in the aqueous solution is then entrapped in the microgels during the synthesis. The highest value is 70,1% of the sample HOB-6%, which could be the worst crosslinking ratio to encapsulate the BSA. As a negative control, EA has been performed on this sample. The experiment showed a calculated amount of 112 mg. This value is definitely higher than the initial amount of BSA in the recipe. An explanation of this value might be the presence of Ammonium Persulfate as initiator. This component of the synthesis was already taken in account in the calculation of the content of Sulfur. Though, it is not possible to compute the conversion rate of this component, so a detailed analysis cannot be performed. In conclusion, EA is not a reliable negative control. Consequently, the values obtained by Fluorescence assay are not trustworthy and the concentration of protein inside the microgels cannot be detected. It has been decided to neglect the calculation of the concentration inside the microgels and put all the efforts on the retaining of the activity.

### 4.3 Activity of encapsulated GOD

In this section, the goal is to characterize the microgels by means of the activity test. For first, the activity of immobilized GOD was tested. The result showed huge loss ratios ( $\sim 99\%$ ) and small values of enzymatic activity. In order to improve the activity, the parameter of the synthesis were tailored. The chosen parameters are:

- **Crosslinking degree  $\eta$ :** changing the crosslinking degree, the encapsulated amount of GOD might change and consequently the overall activity. The chosen  $\eta$ s for the syntheses are 3%, 6% (standard), 9% 12%.
- **Content of organic phase (n-Heptane):** enzymes and proteins usually lose their activity when put in contact with organic solvents. The content of organic solvent has been changed in order to reduce the contact area between GOD and nH. In addition, the variation of composition of the oil phase might affect the size of the microgels. The new syntheses has been carried out with 5, 10, 15 and 25 (standard) ml of n-Heptane.
- **Content of surfactants:** the increasing of content of surfactants there might be less contact time between the organic solvent and GOD. This might prevent the denaturation of the enzyme. In addition, also the size might be affected. Starting from the standard recipe, the amount of surfactants has been increased by a factor 2 (2X), 3 (3X), 4 (4X) and 6 (6X). The ratio between the two surfactants SPAN80 and TWEEN20 has been kept constant.
- **Content of GOD:** since it has been impossible to detect the amount of GOD inside the microgels, new syntheses have been performed in order to define a eventual threshold of encapsulated GOD by means of the activity characterization. The chosen amounts of GOD to encapsulate are 1, 5, 10 (standard), 15, 20 mg.

In the results the nominal activity of the microgels has been detected. In addition, a loss ratio has been defined. The loss ratio is computed taking in account the initial activity of pure GOD. The initial activity has been checked with the activity test. The result is 18,14 U/mg.

#### Variation of the crosslinking degree $\eta$

The first parameter that has been change is  $\eta$ . Four synthesis have been performed with not pure GOD in order to study the effect of the changing of the crosslinking degree. The samples are labeled as HOG-3%, HOG-6%, HOG-9% and HOG-12%. Figure 4.8 displays the bright field images of the four samples.

In all the synthesis the morphology does not change: they are perfectly rounded. While in the the sample 3%, 6% and 9% the surface is smooth, in the sample with  $\eta = 12\%$  some roughness appears on the surface. For what concern the study of the size, Figure 4.9 explain the results.

The microgels are still widely polydisperse. The size range is circa 40-200  $\mu\text{m}$  for all the samples. The average size is almost constant for all the samples. The

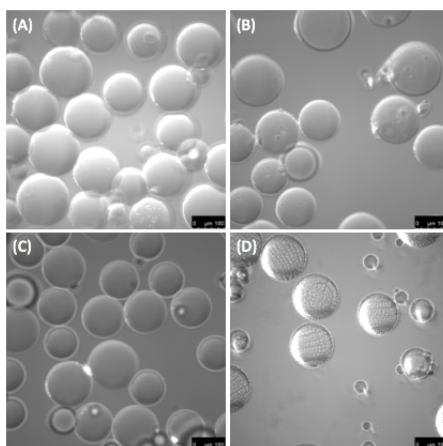


Figure 4.8: The crosslinking degree has been varied: in (A)  $\eta$  is 3%, in (B)  $\eta$  is 6%, in (C)  $\eta$  is 9% and in (D)  $\eta$  is 12%.

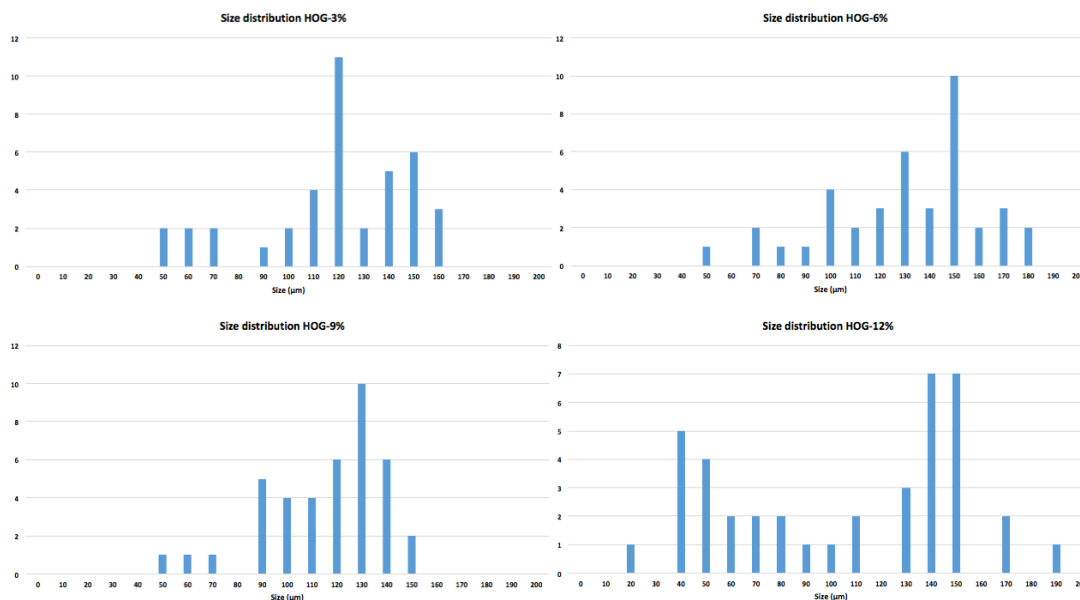


Figure 4.9: The graphs show the size distribution of all the samples with different  $\eta$ .

values are  $113,30 \mu\text{m}$ ,  $125,30 \mu\text{m}$ ,  $110,89 \mu\text{m}$  and  $98,86 \mu\text{m}$  respectively for the samples 3%, 6%, 9% and 12%. For what concern the activity tests the results are shown in Figure 4.10.

In case of crosslinking 3% the best result is obtained. Compared with the standard, the detected activity has a value double. Taking in account also the samples 9% and 12%, it is definitely higher. Even though an increase of activity has been detected, the value is still relatively low ( $0,03 \text{ U/mg}$ ). In parallel, the lowest loss ratio belongs to  $\eta = 3\%$  while it increases for the others. Comparing with the standard, the loss ratio lowers from 96% to 93%. Nonetheless the improvement, it is still high. The result can be explained considering the porosity of the microgels. In fact, with a crosslinking of 3%, the porosity of the microgels is higher and the penetration of GOD inside the microgels during the synthesis

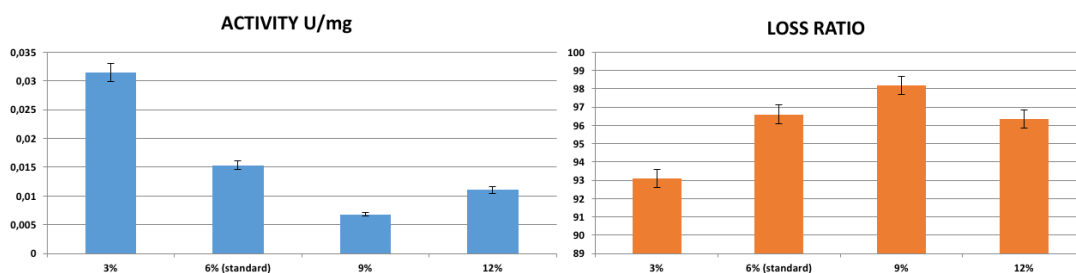


Figure 4.10: Activity U/mg and loss ratio of all the samples with different  $\eta$ .

might be more substantial. With an higher crosslinking rate, less GOD penetrates.

### Variation of the amount of organic solvent n-Heptane

The second parameters that have been tried to change is the content of organic solvent. Other four syntheses have been carried out with different content of n-Heptane in the oil phase. The chosen amounts of nH in the oil phase are 5, 10, 15 and 25 (standard) ml. The samples have been named 5 ml, 10 ml, 15 ml and 25 ml.

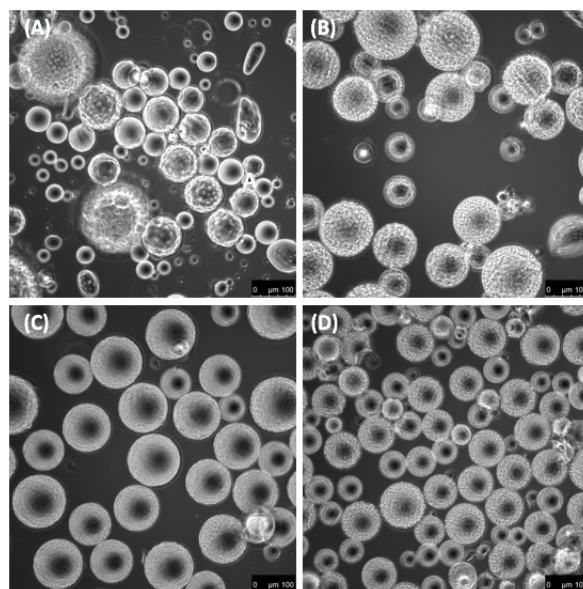


Figure 4.11: The content of n-Heptane has been varied: in (A) it is 5 ml, in (B) it is 10 ml, in (C) it is 15 ml and in (D) it is 25 ml.

As Figure 4.14 displays, the synthesis gave suitable results: the microgels are round-shaped. The roughness of the surface increases, not affecting the final microgels. For what concern the size, the range is 60-180  $\mu\text{m}$ . As indicated by Figure 4.12, the level of polydispersion is still high, as expected.

The calculated average size is 142,54  $\mu\text{m}$ , 96,25  $\mu\text{m}$ , 132,59  $\mu\text{m}$  and 125,30  $\mu\text{m}$  respectively for the samples 5 ml, 10 ml, 15ml and 25 ml. The expected result was a decrease of the size inversely proportional with the content of organic sol-

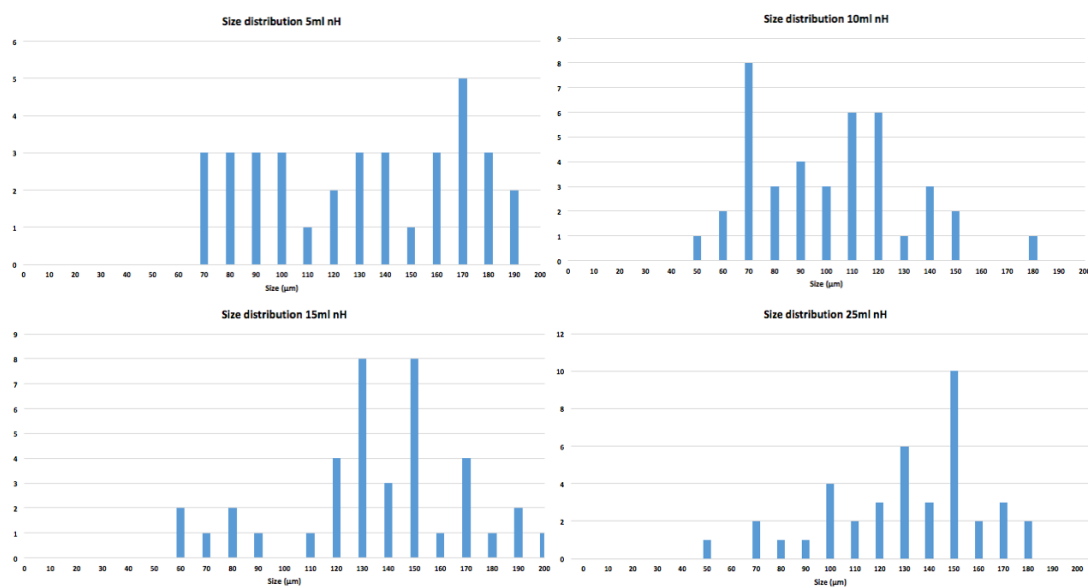


Figure 4.12: The graphs show the size distribution of all the samples with different content of n-Heptane.

vent. It did not occur. For what concern the activity experiments, the obtained histograms are shown in Figure 4.13. In this case the results are incoherent respect to the expected values.

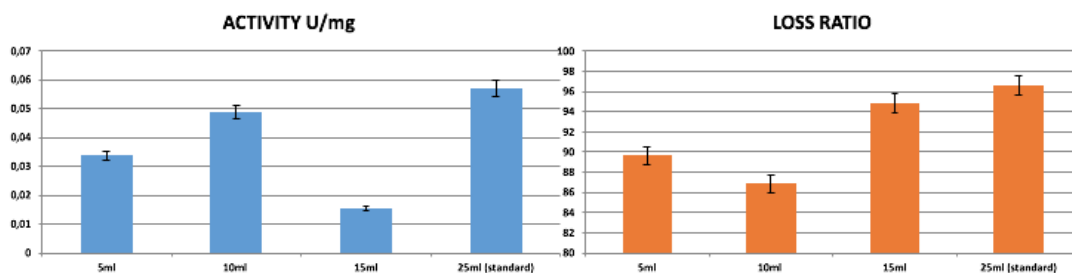


Figure 4.13: Activity U/mg and loss ratio of all the samples with different content of organic solvent.

About the values of activity, the highest peaks are obtained for a content of solvent equal to 10 ml and 25 ml. The sample synthesized with 5 ml of nH still shows a value of activity comparable with the highest peaks. Looking at the the sample 15 ml, the value of activity is definitely lower than the other samples. In general though, the values of activity are still in the range or even lower than the standard sample. A different discussion can be done for the loss ratios. In fact, the two samples with 15 and 25 ml of nH show values of 97% and 94%. A noticeable improvement occurred for the samples synthesized with an oil phase with 5 ml and 10 ml. This improvement is due to the different production rate of the synthesis. The produced amount are shown in Table 4.2.

Table 4.2: Produced quantities of samples with variation of content of organic solvent

| Sample   | Produced quantity [mg] |
|----------|------------------------|
| HOG-5ml  | 555,6                  |
| HOG-10ml | 513,7                  |
| HOG-15ml | 463,5                  |
| HOG-25ml | 401,7                  |

The quantities produced by the first two syntheses (5 ml and 10 ml) are remarkably higher than the last two (15 ml and 25 ml).

### Variation of the amount of surfactants

The third parameter that have been tailored is the content of surfactants. While the ratio of the two surfactants SP80 and TW20 has been kept constant, the overall amount has been multiplied by factor 2 in the sample labeled HOG-2X, by factor 3 in the sample labeled HOG-3X, by factor 4 in the sample labeled HOG-4X and by factor 6 in the sample labeled HOG-6X. In Figure ?? the morphological characterization is shown.

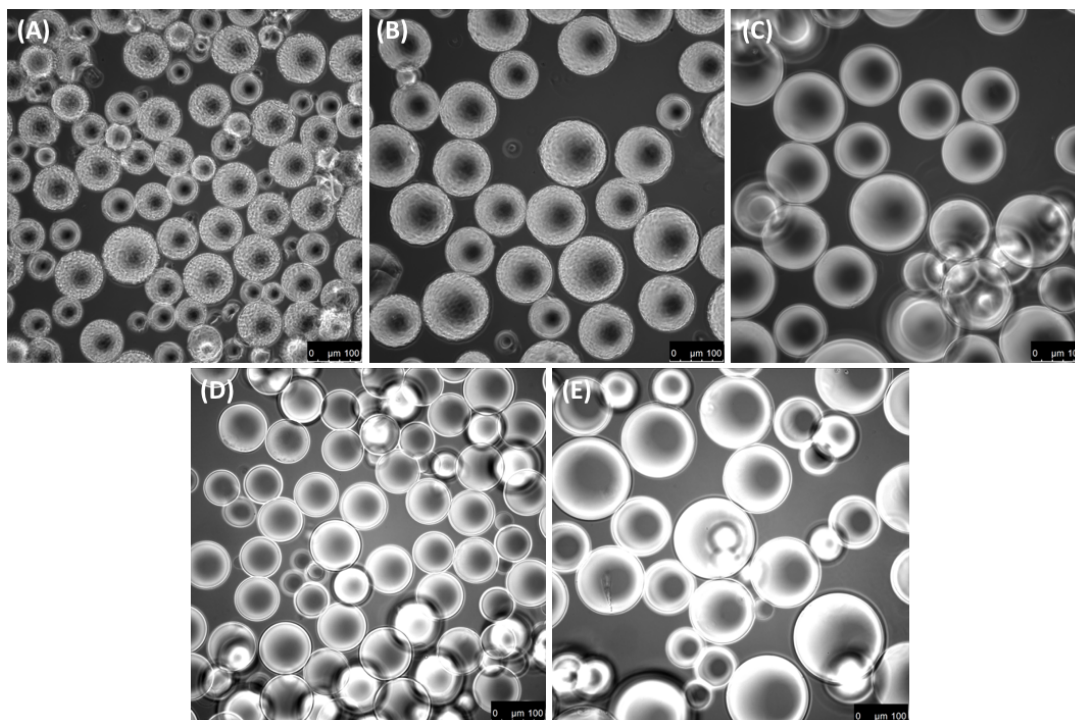


Figure 4.14: The content of surfactants has been varied referring to the standard recipe: in (A) it is standard, in (B) it is 2X, in (C) it is 3X, in (D) it is 4X and in (E) it is 6X.

The morphological analysis confirmed the effectiveness of the recipe. In all the samples the microgels are round-shaped and well defined. Again, the surface roughness is present, but it does not affect the final result. Regarding the size analysis, the problem of polydispersity is still present, as Figure 4.15 displays.

The average sizes of the samples are 125,30  $\mu\text{m}$  for the standard, 105,26  $\mu\text{m}$

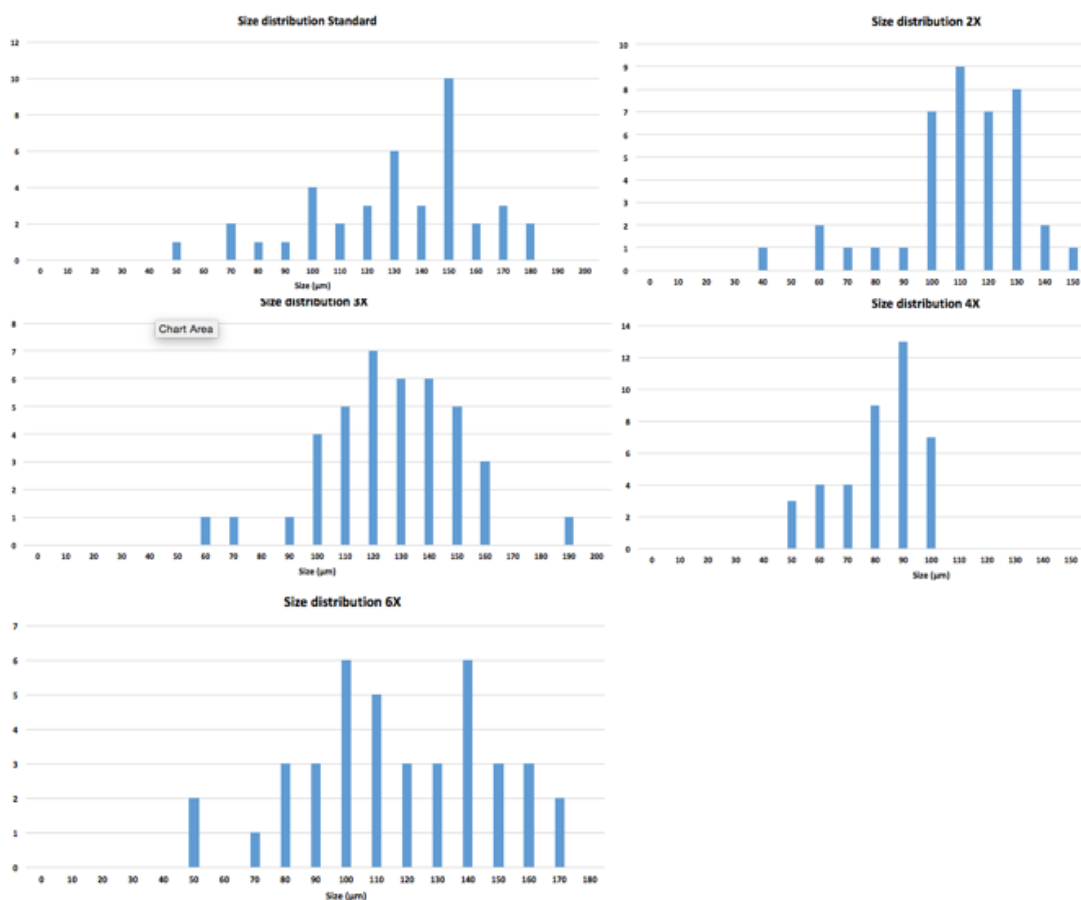


Figure 4.15: The graphs show the size distribution of all the samples with different content of surfactants.

for 2X, 121,32  $\mu\text{m}$  for 3X, 76,55  $\mu\text{m}$  for 4X and 112,43  $\mu\text{m}$  for 6X. The expected result was a possible change of the size of the microgels, since an higher content of surfactant might have produced smaller microgels. The result was totally different since the size range extended between 60 and 200  $\mu\text{m}$ , almost the same of before. About the activity, the values are reported in Figure 4.16.

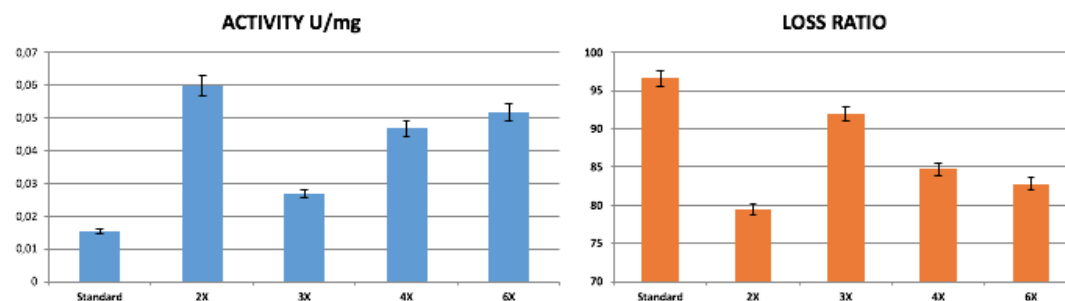


Figure 4.16: Activity U/mg and loss ratio of all the samples with different content of surfactants.

Both the parameters showed positive results. Comparing with the standard

sample, the activity is remarkably higher in all the cases, reaching the values of 0,05-0,06 U/mg. An exception is the sample 3X: the activity is a bit lower but an improvement is still noticeable. Again, the value of activity per mg is far from the original value of GOD. In parallel, also the loss ratios are undoubtedly lower. The lowest loss ratio has been obtained for the sample 2X, with a value lower than 80%. The samples 3X, 4X and 6X presented a loss ratio in the range of 92-82%. The explanation of these results might be found in the contact time of the aqueous with the oil phase. GOD is dissolved in the aqueous phase and then put in contact with the oil phase by means of a pipette. In the time between the mixing and the formation of the droplets the biocomponent is in contact with the organic solvent, a proved denaturing agent for GOD. Increasing the content of surfactants, the number of surfactant molecule increases and thus the probability for the two phase to meet a surfactant molecule and dispose in droplet shape is higher, reducing therefore the contact time of Glucose Oxidase with n-Heptane.

### Variation of the amount of Glucose Oxidase

The last parameter that have been changed is the content of Glucose Oxidase dissolved in the aqueous phase. It was changed since it was impossible to detect the concentration of the enzyme inside the microgels. In the standard recipe the content of GOD was 10 mg. The new attempts of syntheses have been performed with 1 mg, 5 mg, 15 mg and 20 mg. The samples have been named HOG-1mg, HOG-5mg, HOG-15mg, HOG-20mg. In Figure 4.17 the images at the microscope are shown.

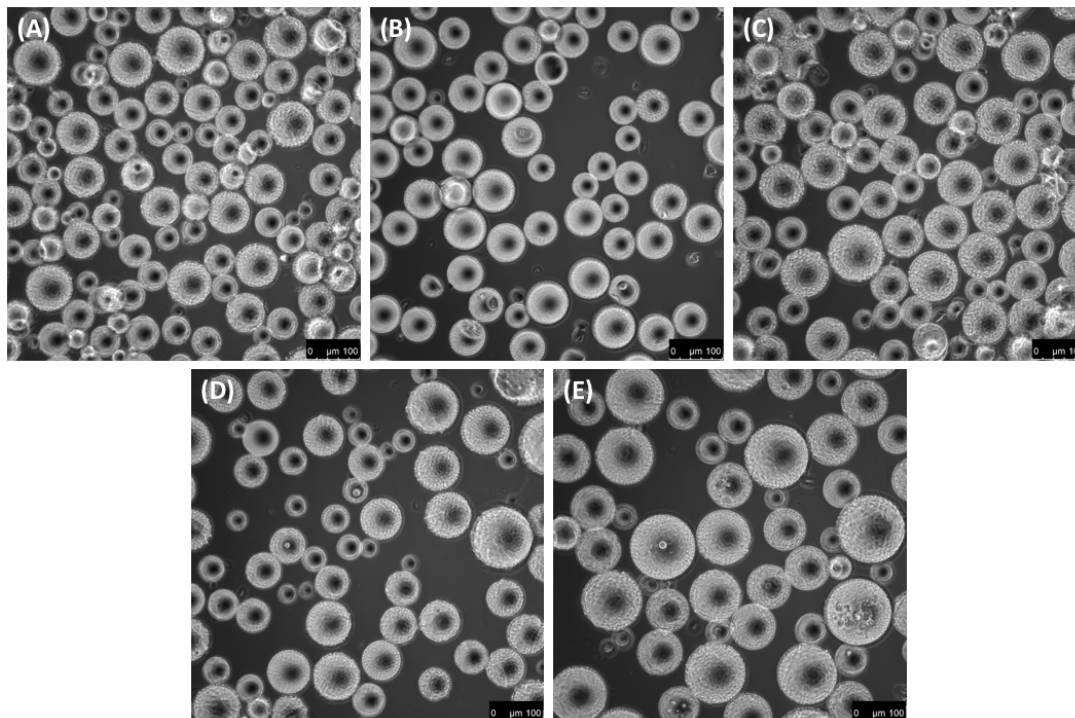


Figure 4.17: The content of GOD has been varied referring to the standard recipe: in (A) it is 1 mg, in (B) it is 5 mg, in (C) it is 10 mg (standard), in (D) it is 15 mg and in (E) it is 20 mg.

The morphological analysis showed again that the microgels are well defined and round-shaped. The syntheses succeeded again even though some surface roughness is present. It does not affect the study. Also the activity tests have been performed. The result are shown in Figure 4.18.

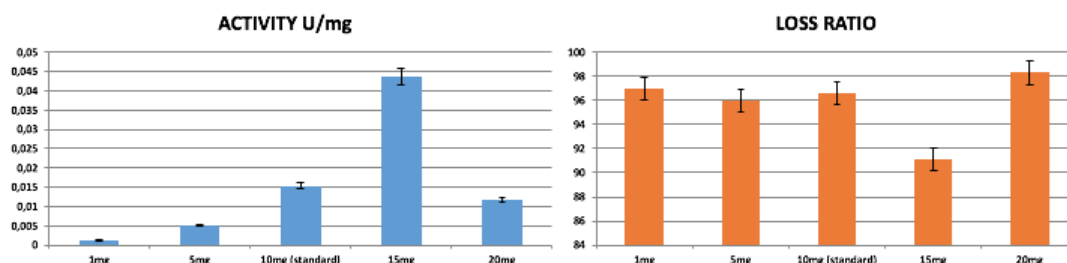


Figure 4.18: Activity U/mg and loss ratio of all the samples with different content of GOD.

Analyzing the histogram about the overall activity, an increase of the characteristic is noticed. From a value really close to zero for the sample with 1 mg, the activity constantly increases until it reaches the maximum value of 0,44 U/mg for the sample with 15 mg of GOD. Some improvements have been done even though the module of the highest activity is still really lower than pure GOD. When the amount of GOD in the synthesis is higher than 15 mg the activity comes back to lower values. Since the synthesis conditions are the same for all the samples, it is possible to directly correlate the increase of activity with the greater amount of GOD encapsulated in the microgels. A threshold of encapsulation can be defined for the synthesis: putting a quantity of GOD bigger than 15 mg in the microgels does not produce an increasing of the activity. On the contrary, the encapsulation of GOD is bad-performing and the overall activity of the microgels decreases. For what concern the loss ratios, every sample shows a value higher than 96%, but the sample 15 mg. This result is in parallel with the one obtained measuring the activity.

The tailoring of the synthesis have given some good results. Now, the best condition of synthesis can be chosen in order to produce the microgels. Nonetheless the obtained results, the experiments showed low values of activity in every occasion. For every sample, the activity was way lower than 0,1 U/mg. A new approach has been taking in consideration from this moment: the supplement of additives in the synthesis. After some literature research, a choice has been taken and the chosen additives were:

- **Trehalose.** The stability of proteins is modulated by the addition of cosolvents. Some cosolvents, such as sugars and polyols act as protein stabilizers, whereas others denature proteins. Trehalose is a sugar that has been proved to be one of the best protein stabilizing compatible solutes found in nature. The chemical formula is given in Figure 4.19.

Its stabilizing property is explained by the preferential hydration theory [122]. The theory explains the ability of trehalose to be excluded from the

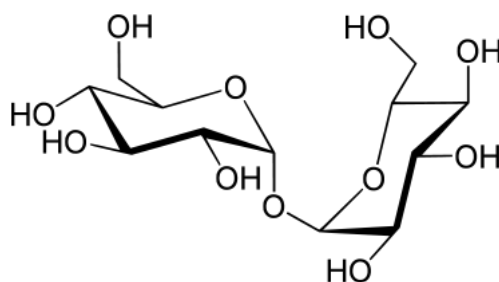


Figure 4.19: Chemical structure of Trehalose.

protein surface and thus, facilitating the interaction between the protein and water molecules. In addition, it has been proposed that trehalose increases the stability of proteins by favoring the most compact folded state, while high viscosity hinders protein domain motions leading to unfolding [123][124]. The synthesis have been performed with 100 M of Trehalose dissolved in the aqueous phase.

- **Glycerol.** This additive is a polyol used as cosolvent for the protein stabilization. Figure 4.20 displays the structure.

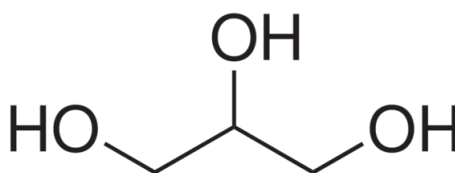


Figure 4.20: Chemical structure of Glycerol.

Usually polyols stabilize protein by means of mechanisms such as excluded volume effect [103] and the solvophobic effect [104], which lead to protein conformations with minimal solvent accessible surface area. By the same mechanisms polyols would promote reversible protein association because the solvent accessible surface area of the protein complex is smaller than that of unassociated proteins. Glycerol is commonly used in protein refolding [125], protein crystallization [126], formulation of biopharmaceuticals [127] and in the food industry [128]. Physically, it induces protein compaction [129], reduces protein flexibility [130] and affects both native and non-native protein aggregation [131], all positive elements for the retaining of the enzymatic activity. The synthesis have been performed with 100 M of Glycerol dissolved in the aqueous phase.

- **Vinylimidazole.** Another way to enhance the activity of GOD inside the microgels was the incorporation of a copolymer during the polymerization. A possibility of denaturation could be the electrostatic interaction between the polymer shell and the enzyme. In order to avoid these interactions Vinylimidazole has been added to the recipe [102]. The structure of VIm is

shown in Figure 4.21. The synthesis have been performed with 5% mol of Vinylimidazole dissolved in the aqueous phase.

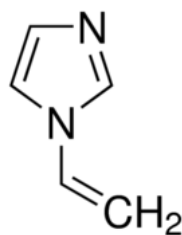


Figure 4.21: Chemical structure of Vinylimidazole.

The new three syntheses have been carried out as well as the activity characterization. The three samples are named Gly, VIm and Trehalose. The morphological results are shown in Figure 4.22.

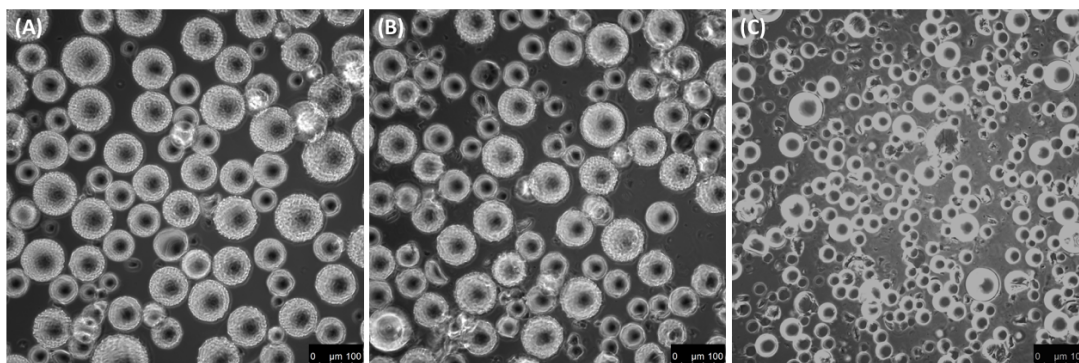


Figure 4.22: The additives have been supplied to the standard recipe: in (A) it is Glycerol, in (B) it is Vinylimidazole and in (C) it is Trehalose.

The size analysis showed results analogous to the previous syntheses. The average sizes are  $61,98 \mu$ ,  $56,96 \mu$  and  $114,09 \mu$  respectively for Gly, VIm and Trehalose. The polydispersity is still high, as it can be seen in Figure 4.23. A difference is the size range: for the sample Gly and VIm it is  $40\text{--}90 \mu\text{m}$ , while for Trehalose it is  $40\text{--}170 \mu\text{m}$ . The microgels in the first two cases are smaller than the third, which has a size range comparable with the previous synthesis.

For what concerns the activity characterization the results are shown in Figure 4.24.

Comparing with the standard recipe, the histograms show that the stabilization of GOD failed in case of Gly and VIm. The amazing result was obtained in case of Trehalose: in this case the activity has the value of  $0,15 \text{ U/mg}$ , more than one order of magnitude higher than all the syntheses ever performed. The stabilization of GOD succeeded, finally the microgels have a good retaining of the activity. The good result is confirmed also by the loss ratio. The value of the loss ratio in this case is  $43,9\%$ . It means that the retaining of the activity per mass of GOD of the microgels is more than doubled respect to the standard syntheses. An explanation of this result might be found in the increase of the

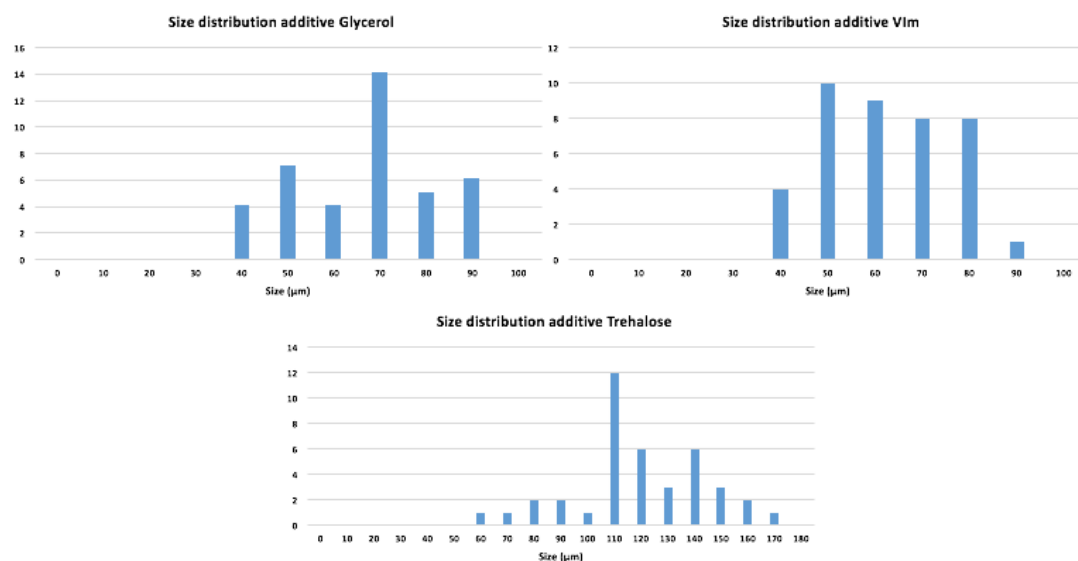


Figure 4.23: The graphs show the size distribution of all the samples with different additive.

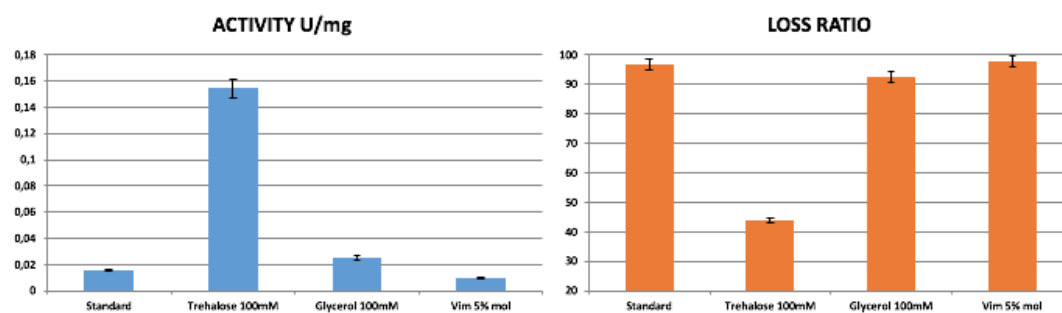


Figure 4.24: Activity U/mg and loss ratio of all the samples with different additives.

temperature right after the initiation of the polymerization. In fact, when initiated, the vessel reached a temperature around 37-40 °C. This temperature is far from the denaturing temperature of GOD ( $\sim 50^{\circ}\text{C}$ ), i.e. the temperature in which enough energy is provided to the protein to unfold, with consequent lost of activity. Though, inside the reaction vessel there might be some points in which the temperature was higher and reached the denaturing temperature. As consequence GOD was losing its enzymatic activity. In order to go further with the study of additivation of Trehalose, different quantities of Trehalose has been put inside the synthesis. The chosen concentration are 50 M, 150 M and 200 M. The syntheses with 150 M and 200 M totally failed. The aqueous phase with the normal composition plus the trehalose was synthesizing before the mixing with the oil phase, becoming a bulk polymer. The formation of the micelles was impossible and consequently the production of the microgels. Nonetheless, the synthesis 50 M succeeded. The morphological result is shown in Figure 4.25.

The activity test was performed on this sample and compared with the standard sample and the Trehalose 100 M. The result is shown in Figure 4.26.

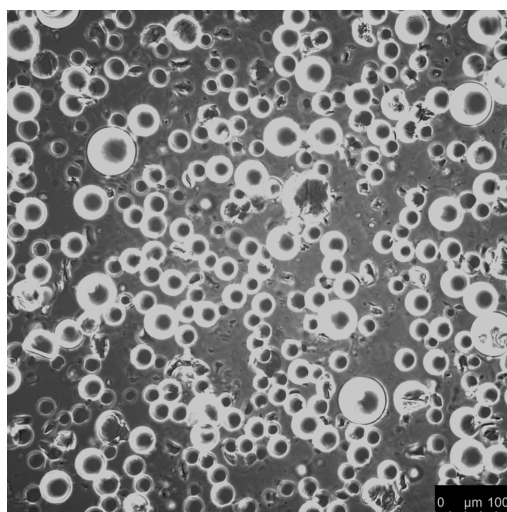


Figure 4.25: Microgels with addition of Trehalose 50 M.

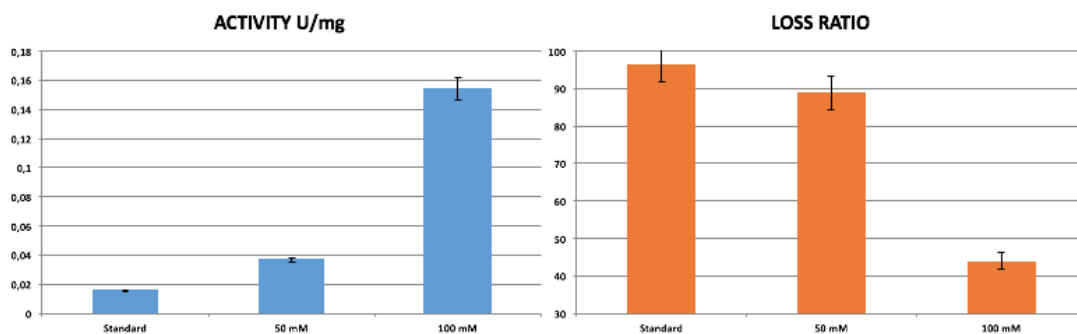


Figure 4.26: Activity U/mg and loss ratio of the sample with Trehalose 50 M.

Compared with the standard and Treha 100 M, Treha 50 M is closer to the standard as results. In fact the activity is low, even if an improvement is noticed. Also the loss ratio is pretty high: with a value of 88%, it is far from the surprising result of Treha 100 M. An improvement is noticed also in this case. In fact 88% is a net enhancement compared with the 96% of the standard sample. This proves that Trehalose is still stabilizing the enzyme and that it is a good stabilizer for GOD.

#### 4.4 Microfluidics

In order to overcome the problem of the polydispersity, the use of a microfluidics setup has been taken in account. All the components to build up the setup have been ordered and a continuous pressure driven production plant has been built. The explanation step by step of the components, the functioning and the building up can be found in paragraph 3.6. In Figure 4.27, the finished setup can be seen.

From the two vessels containing respectively the oil phase (OP) and the aqueous phase (CP), two small tubes (diameter 1 mm) come out and are connected to

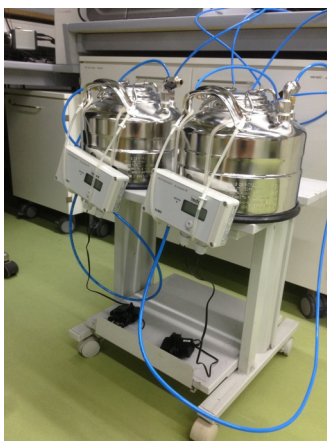


Figure 4.27: Microfluidics setup.

the microfluidics chip. The chip is the pivotal part of the setup. In fact, thanks to the microchannels in which the liquids are pumped the droplets are created. This happens especially at the triple point, in which the continuous and the dispersed phase get in contact and by means of the friction they create a droplet. The droplets are then kept together thanks to surfactant molecules, as explained in paragraph 2.2.1. A schema of the triple point can be seen in Figure 4.28.

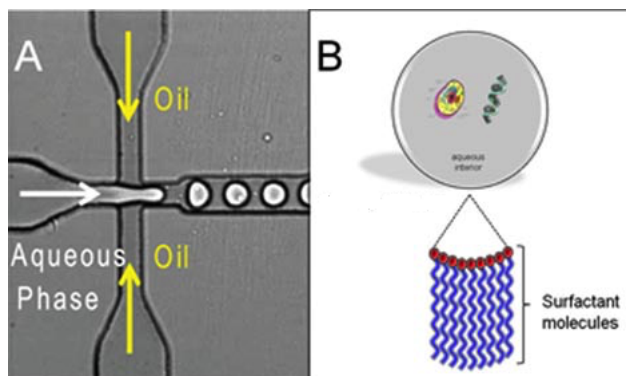


Figure 4.28: This is the point in which the microchannels get in contact as well as the oil and the aqueous phase. The droplets form here and are kept together by the surfactants [132].

The chip is small, so it can be put under the optic microscope and the triple point is easily visible. All the experiments were performed live. The liquids are pumped in two channels: one internal with the aqueous phase and one external with the oil phase. The external one is split in two parts in order to reach the triple point from two different directions. The range that the pressure can reach is 1-1000 mbar, controlled by means of the pressure controller. At the beginning the oil phase is pumped into the chip. Once that the flow is stable, also the aqueous phase is pumped. When the liquid gets in contact three scenarios can occur depending on the pressure of the aqueous phase:

1. If the pressure is too low, the flow of the aqueous phase has not enough strength to brake the friction forces created by the oil phase and it creates a

stable cone that does not produce any droplet. In extreme cases, when the pressure ratio between the two flows is too high ( $OP > AP$ ), the oil phase pushes back the aqueous phase and penetrate into channel. This can be dangerous since contamination of the aqueous phase can occur.

2. If the pressure is too high, the two flows at the triple point go straight out the output channel without mixing or create the droplets. Once they are in the output channel, they emulsify creating random-size micelles.
3. If the pressure ratio between the two phases is correct, the micelles form and go into the output channel reaching the collecting flask.

The first step was to create the droplets. Two flask were prepared: one with water and the other with n-Heptane and the surfactants SP80 and TW20 (same quantities of the protocol synthesis). The flasks were put into the vessel and the Nitrogen valve was released. The pressure was tailored for long time and countless attempts but the result always crashed into the situation 1 and 2 enumerated before; the micelles did not form. In this case the used pressure range encompassed the values between 100 mbar and 1000 mbar. Since any result was obtained so far, the choice to change the oil phase was taken. This decision was reached due to the low viscosity of nH. Consequence of that was the possibility that the friction brought by nH was not enough to brake the stream of water and create the droplets. So, the composition of the oil phase was changed and Petroleum ether (PE) was add to the mixture (ratio 1:1 with nH). The oil phase was now composed as follow:

- Organic solvents: n-Heptane and Petroleum ether ratio 1:1 in volume.
- Surfactants: Span 80 and Tween 20, as in the protocol recipe. The overall quantity is calculated keeping the same ratio used in the protocol recipe with nH, but related to the total volume of organic solvent (nH+PE).

The new oil phase was put in the vessel and the Nitrogen valve was started. In this case, the oil phase was pumped with a really low value of pressure (35 mbar). This value was kept constant while the value of the aqueous phase changed. As soon as the flow was stable, the pressure of the other vessel was raised to 10 mbar and the aqueous phase started to flow. This time, the study of the pressure was done at really low values of pressure ( $< 100$  mbar). at 10 mbar the flow of water could not reach the triple point, so it was slowly increased to 20 mbar, in which the cone at the triple point started to form. Once the cone formed, the next step was to raise more the pressure in order to let the droplet create at the tip of the cone. Therefore, the pressure has been raised again really slowly. The tip of the cone started to change shape: from rounded it became sharper. At the value of 50 mbar the first droplet appeared on the screen. The creation of the droplets became constant. In Figure ??, the screenshots of a video show the formation of a droplets.

In Figure ??, the screenshots of a video show the formation of a droplets. At the beginning the cone is rounded (A). Than the tip starts to sharpen under thanks to the pressure applied by the incoming aqueous phase(B). Moreover, the elongated cone shrinks in the middle, creating the droplet (C)(D). The shrinking

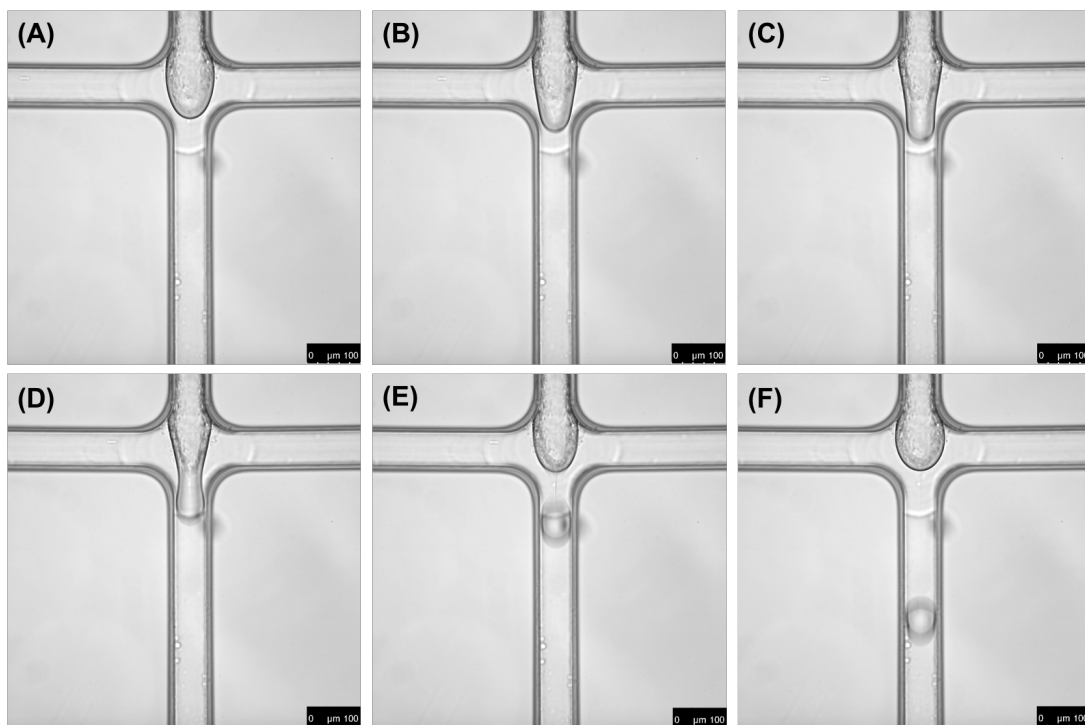


Figure 4.29: From (A) to (F), the cone elongates and the droplet detaches from the flow.

continues until the droplets is detached (E). The created micelle then goes forward in the outlet channel. The production rate of the the setup was really slow, but it could be kept working overnight, since the droplets formation was really stable and constant. Moreover, the problem of polydispersity seemed figured out: the size of the micelles was always the same. The pressure of the water was raised again in order to detect the changes. In fact, the size of the droplets increased, as can be seen in the sequence of screenshots in Figure 4.30, in which the pressure of the water was 100 mbar.

It has been played with the pressure a little bit more and the droplets formation of different sizes were performed. Also different values of oil phase pressure were tried but always starting from the same ratio 35/50 of the first droplet formation. In any case, at the values of  $\sim 140$  mbar for the oil phase and  $\sim 200$  mbar for the aqueous phase the flow of water became constant and the formation of the droplets ceased. The following idea was to synthesize the microgels after the formation of the micelles in the chip. The aqueous phase and the oil phase were kept the same composition of the protocol recipe in order to try the synthesis. Afterwards, the idea was to additivate. Two ways of initiating the polymerization were considered: with APS/TEMED complex activation and with UV light irradiation. Unfortunately, the time was not enough to perform this type of experiments.

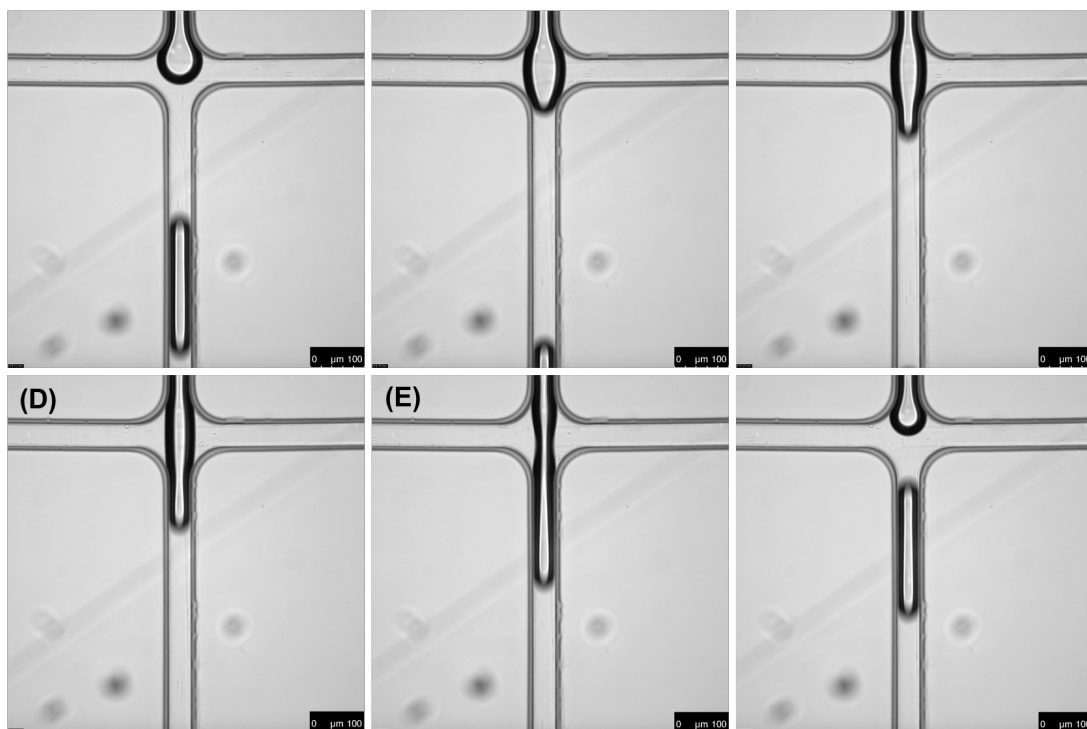


Figure 4.30: From (A) to (F), the cone elongates and the droplet detaches from the flow. In this case the droplet is bigger.

## 4.5 Immobilization

The results explained in this part explain the immobilization of the microgels onto electrode substrates such as Gold wafer and ITO wafer. At the former stage, the procedure has been carried out trying to use eventual physical interactions between the microgels and the substrate. At the latter stage, the attempts have been carried out by means of chemical interactions, trying to build a covalent binding between the substrates and the microgels.

### 4.5.1 Physisorption

The two processes to form a self assembled monolayer (SAM) of microgels onto the substrates by means of physisorption were spin coating and paint on. Only the Au wafers were treated using these techniques. Regarding the spin coating the result are shown in Figure 4.31.

In Figure 4.31(A) the deposition of the microgels is shown. The SAM is not uniform on the surface, some parts microgels did not attach to the wafer. Figure 4.31(B) represents the wafer after the rinsing with water. Almost all the microgels are washed away from the surface. A second rinsing has been performed with acetone: the wafer was as clean as untreated. The failure of the attachment is evident. The physical interaction between the microgels and the surface are not strong enough to resist to the washing. The other attempt was carried out with the technique of paint on. Figure 4.32 displays the result.

Figure 4.32(A) shows the result after the immobilization of the microgels.

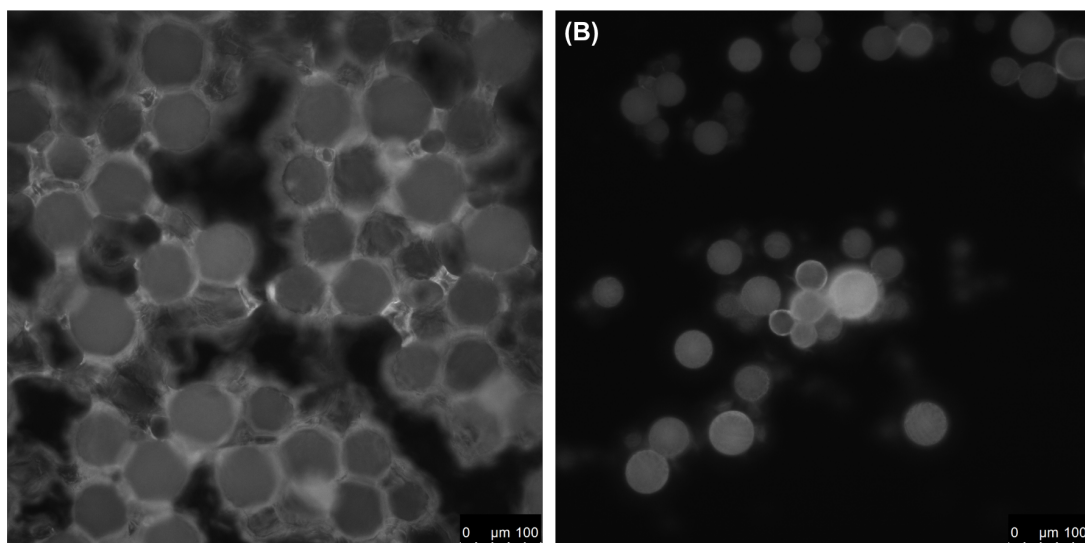


Figure 4.31: Wafer of Gold spin coated: (A) is the picture of the immobilized microgels while (B) represents the wafer washed with water.

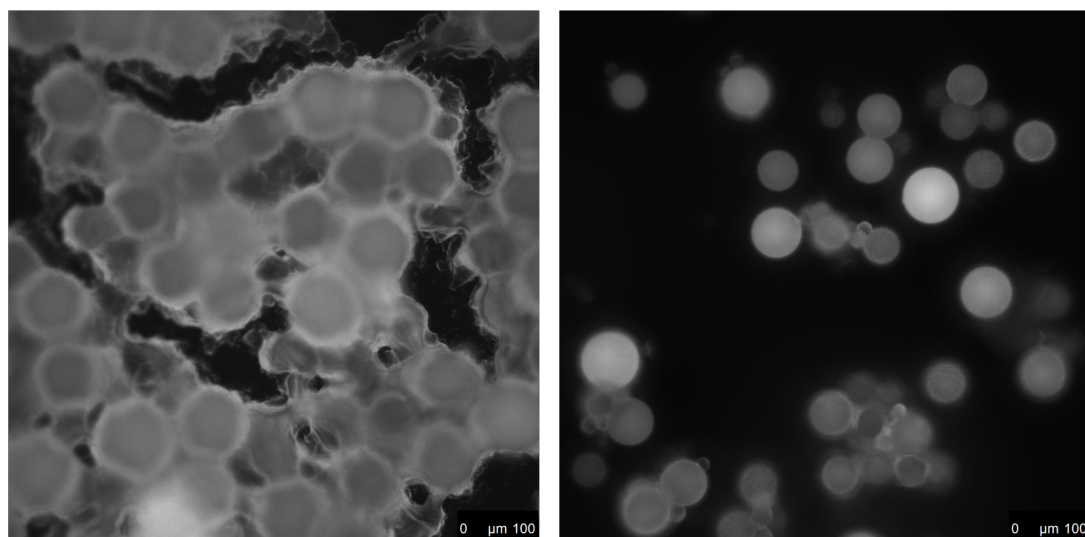


Figure 4.32: Wafer of Gold covered with paint on technique: (A) is the picture of the immobilized microgels while (B) represents the wafer washed with water.

The layer is not uniform and the shape of the microgels is a bit changed. They still look rounded but some merging between each other appeared. When the wafer was washed with water (4.32(B)) almost all the microgels detached from the surface, totally disrupting the layer formed. Once the wafer was rinsed with acetone, it turned completely clean as new. Again, the physisorption failed due to the weakness of the interaction between Au and the microgels.

#### 4.5.2 Chemisorption

Since the physisorption of the microgels onto the Au failed, the way of the chemisorption was undertaken. The former step was to reduce the amide group of

the polyacrylamide in order to obtain an amine groups. In fact, amines are more reactive than amides because they can easily substitute with other hydrolyzed groups with Nitrogen. The latter step consisted in the functionalization of the surface with a chemical with on one side a group that can covalent bond with the wafer and on the other side a group that can hydrolyze and react with the amine group of the microgels. The attempts of chemically bind the microgels was performed on both the Au and ITO wafers.

### Gold wafers.

The Au wafer were functionalised by means of a two step reaction:

1. Attachment with lipoic acid. In fact it has been proved that Gold and Thiol groups can covalently bind. On the other side of the chain of lipoic acid, a carboxyl group is present. The reaction was carried out with four different concentration of lipoic acid in solution: 1 mM, 10mM, 50 mM and 100mM. As first characterization, contact angle measurement was performed on the sample with 10 mM and compared with the one of the untreated wafer. The results are shown in Figure 4.33.

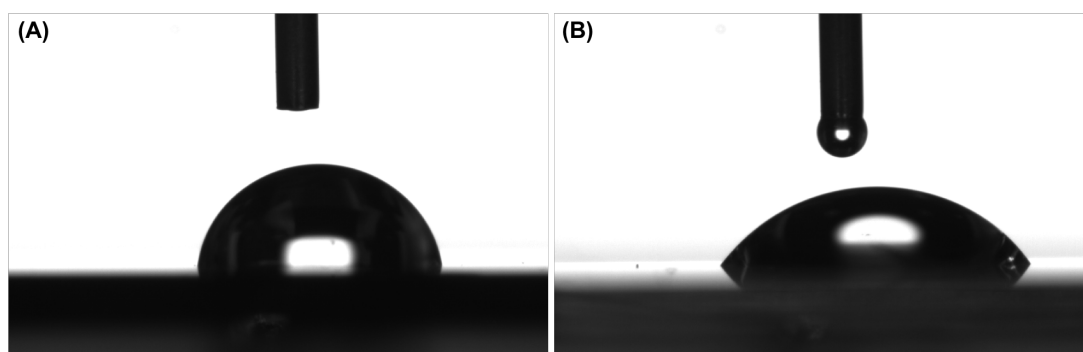


Figure 4.33: Contact angle measurements on untreated Au wafer (A) and lipoic acid functionalised Au wafer (B).

The contact angle measurements gave a value of  $81,15^\circ$  for the untreated Au wafer and  $56,35^\circ$  for the functionalised wafer. The treated samples were characterized with X-ray photoelectron spectroscopy (XPS) in order to check the presence of Sulfur on the surface. The result is shown in Figure 4.34.

In the XPS characteristic the peak of  $S2p_{3/2}$  is shown. The binding energy of a Thiol group with Gold is 162,5 eV, exactly the position in which peaks of all the concentration are present. Since all the peaks have the same size, the number concentration of bonded Sulfur on the surface is equal for all the sample. In the case of the sample 50 mM and 100 mM, another peak at  $\sim 164$ -165 is present. That is the peak of a free Thiol group that did not react and that remained entrapped on the surface. This analysis confirmed the success of the first treatment.

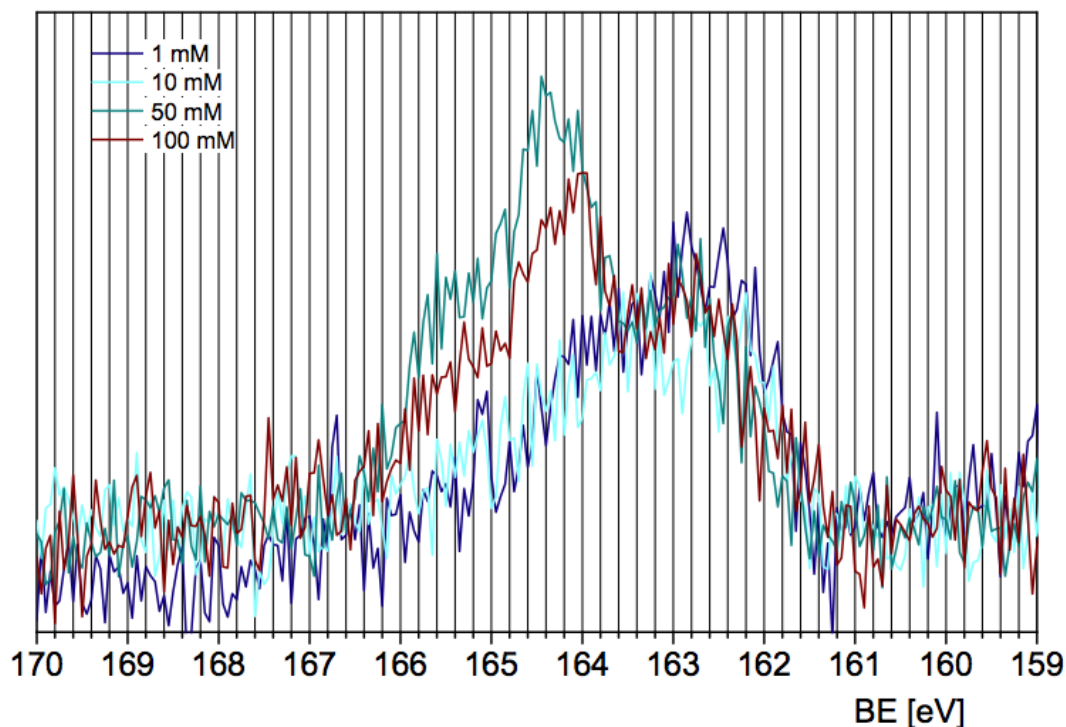


Figure 4.34: Peak S2p of XPS spectrum of Gold wafer functionalised with LA at four different concentration: 1 mM, 10 mM, 50 mM and 100 mM.

2. EDC-NHS coupling. As done for the fluorescence of the protein, the carboxyl group on lipoic acid were coupled with EDC-NHS reaction in order to have a hydrolyzing group that binds with the microgels. The wafer was then characterized with XPS. The spectrum is in Figure 4.35

Two peaks are detected in the spectrum: the first one at energy  $\sim 400$  eV is the characteristic of N1s. The second one at 402,5 eV, might be some  $NSiO_2$  created during the reaction. Anyway, the presence of Nitrogen on the surface assure the success of the reaction.

The reaction with the microgels was carried out. The result was totally disappointing: not even a single microgel attached to the surface. An possible explanation to this event is the not success of the reduction reaction. In fact, the yield of the reaction is quite low and the amines converted on the microgels surface might be not enough to interact with the hydrolyzed NHS.

### Indium Tin Oxide wafers.

The ITO wafers were functionalised with IPTES. This molecule has on one side ethoxysilane groups that react and bind with ITO. On the other side, a cyanate group is present. This group hydrolyzes in water and make the substitution with the amines of the microgels possible. After the reaction of functionalization of the surface, contact angle measurement has been performed and compared with the untreated wafer. The result is shown in Figure 4.36.

The measurement showed a first good result: the untreated wafer contact

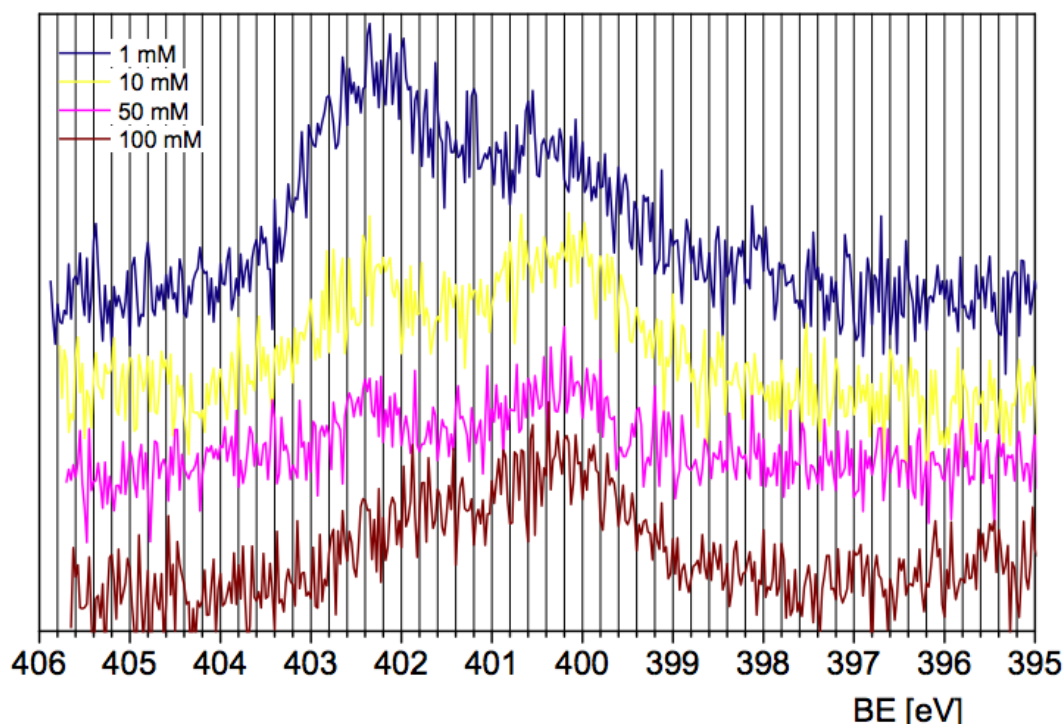


Figure 4.35: Peaks of N1s of XPS spectrum of Gold wafer functionalised with LA at four different concentration (1 mM, 10 mM, 50 mM and 100 mM) and EDC-NHS.

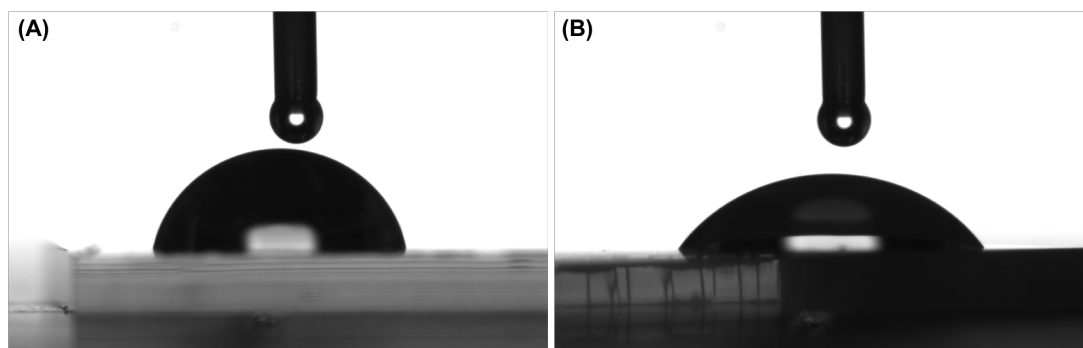


Figure 4.36: Contact angle measurements on untreated ITO wafer (A) and IPTES functionalised ITO wafer (B).

angle had a value of  $78,07^\circ$  while the treated wafer had a value of  $52,05^\circ$ . The hydrophilicity of the surface increased. In order to be completely sure about the treatment, an XPS experiment has been carried out on the wafer in order to detect the Silicon. The measurement was performed on four samples.

As showed in Figure 4.37, the presence of Si2p is detected by the peak at energy  $\sim 102$  eV. In two samples the peaks are a little bit shifted, this might be due to a possible oxidation of the Silicon during the syntheses. In any case, the XPS measurement confirmed the presence of Silicon on the surface of the wafer and the success of the treatment. Successively, the wafer were put into a vessel in order to react with the microgels. Again, none of the microgels attached to

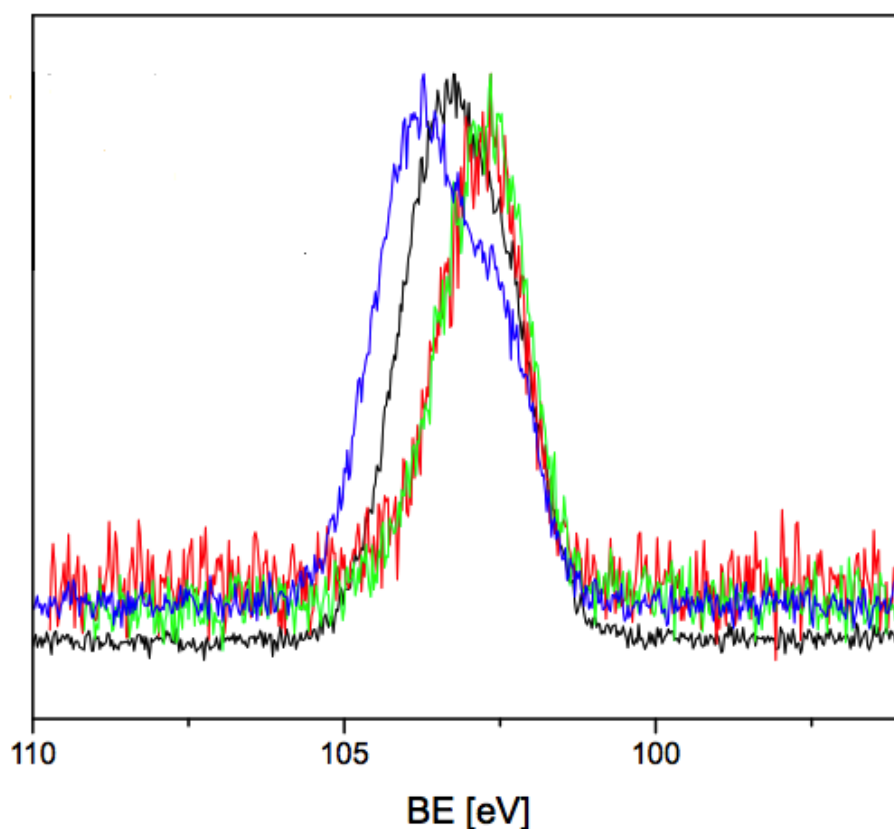


Figure 4.37: Peaks of N1s of XPS spectrum of Gold wafer functionalised with LA at four different concentration (1 mM, 10 mM, 50 mM and 100 mM) and EDC-NHS.

the wafer. The explanation is the same of the case of Au wafer: the yield of the reduction reaction is too low to permit the microgels to interact with the functionalised surfaces.

## Chapter 5

### Conclusions and perspectives

The first obtained result is a standard and useful recipe to synthesize Polyacrylamide microgels. The synthesis has been thought to be easy to perform and reproduce and with an high rate of production. The performed syntheses showed a high reproducibility and a rate of production of hundreds of milligrams. In parallel with those two characteristic, also a good quality product has been synthesized. In fact, the microgels are always well rounded and well defined. The monodispersity by means of batch synthesis has not been achieved. So far, polydispersity in batch synthesis has been categorized as a characteristic of such as syntheses. The second goal that have been successfully reached is the encapsulation. The coupling with a fluorescent dye and the characterization by means of fluorescence microscopy clearly show that both BSA and GOD are well present inside the microgels. Unfortunately, none of the used assay and techniques have been able to reveal the concentration of the entrapped protein inside the microgels. Though, this is a result in concordance with the literature. In fact, in the scientific panorama still does not exist a technique to measure the content of bio-species inside the microgels. Nonetheless this negative result, an explanation has been presented: likely the scattering in the well and with the microgel interferes with the measurements.

The third and most important goal was to stabilize GOD when entrapped and retain its enzymatic activity. Tailoring the different parameters, it has been possible to retain the activity and decrease the loss ratio of GOD. It is possible now to synthesize PAA microgels using the most convenient condition for what concern the crosslinking degree, the content and type of surfactant, the content of organic solvent in the oil phase and the maximum content of GOD that can be encapsulated. Combining all the best parameters, it is possible now to immobilize GOD with satisfactory results. Another step further in the retaining of the activity has been done with the use of additives. In fact, the study revealed that Trehalose is a really suitable thermal stabilizer for the encapsulation of the microgels. During the synthesis, it prevent the denaturation of GOD by thermal unfolding. In this case, the best loss ratio has been reached, with a value of 43%. About the fourth goal, the monodispersity, the microfluidic synthesis has been thought. The setup has been prepared and built and the droplet formed. Though, the available time was not enough to perform satisfactory experiments. This task will be hand down to the posterity. The immobilization of the microgels onto Au and ITO electrodes has been tried with several techniques. The physisorption failed as well as the chemisorption. Though, the functionalization of the surfaces worked and we look forward to find another suitable way to reduce the microgels and reach the covalent attachment.

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