Chapter 13

Macromolecular Biosensors Based on Proteins Involved in Bile Stones Formation

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1. Introduction

Biomineralization refers to the process of obtaining biominerals in living organisms, and can be both a pathological and a non-pathological process [1]. This term was coined at the beginning of the eighties of the past century, and opened a new way of inspiring materials for biomedical applications [2].

Nowadays, the understanding of many diseases, like gallbladder stones related to pathological aspects of biominerals' formation in living organisms, is based on the knowledge of the chemical recognition between biological macromolecules, calcium salts and cholesterol molecules [3-5]. This kind of chemical interaction has played an important role in the development of biologically inspired biosensors. A biosensor is an analytical device consisting of two elements in spatial proximity: (1) a biological recognition element able to interact specifically with a target; (2) a transducer able to convert the recognition event into a measurable signal [6].

According to the mechanism of biological signaling used, biosensors are classified into five major types, one of them is the biomimetic one; in this sense, a biomimetic biosensor is an artificial or synthetic sensor that mimics the function of a natural biosensor [7]. Some examples of these types of sensors are the quercetin-modified wax-impregnated graphite electrode (Qu/WGE) for the purpose of detecting uric acid (UA) in the presence of ascorbic acid

(AA) [8]; a cell-based biosensor platform of neuron silicon interface with acid-sensing taste receptor cells cultured on light addressable potentiometric sensor (LAPS) [9]; a Langmuir-Blodgett film of tyrosinase incorporated in a lipidic layer with lutetium bisphthalocyanine as an electron mediator for the voltammetric detection of phenol derivatives [10]; a mixed self-assembled monolayers (SAMs) functionalized with specific olfactory receptors (ODR-10) constructed on the sensitive area of surface acoustic wave (SAW) chip [11]. Recently, a new type of chemical biosensor based on intramineral proteins of eggshells for carbonate ions detection has been published elsewhere [12].

The precipitation of the cholesterol and the calcium salts are commonly found in the bladder (bile), whereas the calcium carbonate, which is the major component, is normally found in the pancreas, at alkaline pH [13]. This formation usually follows the principles of crystal growth and the simple chemical solubility rules. However, it has been recently observed that the formation of gallstones or minerals of biogenic origin (grown in these biological vesicles) leads the growth process in extreme conditions, even devoid of water in some cases. It is not clear whether some of the genes (mostly in mammals) could be involved in most of the pathological processes, however, it has been proved the ethnical influence on the medical diagnosis [14]. Recent publications have shown that there are genes already identified (mainly in marine organisms) whose role in activating biomineralization processes has been tested by molecular biology techniques [15]. Nowadays, the knowledge about the genes involved in the formation of certain skeleton in marine spicules is, in general clearly identified. However, our understanding of the role of any of the proteins in biomineralization is scarce, so is our understanding of the role of the matrix proteins as well as protein-protein, and protein-mineral interaction [16].

Concerning the crystallization of cholesterol in human bile, there are some proteins from the serum involved in this matter, the immunoglobulines IgM, IgA, IgG as well as the proteins α_1-Acid Glycoprotein (AAG, usually called Orosomucoid), Phospholipase C and Aminopeptidase N. According to a recent publication, only three proteins from this list showed a potent enhancement and a promoting effect on the crystallization of cholesterol: IgM, IgA and AAG protein [17]. Particularly, orosomucoid a protein of 42 kDa, is one of the most abundant proteins of serum proteins with no well-known physiological function. However, a number of biological activities have been described for orosomucoid, such as promotion of collagen fibril formation, inhibition of platelet aggregation, inhibition of heparin accelerated antithrombin III mediated activation of thrombin and factor Xa, binding of Δ-4 Ketosteroids, sequestration of a glycosaminoglycan cofactor in the lipoprotein lipase reaction and the preferential binding of more than 60 cationic therapeutic drugs [18]. The orosomucoid protein has an unusual pI of around 3 and an extraordinary carbohydrate content of approximately 40% (w/w), comprising five N-linked glycans [19]. However, although the importance of this orosomucoid in human physiology seems to be high, there are no more structural data from the native glycoprotein obtained at high resolution. The orosomucoid was firstly crystallized in 1984 [18], and since then no more structural data of that native glycoprotein at high resolution have been published. The high-resolution crystallographic structure was not available for a long time. However, the three-dimensional structure has

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been recently available at 1.8Å resolution of a recombinant AAG protein crystallized in a tetragonal $P4_12_12$ space group. This recombinant human AAG produced as monomeric, yet unglycosylated protein in E. coli was obtained via secretion into the bacterial periplasm, where formation of its two-disulphide bonds was facilitated in the oxidizing environment [20]. The former crystals of the native AAG protein were obtained in a hexagonal P622 or $P6_222$ or the enantiomorph $P6_422$ space group in the presence of chlorpromazine at 18 °C [18]. Interestedly, the co-crystallization of the tetragonal AAG by soaking method in the presence of the well-known ligands (phenothiazine tranquilizers) like chlorpromazine, bromazepam or diazepam, failed [20]. This is due to the unglycosylated 3D structure. It seems that the carbohydrates play an important role into the 3D structure-function of the native glycoprotein when making the chemical recognition between this AAG protein and some common drugs.

In this chapter we evaluate, from the electro-analytical point of view, the plausible role into the chemical interaction and chemical recognition of sodium carbonate, bilirubin, cholesterol with α_1-Acid Glycoprotein (AAG) usually found in biogenic minerals in bile. Additionally, we show the effect of different gel media on the crystallographic habits of synthetically grown crystals of cholesterol. These crystals were characterized by X-ray powder diffraction. Finally, based on our results, we propose a new design of a biologically inspired biosensor.

2. Experimental

Cyclic Voltammetry. All the electroanalytical assays to investigate the analyte-protein interaction for example, sodium carbonate, cholesterol, and bilirubin with the α_1-Acid Glycoprotein were performed using a Potentiostat/Galvanostat PG580 from UNISCAN Instruments (UK). The potential was ranging from 0 to 1.80 Volts versus a Saturated Calomel Electrode (as a reference electrode). The velocity for this electrochemical analysis was 50 mVs^{-1}.

Figure 1. Electrochemical cell used for cyclic voltammetry experiments.

Figure 1 shows the electrochemical cell used for this determination. Three electrodes were used along the experiments. The working electrode (5 mm diameter) was a gold microelectrode Au10 from Autolab Electrochemical Instruments (USA). The auxiliary electrode was a platinum wire of 0.5 mm in diameter. The gold electrode was polished using a diamond paste of 0.25 microns in particle size. The gold electrode was sonicated several times in order to remove the impurities. The auxiliary electrode was cleaned with a proper fine texture sandpaper to eliminate oxides on the surface. As an inert electrolyte 0.5M Potassium Chloride (Strem Chemicals, Inc. Newburyport, Code 7447-40-7) was used with a purity of 99.999%. The highly pure α_1-Acid Glycoprotein (AAG protein) was purchased from Sigma (Code G9885) without further purification. The electroanalytical plots, as those shown in Figure 2, were obtained taking into account the gold electrode saturation for each scanning cycle as well as removing the background from the oxidation of gold at 1.3V. This value corresponds to a maximum peak into the anodic response of the voltammogram (right-hand side of the plot current versus potential) at each analyte concentration. This AAG protein (50 μg) was absorbed on the gold electrode until the water was evaporated. For each analyte five different concentrations were done as well as duplicates for each single concentration. The electrochemical experiment is carried out until the analyte is put into contact with the protein and after an equilibrium time of 2 minutes. Before running any of the experiments the oxygen was degased by bubbling pure nitrogen for 15 minutes. All dissolutions were prepared in a glass beaker of 5 mL with 0.1, 0.2, 0.3, 0.4, and 0.5 mM of each electroanalyte. The dissolutions were then transferred to the electrochemical cell. However, as bilirubin is not soluble in water, it had to be prepared in DMSO as follows: 2.92 mg were dissolved in 1 mL of DMSO-Water 80:20 and keeping the solution away from oxidation inside of an amber container. Cholesterol was prepared dissolving 1.93 mg in 1 mL of ethanol-water 80:20 and sealing it carefully to avoid any alcohol evaporation.

In order to investigate the chemical interaction between cholesterol and AAG protein, the cholesterol crystals were grown in different crystal growth media, solution, and two types of hydrogels in the Granada Crystallization Boxes via counter-diffusion methods [21]. In solution method, it was used an ethanol solution in the classic evaporation process; and in the gel method tetramethyl orthosilicate (TMOS) hydrogel was prepared by polycondesation reaction [22], and agarose hydrogel was prepared by heating and cooling method [23]. After obtaining the gel phase, the solution of cholesterol 0.5% w/v in ethanol was poured onto the top of each gel allowing cholesterol molecules to diffuse into the gel network for one week at 18 °C. After this week, the solution was removed and replaced with water to produce the crystal growth of cholesterol by reducing the solubility in water, changing the dielectric constant.

X-ray powder diffraction. Cholesterol crystals were characterized by using an Empyrean XRD system from PANalytical Instruments (Netherlands), into the following conditions: Cu K_α radiation, monochromator, time step = 0.004s, step size = 19.68, 2θ = 4 to 50 deg, at room temperature.

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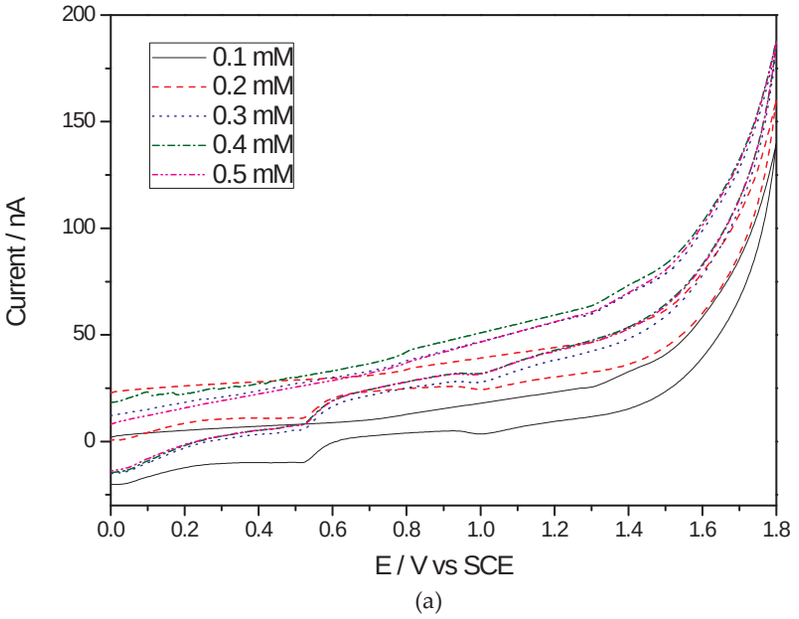
3. Results and discussion

The cyclic voltammetry for AAG protein showed an anodic response when interacting with carbonate, cholesterol and bilirubin (Figures 2a, 2b, and 2c). The protein alone did not show any electrochemical response in an aqueous solution of KCl 0.5M (curve not shown). This protein-substrate response was dependent of the concentration, and is characterized by an increment of current with the sequential increment of the analyte concentration. Only two of the three analytes (carbonate ions Figure 2a and bilirubin Figure 2c) investigated showed a strong protein interaction with them and generated a characteristic plot with increments of current. A small peak is observed at a potential value of 0.54 V, which corresponds to the oxygen reduction of the small amount of water electrolysis that occurs during the electrochemical response at E > 1.6 V. The peak is more evident for the cholesterol (Figure 2b) that interacts in minor proportion with the protein. This behavior decreases the strength of the interaction, which is almost imperceptible for bilirubin (Figure 2c). This demonstrates that covered electrode with a stable protein-analyte layer generates a cleaner analytical signal avoiding the water electrolysis as a parasite reaction.

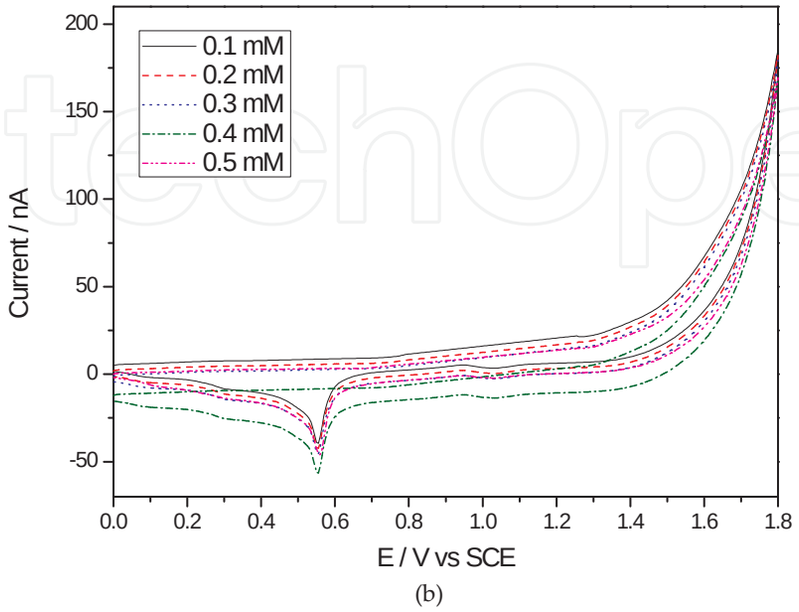
In order to have a clearer visualization of the effect, the electrochemical response of the protein was measured on the gold electrode without analyte (I_o). Then this curve was used to obtain a normalized plot I/I_o, which allowed standardizing all the experiments respect to the chemical interaction to the analyte (see Figure 3). The chemical interaction between the AAG protein and the specific analytes can be detected by either a big difference between I/I_0 or a big change value on the slope. When obtaining the normalized plots as shown in Figure 3, bilirubin showed the strongest chemical interaction, less intense followed by the carbonate ions. This behavior is particularly interesting since most of the preliminary investigations have claimed that AAG protein recognizes cholesterol molecules or works as a crystallization promoter in human bile, as that observed for different immunoglobulins like IgM, IgA, IgM or for phospholipase C and aminopeptidase N [17], but according to these results the interaction with bilirubin must not be discarded.

From these results it is clear that supersaturation of cholesterol in bile is a necessary condition though not sufficient, for the formation of cholesterol gallstones [3]. Biliary proteins, which are capable of affecting the rate at which cholesterol crystallization occurs, are important factors in pathogenesis of cholesterol gallstone diseases [3]. The pathway is as follows: (1) AAG-bilirubin complex formation, (2) cholesterol nucleation and (3), cholesterol crystal growth. The solubility of cholesterol is quite an interesting issue to take into account, when trying to investigate this chemical recognition in vivo experiments. Cholesterol in vitro is soluble in alcoholic solutions, but in vivo, cholesterol molecules should be solubilized by aqueous micellar solution or transported either by apolipoproteins or by any other biomolecules in order to make it soluble. The cholesterol can be crystallized inside the bile, or be secreted to the blood stream or sent directly to the stools [4]. This process based on the electrochemical observations, is in agreement with the enterohepatic circulation from the liver to the bile [11]. In this physiological process bilirubin needs the chemical interaction with cholesterol. Bilirubin is firstly conjugated with glucoronic acid in the liver by the en-

zyme glucuronyltransferase making it soluble in water, and perhaps with cholesterol too. Therefore, it makes sense that the first crystals of AAG protein were obtained by Schmidt in 1952 using ethanol as precipitating agent [24]. The α_1-Acid Glycoprotein is a cholesterol crystallization promoter conjugated with a complex bilirubin-cholesterol as shown in the electroanalytical results in Figure 2.



(a)



(b)

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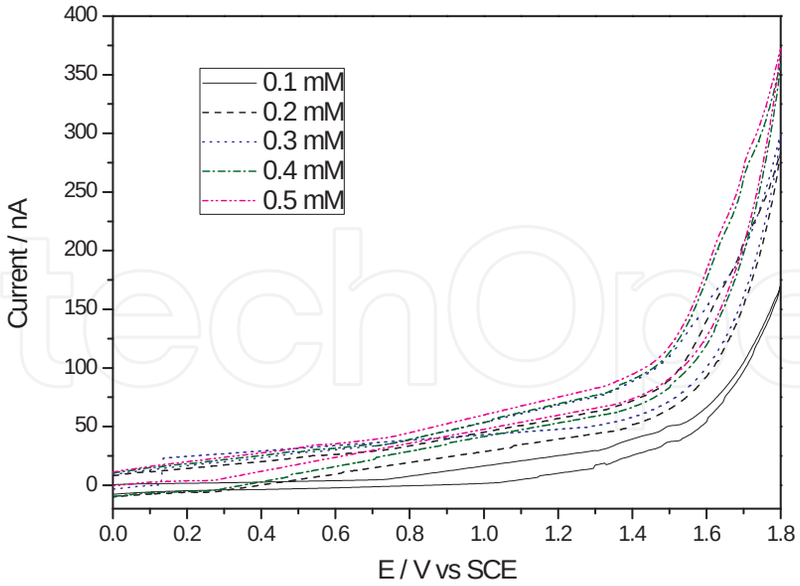

(c)

Figure 2. a. Cyclic voltammogram of AAG – KCl / Na_2CO_3 ranging the carbonates concentration from 0.1 to 0.5 mM scanning speed of 50 mVs^{-1}. b. Cyclic voltammogram of AAG – KCl / Cholesterol ranging the cholesterol concentration from 0.1 to 0.5 mM scanning speed of 50 mVs-1. c. Cyclic voltammogram of AAG – KCl/Bilirubin ranging the bilirubin concentration from 0.1 to 0.5 mM scanning speed of 50 mVs-1

In humans, gallbladder sludge (gel-like media), a reversible pre-gallstone phase, consists of cholesterol crystals and bilirubin granules in a mesh of mocus called mucin. Mucin a glycoprotein commonly observed in bile, is the constituent of the core and non-cholesterol matrix of cholesterol gallstones. It is highly likely that gallbladder mucin is actively involved at a number of stages in cholesterol precipitation [25].

On the other hand, calcium salts are present in all pigment gallstones as compounds of one or more of the anions in bile: (i) carbonate; (ii) bilirubinate, and (iii) phosphate. In addition, since cholesterol stones have been found to contain pigment stone centers, perhaps bilirubin and AAG protein are complexed. We can postulate that the presence of calcium salts precipitation in bile is a critical event in the initiation of cholesterol gallstones, so that the latter should be considered a two-stage process: (i) precipitation of calcium salts chemically bonded to AAG protein to form a macromolecular complex plus some components of the bile, for example mucin as a growth media, and then (ii) crystallization of cholesterol from its supersaturated state on this gel-like environment. It has been published that in vivo experiments cholesterol precipitation starts with thin filamentous structures, which evolve into helical and then tubular forms before breaking into characteristic flat cholesterol monohydrate plates [26].

From the crystal growth point of view, the crystal habit of cholesterol grown in vitro and in three different media (two of them hydrogels used to emulate mucin, a gel-like component usually found in bile) shows the vicissitudes that a crystal goes through when growing (Figure 4). The crystallographic faces, shown on each gel media, describe the plausible shapes that cholesterol can display according to the transport and to the crystal growth processes,

(the cholesterol keeps the same space crystallographic group). X-ray powder patterns of cholesterol lamellar-like crystals are shown in Figure 5. They have a good agreement with the pattern reported in the Powder Diffraction File (PDF, file 7-742), which corresponds to the triclinic phase, as well as those reported by several authors [26-28]. These cholesterol crystals should be grown in the presence of different combinations of AAG protein plus bilirubin, in order to check the protein as a promoter (nucleant) of cholesterol molecules.

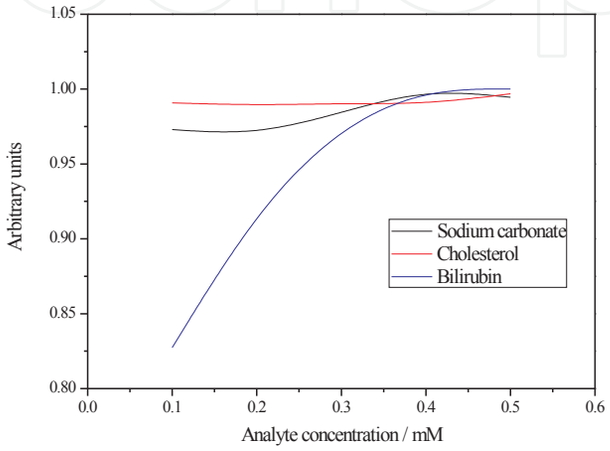

Figure 3. Normalized plot (I/I_o, see text for details) for anodic peaks corresponds to the maximum response of each voltammogram AAG-KCl, at different molar concentration for each analyte: sodium carbonate, cholesterol and bilirubin according to Figures 2a, 2b and 2c respectively.

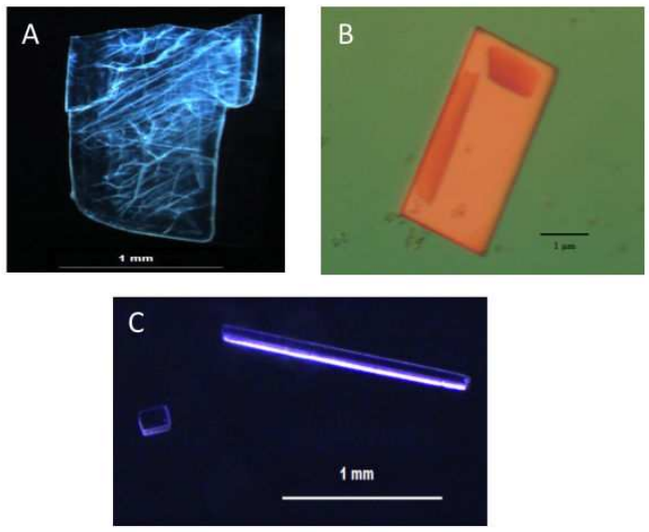

Figure 4. Optical images of Cholesterol crystals grown in: A) Solution Ethanol/Water, B) Tetramethyl orthosilicate hydrogel, C) Agarose hydrogel. The pictures (A) and (C) were taken between 90 degrees overcrossed-polarizers, while (B) picture corresponds to unovercrossed-polarizers. Different scale bars are included as inset on each picture.

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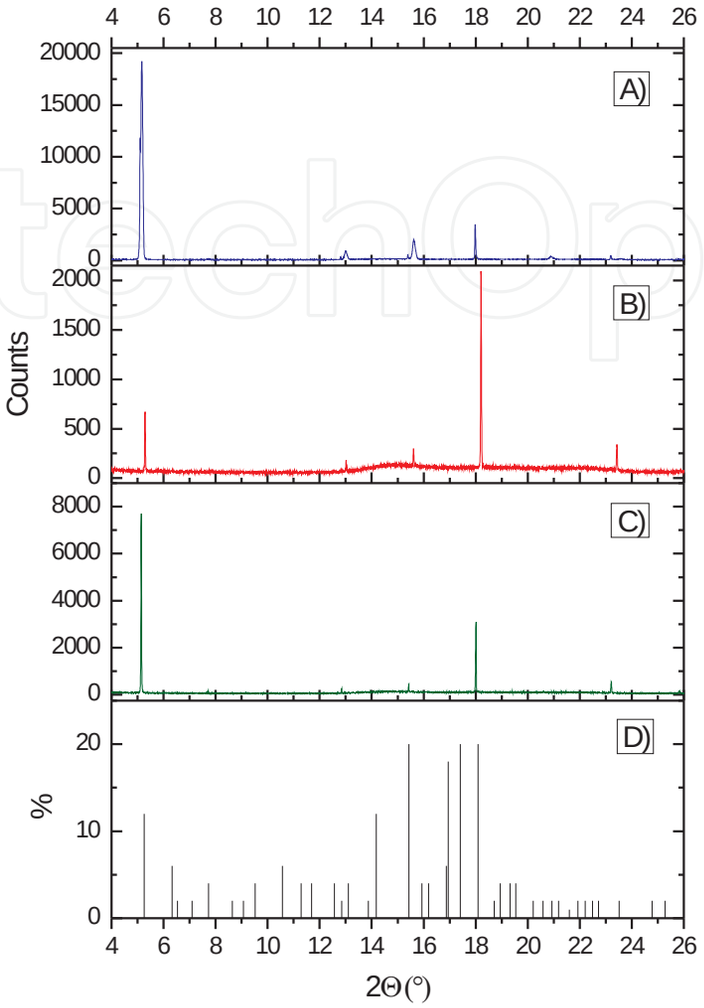

Figure 5. X-ray powder diffraction characterization plots of cholesterol crystals grown in three different media: A) alcoholic solution by evaporation method, B) tetramethyl orthosilicate hydrogel, C) agarose hydrogel and D) X-ray diffraction pattern (PDF file No. 7-742) of a cholesterol of the triclinic phase P1.

In the near future, cholesterol crystals could be grown in the presence of bilirubin, glucoronic acid and most of the components of the bile extract, in order to see whether these crystals will show the same crystallographic faces or not. These crystals could be re-dissolved in alcoholic solutions and the proteins extracted. These proteins could be purified and characterized from the cholesterol crystals in order to check their similarity to the AAG protein.

Finally, the AAG protein should be co-crystallized in the presence of bilirubin-cholesterol searching for the specific bonding-sites into the crystallographic structure either by X-ray Crystallographic methods or by NMR, as performed in the crystallographic projects nowadays. In future investigations we might be looking for inhibitors for the AAG Protein, like the promising cholesterol antinucleating 120kDa glycoprotein found by Ohya et al. [29]; or

look for strategies to turn the genes off related to the over-expression of this AAG protein in vivo to control the cholesterol crystallization.

4. Biosensor's design

From these electroanalytical results, the most plausible way of producing an ad hoc cholesterol-AAG/BR biosensor based on biological macromolecules like AAG, should follow the steps from A to D as shown in the box-diagram of Figure 6. The first step (A) corresponds to the gold electrode. The AAG protein would then be deposited on the gold electrode by using a thin film of the protein, this is the second step called (B). In the following step, (C) the protein should be complexed with bilirubin either by electrochemical interaction, or by Langmuir-Blogget isotherms as proposed by Xie et al., [30]. Finally, (D) the amperometric biosensor could be tested in the presence of different concentrations of alcoholic solutions of cholesterol, based on the strategy described in the experimental set up on this contribution.

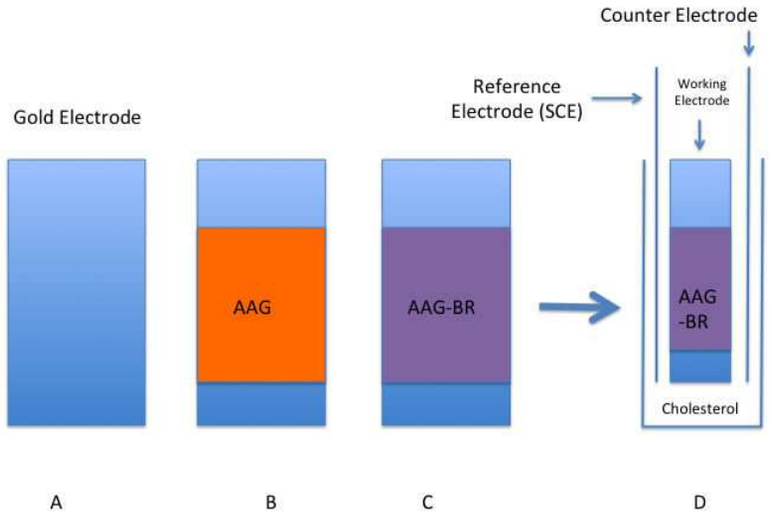

Figure 6. It shows different steps in order to produce an ad hoc Cholesterol-Bilirubin-AAG Biosensor.

The electrochemical response using this biosensor will be used to optimize the detection limits to check cholesterol in different media either in solution or in gel (this latter which would emulate the tissue texture or the mucin usually found in the bile).

5. Conclusions

We can observe from this research, that there is no a direct interaction between α_1-Acid Protein (AAG) and cholesterol molecules. The perfect biosensor for the evaluation of this chemical interaction should take into account that bilirubin is the intermediate molecule between

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cholesterol, and the AAG protein. This protein works as promoter, but needs some intermediate molecules like bilirubin to solubilize cholesterol in aqueous solutions when precipitating gallstones. Future investigations should be focused on co-crystallizing these proteins in the presence of bilirubin/cholesterol. The crystallographic 3D structure of the native glycoprotein can give answers about the specific sites (amino acids), where cholesterol molecules could be attached to the protein molecule working as nucleation precursor.

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