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Chapter XX. Immobilization of enzymes in protein films

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Abstract

Heterogeneous biocatalysis usually involves the use of immobilized enzymes on solid supports. Enzymes have suitable properties in terms of efficiency and selectivity for use as immobilized catalysts. Different approaches have been developed for effective immobilization, including adsorption, covalent binding, entrapment, encapsulation and cross-linking. Those systems offer some advantages with regard to homogeneous catalysts in solution, such as low costs, easy separation and recovery of the catalyst, reusability and enzymatic stability. Here, we describe a new approach for the immobilization of active enzymes into homogenous films composed solely of scaffolding proteins, that differs from the standard methods of enzyme immobilization on solid supports.

Key words: Enzyme, Enzyme immobilization, entrapment, protein scaffolds, biomaterials, protein materials, heterogeneous catalysis.

1. Introduction

The current state-of-art in heterogeneous catalysis focuses on the implementation of enzymes attached to a solid support. Enzymes present specific efficiency, selectivity and high activity that make them desirable as catalysts and for implementation in biocatalytic processes. Heterogeneous biocatalysis allows the use of insoluble catalysts in the reaction mixture, involving the confinement of enzymes in a solid matrix mainly made of organic or inorganic compounds. Some of the advantages of this strategy compared to homogeneous catalysis using soluble enzymes are related to the economic cost, the enzyme reusability, and the improvement in stability [1, 2]. Moreover, after binding of the enzyme molecules, the catalysts change from a homogeneous to a heterogeneous form, which facilitates simple separation of the biocatalytic system from the reaction mixture and results in products of higher purity.

Several methods have been carried out up to date, such as adsorption, covalent binding, affinity immobilization, entrapment or encapsulation and enzyme cross-linking [3, 4]. Most of them require carrier matrices made of inert polymers and inorganic materials, even though alternate carrier-free immobilization has been showed to be as efficient and useful, providing the advantage of cutting down production cost and increasing enzymatic activity [5]. However, each method displays its drawbacks and advantages, and therefore, the choice of a particular method of immobilization depends on the intended purpose. Moreover, the effect of a specific matrix

compound may disturb enzyme activity and/or substrate diffusion [3]. Therefore, new strategies to develop novel heterogeneous biocatalysts are desirable [6].

In the recent decades, biopolymers have been successfully used in different fields with the aim to develop cost-efficient and sustainable processes. These approaches require properties such as biocompatibility, biodegradability and non-toxicity [7, 8]. In this context, proteins can serve as a basis of the polymeric supports because, apart from fulfilling all the aforementioned requirements, they can also provide diverse reactivity and specific environment suitable for the enzyme. Moreover, the use of proteins as scaffolds will potentially allow the control over the spatial organization of active elements without drawbacks present in synthetic materials.

Repeat proteins are a family of proteins whose major role is to mediate protein-protein interactions and to organize multiple proteins into functional complexes. Repeat proteins are composed of tandem arrays of basic structural motifs and their extended structure makes them suitable candidates to be used as molecular scaffold [9, 10].

In particular, tetratricopeptide proteins (TPR) have been demonstrated to be an exceptional scaffold since consensus TPR (CTPR) proteins form ordered films, due mainly to the side-to-side and head-to-tail interactions, that can also be observed in the crystal structure.[11] The basic CTPR unit consists of 34 amino acids with helix-turn-helix motif. CTPR proteins have a modular structure in which the repeats can be combined in tandem to form highly stable CTPR proteins that form a continuous right-handed superhelical structure, with eight repeats per one full turn of the superhelix [12, 13]. These features make the repeat protein scaffolds ideal building blocks for numerous applications [14], including the generation of functional protein-based materials[15, 16]. Therefore, scaffolds and materials composed of CTPR proteins show great potential for the ordered entrapment and immobilization of enzymes toward the generation of novel heterogeneous biocatalysts.

In this chapter, we describe a methodology for enzyme immobilization on a repeat protein-based scaffold (Figure 1). We describe the immobilization of enzymes in protein-based materials by entrapment and covalent cross-linking, as primary methods. All steps from protein purification to film characterization are described in the current chapter.

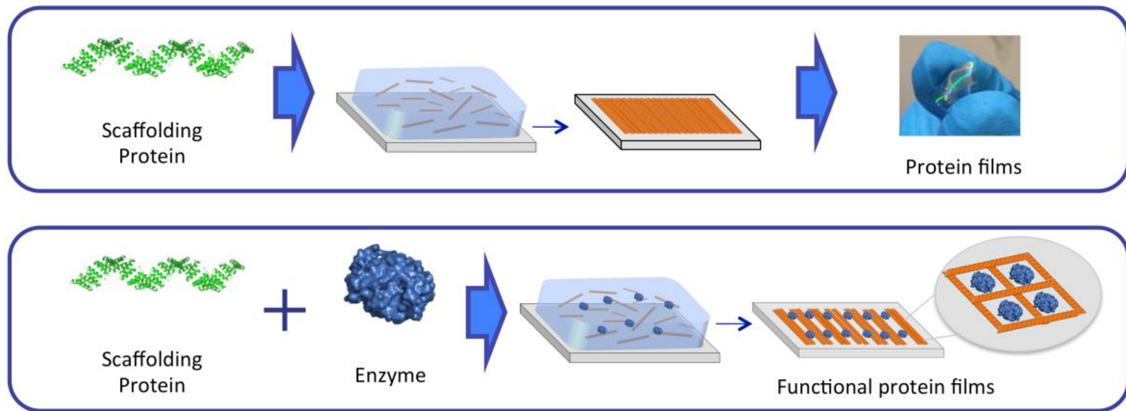

Figure 1. Schematic representation of the methodology for protein-based film formation (top) [11] and enzyme immobilization in the protein films.

2. Materials

All solutions were prepared using MilliQ water except those for bacterial culture in which double distilled water was used.

2.1 Expression and purification of film scaffolding protein

- Bacterial culture: dissolve 15.5 g LB (Luria-Bertani broth, Thermo Fisher) and 9.5 g NaCl in 1 L of double distilled water in a 2 L Erlenmeyer. Alternatively, prepare LB broth by dissolving 10 g Tryptone, 5 g yeast extract, and 10 g NaCl. Seal all flasks properly with aluminum foil. Sterilize all flasks in the autoclave (20 min at 121°C). Store the sterilized flasks at 4°C until use in order to avoiding contamination.

- Over-night culture: sterilize empty flasks (Erlenmeyer of 100 ml) properly sealed with aluminum foil.

- Ampicillin stock solution: prepare a solution of ampicillin sodium salt (Fisher BioReagents) at 100 mg/ml in water. Filter the resulting solution using a 0.22 µm filter under sterile conditions either using a flame or a laminar flow hood. Store 1 ml aliquots at -20°C.

- Isopropyl-β-D-1-thiogalactopyranoside (IPTG) solution: prepare a stock solution of IPTG (Fisher Bioreagents) at 1M (238 mg/ml). Sterilize and store as described above.

- UV-vis spectrophotometer (ThermoFisher).

- Plastic cuvette to measure optical density (OD).

- Top-loading balance (OHAUS).

- Analytical balance (Sartorius).

- 50 ml Falcon tubes (VWR).

- 15 ml Falcon tubes (VWR).

- 4x SDS-PAGE sample loading buffer: mix 1.5 ml of 1 M Tris-HCl pH 6.8 with 3 ml of 1 M dithiothreitol (DTT), 0.6 g of sodium dodecyl sulfate (SDS), 0.03 g of bromophenol blue and 2.4 ml glycerol in a beaker. Stir the solution until a homogenous mixture forms. If solution is orange/yellow, add 1 drop of 5 M NaOH to adjust pH until it becomes blue.

- Electrophoresis cuvette (VWR).

- Power supply (VWR Power Source 300V).

- Electrophoresis gels at 10%-15% acrylamide.

- Glacial acetic acid (VWR).

- Ethanol 96% (Scharlau).

- Coomassie staining solution: dissolve 1.25 g Coomassie Brilliant Blue in 225 ml ethanol, 25 ml glacial acetic acid and 250 ml water.

- Destaining solution: mixture of 600 ml water, 300 ml ethanol and 100 ml glacial acetic acid.

- Sterile tips (VWR).

- DNase commercial solution (Thermo Scientific DNase I, RNase-free, 1000U).

- Protease inhibitor (commercial cocktail).

- Sonicator Sonics (Vibra cell™).

- HisTrap HP Ni-NTA affinity columns (GE Healthcare).

- Centrifuge and centrifuge bottles. Beckman J-26XP (Beckman).

- Orbital shaker. Excella E24R Incubator Shaker Series (New Brunswick Scientific).

- Lysis buffer: 300 mM NaCl, 50 mM Tris pH 8.0.

- Wash buffer: 300 mM NaCl, 5 mM imidazole, 50 mM Tris pH 8.0.

- Elution buffer: 300 mM NaCl, 300 mM imidazole, 50 mM Tris pH 8.0.

- Spectra/Por®1 Dialysis Membrane. Standard RC Tubing MWCO: 6-8 kDa (VWR).

- Dialysis clamps (VWR).

- Dialysis solution: 150 mM NaCl, 50 mM Tris pH 8.0.

- *Fast Performance Liquid Chromatography* (FPLC). AKTA pure chromatography system from GE Healthcare Life Sciences. Pick the suitable column depending on the size of protein to purify; for proteins in the range from 3 kDa to 70 kDa use HiLoad Superdex 75 PG column, for proteins of higher MW select the suitable column with a resolution in the desired molecular weight range. Regarding the buffer use the dialysis solution.

2.2 Scaffold-enzyme conjugation

- Low-salt buffer: 10 mM NaCl, 10 mM sodium phosphate pH 7.5

- CTPR10 stock solution for entrapment method: Concentrate protein solution up to 1 mM in Low-salt buffer.

- CTPR10-Cys stock solution for conjugation method: 1 ml at 70 µM in 50 mM sodium phosphate pH 7.5.

- MilliQ water.

- DMSO commercial solution.

- 2,2'-Dithiodipyridine (aldrithiol): prepare a stock solution of 44 mM aldrithiol (10 mg in 1 ml of DMSO).

- Traut´s reagent (2-Iminothiolane): prepare a stock solution at 200 mM (27.5 mg in 1ml of water).

- DTT stock: prepare a stock solution at 1 M (3.9 mg DTT in 25 ml MilliQ water) in a fume hood because of the toxicity of the reagent. Filter through 0.22 µm filter.

- PD-10 pre-packed columns (Sephadex™ G-25 M) GE Healthcare.

- Centrifugal Filters, Amicon Ultra-0.5 ml 10 kDa Molecular Weight cut-off (MWCO).

2.3 Protein film deposition

- Low-salt buffer: 10 mM NaCl, 10 mM sodium phosphate pH 7.5

- Surfaces: depending on the downstream applications, silica, quartz, gold, glass, silver or hydrophobic surfaces can be used.

- CTPR10 solution: prepare a protein solution of at least 300 µM (13.6 mg/ml) in Low-salt buffer. If the protein is stable without salts in water, it would be better to remove the salt to avoid its precipitation in the films.

- PEG-400 commercial solution.

- Spin-coating device. Laurell technologies corporation (Model WS-400B-6NPP/LITE)

- UV/Ozone ProCleaner™ (Bioforce nanoscience).

- Electronic Diener (Plasma surface technology).

- Ethanol 70%.

- Acetone.

- 2% sodium dodecyl sulfate solution (SDS): dissolve 10 g of SDS in 500 ml of MilliQ water with stirring. Filter solution with 0.22 µm filter.

- γ-Butyrolactone (GBL) commercial solution (Sigma-Aldrich).

- 10% w/v Poly(methyl methacrylate) (PMMA) stock solution: mix 500 mg of PMMA powder with 4500 mg of GBL. Stir the solution at 70°C for 1h. The viscosity of the solution is sensitive to small variation of PMMA content, potentially affecting the ease of sample manipulation.

- 1% Glutaraldehyde GA stock solution (50 wt.% in water, Merck): For a final volume of 2 ml, take 60 µl of 50 wt.% and add water up to 2 ml. Mix properly.

- NHS-diazirine solution (100 mM): weight 2.25 mg of compound, add 100 µl of DMSO and re-suspend it using a pipette.

- UV-lamp: UVP UVLM-28 EL series UV Lamp, 8 watt, 2UV 302/365 nm, 0.32 Amp/230v – 50 Hz.

2.4 Protein film characterization

- Circular dichroism. Spectropolarimeter Jasco-815 (JASCO).

- Quartz cuvette light path 0.1 mm (Hellma Analytics).

- Ellipsometer. VASE Ellipsometer (J.A. Woollam Ellispsometry Solutions).

- Plate-Reader. Varioskan Flash (Thermo Scientific).

- Horseradish Peroxidase (HRP) (Sigma-Aldrich)

- Cell culture plates, 24-well (VWR) to carry out the enzymatic reaction.

- 96 flat bottom Well UV transparent plate.

- Hydrogen peroxide solution (30 % w/v).

- Hydrogen peroxide stock solution (2 mM): mix 682 µl of hydrogen peroxide stock at 30 % w/v with 318 µl of MilliQ water.

- 2,2'-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) green powder (ABTS), (Sigma).

- PB: 100 mM phosphate, pH 6.0.

- ABTS stock solution (2 mM): weight 3.3 mg of ABTS and diluted with 3 ml of PB.

- HRP reaction mixture: mix 2.5 ml of hydrogen peroxide stock solution (2 mM) with 2.5 ml of ABTS solution (2 mM).

3. Methods

Proteins should be kept on ice throughout the procedures, unless indicated otherwise.

3.1 Expression and purification of scaffolding protein

3.1.1 *Protein expression*

Place all materials, including tips, 100 µl automatic pipette, LB solution and a 100 ml flask, in a laminar flow hood and turn the UV lamp and the fan, and wait 10 min. Alternatively, the work can be done under flame. After 10 min, turn off the UV lamp and clean hands with 70% ethanol before starting to work. Add 100 ml of LB solution to the flask, add 100 µl of amplicillin at 1 mg/ml and mix it properly (final Amp concentration of 1 µg/ml). Inoculate the liquid culture with the bacterial cells that contain the plasmid encoding the protein for recombinant expression. The inoculation can be performed from a glycerol stock of the bacteria or from a single colony of freshly transformed bacteria. Place the flask in an orbital shaker at 37°C and 220 rpm overnight.

After overnight growth, prepare 1 L LB flasks and using either the laminar flow hood or flame, to maintain sterile conditions. Put 1 ml of amplicillin (1 mg/ml stock) in each 1 L flask and 10 ml of the overnight saturated culture prepared the day before (*see* **Note 1**). Incubate the flasks in the shaker at 37°C and 220 rpm until the culture reaches an OD at 600nm between 0.6-0.8 (*see* **Note 2**). Considering that the bacterial doubling time is approximately 30 min, it is possible to estimate form the first OD measurements when the culture will reach the target OD value. In any case, it is convenient to measure the OD every 30 min after the first two hours after inoculation. In order to measure the OD, take a sample of 1 ml of each flask, after running the blank with the LB solution. Repeat the process each 30 min until the culture reaches an OD in the 0.6-0.8 range. Once the required OD is reached, add 600 µl of IPTG at 1M to induce protein expression and lower the temperature to 30°C and incubate during 5 hours under constant shaking (see **Note 3**). For large CTPR proteins (above 20 repeats), or when protein expression and solubility problems are identified, it is recommended to perform the expression at lower temperature (20°C and overnight) to improve the expression yields.

Once the determined expression time has passed, transfer the culture from each flask into a centrifuge bottle and carefully balance the centrifuge tubes. Place each pair of balanced bottles into the centrifuge and centrifuge for 15 min at 4500 rpm, 4°C. Discard the LB supernatant and resuspend the pellet from 1 L of culture in 30 ml of lysis buffer. Transfer the suspension to a 50 ml falcon tube. The cell suspensions can be stored at -20°C or -80°C.

3.1.2 *Protein purification*

If resuspended cells were frozen prior to protein purification, thaw the tubes either by leaving them at room temperature or on ice, depending on how temperature-sensitive the expressed proteins are. CTPR proteins are generally stable and the cell suspensions can be thawed using warm water to accelerate the process. Immediately after thawing, add 30 mg of lysozyme for a final concentration of 1mg/ml, 5 µl of DNase and 35 µl of protease inhibitor cocktail. Mix and leave on ice for 30 min. After that time, sonicate the mixture using a sonicator at 40% of amplitude for 15 min (0.5 sec on/off cycles) maintaining the sample on ice during the entire sonication process (*see* **Note 4**). After equilibration of the falcon tubes, centrifuge the sample at 10000 rpm for 45 min at 4°C.

Meanwhile, rinse a HisTrap HP column with MilliQ water and then equilibrate it with lysis buffer. Load the supernatant from the previous centrifugation step onto the column (*see* **Note 5**). After loading the sample, wash the column with 50 ml of Wash buffer in order to eliminate nonspecific proteins bound to the resin. Finally, elute the protein using 25 ml of Elution buffer.

Set up a dialysis with the eluted fractions in order to remove the imidazole form the solution. First hydrate the dialysis bag by dipping into water, lock one opening of the bag with a clamp and pour the protein solution into the dialysis bag using a pipette, then close the bag with the other clamp. Place the dialysis bag in dialysis buffer (2L) and leave the sample under constant stirring at 4°C (*see* **Note 6**).

Check the protein purity by SDS-PAGE gel electrophoresis. Mix 15 µl of protein solution with 5 µl of 4x loading buffer and boil the sample for 5 min (*see* **Note 7**). Place the gel in the cuvette, add running buffer and load the protein samples. Run the gel at 150 V until the bromophenol blue front reaches the end of the gel. Place the gel in Coomassie staining solution for 1h, then wash the gel in Destaining solution until the protein bands are clearly observed.

If the gel electrophoresis shows that the sample is not pure enough and contains other proteins, purify by a gel filtration chromatography using FPLC on an appropriate column, as mentioned in Materials section. Filter the sample using a 0.22 µm filter in order to avoid protein aggregate injection onto the column (*see* **Note 8**). Select a sample loop of the same volume of the protein sample to be injected. After collecting the FPLC fractions, run an electrophoresis gel on each fraction. Select the fractions containing pure protein, concentrate using Amicon of 10 KDa MWCO. Determine protein concentration by measuring the absorbance at 280 nm and calculating the extinction coefficients from the amino acid composition [17].

3.2 Strategies for enzyme immobilization in protein films

In order to immobilize a desired enzyme into a CTPR protein film three strategies can be applied. The examples below illustrate the protein-enzyme sample preparation for film casting using a defined enzyme:protein ratio of 1:50, this standard ratio can be modified in the range of 1:10 to 1:100 considering that the scaffolding protein concentration is needed between 100 and 400 µM.

3.2.1. Immobilization by entrapment

Using this method the enzyme is entrapped in the protein film through entanglement with the scaffold by mixing both the scaffolding protein (CTPR10) and the enzyme solutions in a suitable ratio to form a functional film. The ratio depends on the type of enzyme and the downstream application, since each enzyme has different activity and the application will require a specific amount enzyme in the film.

In general, a 1:50 enzyme:protein ratio is used as standard. For final volume of 100 µl, mix 7 µl enzyme solution at 5mg/ml stock solution, 63 µl Low-salt buffer and 30 µl CTPR10 stock solution at 1 mM.

3.2.2. Immobilization by covalent binding

In this strategy the enzyme is covalently linked to the scaffolding protein (CTPR10). In this example the conjugation using thiol chemistry to generate a disulfide bond between the two proteins is described, but other conjugation chemistries are valid and could be considered in order to attach selected enzymes to the scaffolds, such as carboxyl-to-amine crosslinking or click chemistry (considering non-natural amino acids).

In this case, CTPR10-Cys, a CTPR mutant containing a single cysteine residue, is used as scaffolding protein. The cysteine residue is activated using aldrithiol (Ellman's reagent can be used instead). The activation allows the monitoring of the amount of activated protein and, subsequently, the conjugated protein by UV-vis spectroscopy (*see* **Note 9**).

Take 1 ml of CTPR10-Cys protein solution at 70 μM and add 10 μl of 1 M DTT to freshly reduce the cysteine residues. Leave the sample for 15 min. Add 24 μl of 44 mM aldrithiol stock solution to a 15 ml falcon tube (*see* **Note 10**), the final concentration of aldrithiol in the activation reaction should be approximately 5 times the protein concentration. After the reduction of the CTPR10-Cys, remove the DTT from the protein solution by gel filtration. Equilibrate a PD-10 desalting column with 5 bed volumes of Low-salt buffer, and load 1ml of protein solution. Elute the sample with the same buffer and collect the protein into the 15 ml falcon tube with the aldrithiol. Close the tube properly and incubate it overnight under constant shaking at 37°C.

To determine the yield of the activation reaction, measure the concentration of the released pyridinethione ring by its absorbance at 343 nm and using the 4-thiopyridine molar extinction coefficient (8080 $M^{-1}cm^{-1}$). The yield of activation should be around 90%. After the quantification, perform a dialysis to remove the excess 4-thiopyridine. Concentrate the sample using Amicon of 10 KDa MWCO and measure the absorbance at 280 nm in order to determine the concentration of activated scaffolding protein, in order to determine the amount of enzyme necessary to achieve the desired enzyme:protein ratio.

For the activation of the enzyme selected for immobilization a general strategy of primary amine activation is described since it is generally applicable to all enzymes. In a first step the enzyme's primary amines are modified using 2-iminothiolane (*see* **Note 11**). This reaction results in the introduction of thiol groups on the surface of the protein that can form disulfide bonds with the activated CTPR10-Cys scaffolding protein. For enzyme modification, add 100 μl of 2-iminothiolane stock solution at 200 mM (20-fold molar excess) to 1 ml of enzyme solution at 1 mM (*see* **Note 12**). Leave the reaction overnight at room temperature. After the overnight incubation remove the excess of 2-iminothiolane using a PD-10 desalting column.

Mix the activated enzyme and the reactive CTPR scaffolding protein in a 1:1 moral ratio. Add the solution of the thiolated enzyme at 30 μM to an equal volume of the CTPR10-Cys activated protein at the same concentration. Leave the reaction overnight at 37°C under constant shacking. Measure the released 4-thiopyridine at 343 nm to determine the yield of the conjugation reaction and perform a gel electrophoresis to verify the molecular weight of the conjugated product. Dialyze the sample to remove the released 4-thiopyridine and to exchange the reaction buffer with the Low-salt buffer for film formation. Finally, concentrate the enzyme-CTPR sample up to 30 μM add CTPR10 to reach the desired final enzyme:scaffolding protein ratio of 1:50. For a 100 μl solution, mix 30 μl of CTPR 10 stock solution at 1 mM with 10 μl of the conjugate at 30 μM and bring it to 100 μl with MilliQ water or Low-salt buffer.

3.2.3. Expression as fusion protein

The expression of the enzyme and the scaffolding protein as a chimeric protein is another alternative to consider. It implies the application of genetic engineering in order to fuse the genes. In order to apply this methods both proteins should be fused through a flexible linker and

the molecular weight of the final construct should be considered since a large fusion protein may be difficult to express with good yields.

The purified CTPR-enzyme fusion protein must be doped with CTPR protein scaffolding in order to form a protein film. Using the standard 1:50 ratio, mix 10 µl of fusion protein at 30 µM with 30 µl of CTPR10 1 mM and 60 µl MilliQ water.

3.3 Protein-enzyme film fabrication

Proteins used for film casting should ideally be dissolved in Low-salt buffer (e.g. 10 mM NaCl, 10 mM sodium phosphate pH 7.5). The buffer composition will also depend on the scaffolding protein and enzyme requirements to preserve the stability and activity (*see* **Note 13**).

It is noteworthy that the thickness and the area of the film will depend on the concentration and volume of the protein scaffold used in the casting. Protein concentrations between 200-400 µM and volumes of at least 50 µM will provide the film size and thickness required for easy handling.

3.3.1 Surface cleaning

The chosen surface depends on the application. Therefore, cleaning methods may differ or not even be necessary. Here are displayed some common substrates and effective cleaning methods.

3.3.1.1. Silica or similar substrates

First, rinse the surfaces with acetone, MilliQ water, 70% ethanol and finally wash with MilliQ water. Put the surfaces in 2% SDS solution for 30 min. Rinse with MilliQ water and with ethanol. Dry the surfaces under a nitrogen flow and finally put them in the ozonolysis system for 30 min. Alternately a plasma exposure can be also applied to clean the surfaces (*see* **Note 14**).

If the surfaces are reused after protein deposition the acetone rinse is not necessary but a sonication step will be needed to remove the remains of organic compounds. A sonication cycle of 30 sec should be enough but it depends on the material. Consider that the sonication can be very aggressive and damage certain surfaces.

3.3.1.2. Oxidizable substrates

In the case of oxidizable surfaces such as silver or gold substrates, oxidant cleaning methods, such as ozonolysis that damages those surfaces, must be avoided. Plasma cleaning can be used instead, following the protocol described in the previous section.

3.3.2 Protein deposition methods

Currently there are several methods in nanotechnology to carry out the deposition of organic materials on surfaces, such as doctor blade coating, spin-coating, drop casting, dip-coating, spray coating and Langmuir-Blodgett deposition. Among those, drop casting and spin-coating are the most commonly used and the most suitable for protein deposition.

Protein samples have to be deposited immediately after cleaning the surface since otherwise the surface will be contaminated by particles present in the air. In all the cases, add 1% w/v PEG-400 as plasticizer to the protein solution before the protein deposition.

3.3.2.1. Drop casting

Take the enzyme-scaffolding peptide mixtures prepared in sections 3.2.1., 3.2.2., or 3.2.3. Deposit 100 µl of the solution over a selected clean surface. Let it dry overnight at room temperature.

In order to facilitate the manipulation of the films sometimes is convenient to add an additional layer of PMMA over the protein film. However, this procedure can only be applied for thermostable enzymes in which high temperature does not compromise the enzymatic activity, since there is a curing step at 70°C. For this procedure, add 50 µl of 10 % w/v PMMA solution over the protein film and incubate at 70°C until the PMMA cures to a hard consistency (*see* **Note 15**). PMMA solution is rather viscous even at 10 % concentration, so in order to pipette the solution properly cut the end of the tip using scissors before pipetting the required volume (*see* **Note 16**).

3.3.2.2. Spin-coating

Place a silica surface on the spin-coating platform as centered as possible. Add a drop of 20 µl enzyme-scaffolding protein solution at a protein concentration between 200-400 µM over surface. Spread the sample in order to cover the surface completely. Turn the nitrogen and the pump on and spin coat the sample for 5 min at 600 rpm. Using this method the thickness of sample will be approximately 300 nm.

3.3.3 Crosslinking of protein films

The proteins films generated by control deposition of proteins are stable in dry environments, however, for many applications the films are required preserve their structural integrity in aqueous solutions. For this purpose, crosslinking methodologies have been developed

3.3.3.1. Glutaraldehyde crosslinking

The films are subject to a gentle cross-linking methodology in which the GA is introduced by vapor diffusion. Add 500 µl of GA at 1% in a well of the 24-well cell culture plate, cover the well with cover slide on which the protein film has been deposited (facing down) and seal the well properly using tape. Leave the crosslinking reaction for 24 hours at room temperature. After 24 hours, open the well to remove the crosslinked film and let it dry for several minutes (*see* **Note 17**).

3.3.3.2. NHS-diazirine crosslinking

In this procedure the crosslinking agent is integrated in the film prior to formation and the crosslinking is photoactivated once the film is formed. Add 70 µl of NHS-diazirine solution at 100 mM (10-fold molar excess) to 1ml of protein solution (containing the enzyme- and scaffolding protein at the desired ratios) at 30 µM in 150 mM NaCl, 50 mM sodium phosphate pH 7.5 buffer. Incubate the reaction overnight at room temperature allowing the NHS-diazirine to react with

the protein. Remove excess of cross-linker from the protein sample using PD-10 desalting column with Low-salt buffer. Concentrate the sample to approximately 300 µM protein concentration. Deposit 20 µl of the sample on the substrate of interest and let it dry for several hours to form the protein-enzyme film. Finally, expose the film to 365 nm ultraviolet light for at least 2 hours for photocrosslinking (*see* **Note 18**).

3.4. Protein films characterization

The characterization of the enzyme-protein films includes the determination the optical and structural properties of the material and its enzymatic activity.

3.4.1 Structural characterization

3.4.1.1. Circular dichroism (CD).

Circular dichroism is used to determine the secondary structure of the integrated protein units in the solid films. For this measurement the films are directly cast on a quartz surface by drop casting or spin-coating. The thickness of the film is a critical parameter, since films thicker than approximately 10 µm produce scattering and hamper the acquisition of suitable CD spectra. When using drop-casting to deposit the film a drop of 20 µl of protein solution should be deposited and spread over an area of around 1 mm^2 to result on an adequate thickness. After drying of the film, acquire the CD spectrum using the following parameters: high sensibility, data pitch of 0.1 nm, from 5-10 accumulations, continuous scanning, scanning speed of50 nm/min, respond time of 8 sec, and band width of 1 nm.

3.4.1.2. Ellipsometry.

Ellipsometry is used to determine the thickness of the film and the optical parameters, including the refractive index or the extinction coefficient. For this measurement, a homogeneous thin film is required, thus is recommended to deposit the film using spin coating on silica or quartz surfaces. Once the film is formed, place the sample in the holder, fit the lenses and measure either just a measurement at 70 degrees or multiple measurements in the 60 to 85 degrees range. Fit the plot to the Cauchy equation and verify that the Mean squared error (MSE) value is around 1 (< 10 is still acceptable), otherwise the measurements are not reliable.

3.4.2. Functional characterization of films: enzymatic activity

The measurements of enzymatic activity will depend on the enzyme used. Here is presented as an example the particular case of horse radish peroxidase (HRP).

Add 2 ml of the HRP reaction mixture to each well to initiate the reaction and record the absorbance increase at 415 nm (ΔA_{415nm}), use reaction solution as control and also a film of protein without enzyme. Take samples each 30 sec during approximately 10 min. A change of color can be observed overtime due to the oxidation of ABTS.

To calculate the difference in absorbance (ΔA_{415nm}) per minute for the maximum linear rate consider the change in absorbance from the blank sample as follows:

ΔA_{415nm} /min = ΔA_{415nm} /min (sample) – ΔA_{415nm} /min (blank)

Use the following equation to obtain the relative enzymatic activity:

$$Relative\ EA = \frac{\Delta\ A_{415nm} \times V_{tot}}{\varepsilon \times l \times V_{enz}} \times DF$$

where V_{tot} = 2 ml (assay's total volume), ε = 36.8 $mM^{-1} \cdot cm^{-1}$ (ABTS extinction coefficient at 415 nm), l = 0.5 cm (optic pathlength), V_{enz} = 0.010 mL (sample volume) tot, DF= Protein Dilution factor (assay and sample dilutions).

Notes:

1. Depending on the amount of protein desired determine the liters of bacterial cell cultures required considering an average yield of 30-50 mg protein per liter of cell culture.

2. In case the OD value goes above 0.8 it is recommended to restart the process by diluting the overnight culture in fresh 1 L flasks, since the protein expression can be low if the induction is performed at high OD values.

3. Consider that the inducible *lac* promoter is one of the most commonly used promoters for recombinant protein expression in *E. coli*, and that IPTG is the most efficient molecule to induce this promoter's transcriptional activity. However, other reagents might be used to induce the expression depending on the system selected, for example arabinose is used with pBAD plasmids that carry the arabinose-inducible *ara* promoters.

4. Exceeding the sonication time or increasing the amplitude can result in an increase in temperature, resulting in protein damage and aggregation.

5. If the sample centrifuged insufficiently or is left for a prolonged period of time after centrifugation before the separation of the supernatant and the pellet, part of pellet can become resuspended resulting in contamination of the sample and /or damage of the affinity column.

6. Leave enough extra space in the dialysis bag above the clamps in order to open the bag in a straightforward manner to recover the protein after dialysis.

7. If the gel electrophoresis sample changes color from blue to orange it is because the protein solution was too acidic. In this case add 1 μL of 1 M NaOH solution to neutralize the sample before loading it onto the gel.

8. Do not inject protein solution at high concentration (above 100 μM) to avoid the aggregation and potential precipitation of the protein in the column. Moreover, the injection of highly concentrated protein might provide unresolved peaks.

9. In order to apply this strategy the scaffolding protein must have an accessible cysteine.

10. The adrithiol reagent is dissolved in DMSO. A high percentage of DMSO might affect the protein structure, thus the concentration of DMSO should not be higher than 10% (v/v).

11. Avoid using buffers containing primary amine during the activation and conjugation because they will compete with the desired reaction.

12. The sulfhydryl groups introduced in the enzyme may recyclize or oxidize to disulfide over time. Therefore, proceed with the reaction with the scaffolding protein immediately after activation.

13. Low salt content in protein solutions is necessary to obtain protein films with low salt concentration that are homogeneous and easy to manipulate. Salt precipitation in the film might lead to the disruption of the film and also result in heterogeneous macrostructure.

14. Sonication, plasma chamber and ozonolysis are quite aggressive methods and can damage certain surfaces.

15. Avoid using PMMA solutions above 10% concentration because the high viscosity of the solution complicates accurate pipetting and deposition.

16. It is advisable to spread the PMMA solution beyond the area of the protein film for better handling of the material.

17. Long incubation times with GA can damage the proteins.

18. Keep the protein sample in the dark after the reaction with NHS-diazirine until the crosslinking of the solid film with UV-light.

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