Separation and Purification Technology 143 (2015) 161–168

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

# Affinity separation by Langmuir–Blodgett deposition of bovine serum albumin using arachidic acid as specific ligand

Patricia Pedraz [a], Julia Cortés [a], Oliver Hilgendorf [b], Sophie Rassid [c], Cécile Bogaert [c], Ophélie Herouard [c], Francisco J. Montes [a], M. Elena Díaz [a,*], Ramón L. Cerro [d]

[a] Department of Chemical Engineering, University of Salamanca, Plaza de los Caídos 1-5, 37008 Salamanca, Spain
[b] Technische Universität Berlin, Fachgebiet Verfahrenstechnik Sekr., MA 5-7, Straße des 17, Juni 136, D-10623 Berlin, Germany
[c] Ecole Nationale Supérieure de Chimie de Lille (ENSCL), Cité Scientifique, Bâtiment C7, Av. Dimitri Mendeleiev, BP 90108, 59652 Villeneuve d'Ascq cedex, France
[d] Department of Chemical & Materials Engineering, The University of Alabama in Huntsville, 301 Sparkman Drive, 130 Engineering Building, Huntsville, Alabama 35899, USA

ARTICLE INFO

*Article history:*
Received 2 September 2014
Received in revised form 5 December 2014
Accepted 15 January 2015
Available online 7 February 2015

*Keywords:*
Biofilms
Bioseparations
Proteins
Affinity separation
Langmuir-Blodgett
Arachidic acid

ABSTRACT

Affinity separation by Langmuir–Blodgett (LB) deposition is a high resolution bioseparation procedure that takes advantage of the affinity between biomolecules and surface active materials, inducing the formation of a Langmuir-biofilm (L-biofilm) which is later removed from the air–water interface by deposition onto a solid substrate. This novel method is here applied to the separation of bovine serum albumin (BSA) by using arachidic acid (AA) as a ligand. Optimization of the experimental procedure choosing those conditions which favor Y-type multilayer deposition and low protein solubility is key. Both goals are achieved simultaneously by using $Cd^{2+}$ as a counterion and a pH equal to the counterion $pK_A$, that is pH = 5.6. The selection of a target area of 22 $Å^2$/molecule, a value close to the footprint of AA in solid state, ensures that the protein will be squeezed out of the interface and bounded to the ligand. The transfer ratio (TR) values obtained during depositions for up to 6 layers show that the deposition was successful, and spectrophotometric analysis show that a 48% of the protein initially present at the subphase is removed. Additionally, the efficiency of separation for each layer is close to 100% with respect to the maximum amount of BSA that could fit in the film, assuming that the molecule stands vertically in the L-biofilm structure (long axis perpendicular to the solid). Atomic Force Microscopy (AFM) analysis qualitatively confirms the presence of the BSA in the deposited biofilm. The images illustrate that the presence of BSA strongly affects the topography of the surface, in the form of roughness and protrusions, unlike a pure arachidic acid monolayer. Roughness clearly increases when BSA is present in the deposited film. In summary, experimental evidence indicates the high potential of LB as a method for purification of proteins.



## 1. Introduction

Separation processes, in general, constitute an important part of the unitary cost of a chemical product. This subject requires more consideration in the particular case of the biochemical/biotechnology production plants, because most bioseparation methods are not fully operative at industrial scale and require more expensive chemicals and equipment than classical industrial-scale separations. The emergence of genomics and proteomics has shifted the emphasis for bioengineering research to increase efficiency and throughput of separation and purification of proteins. Even though separation of most proteins can be accomplished by several well-known standard methods, it is actually purification what constitutes the bottleneck, both in productivity and cost, of the global production process [1].

Among the broad range of bioseparation methods, affinity separations are among the most selective biomolecule purification methods [2]. Combining the strategy of an affinity separation with the highly structured properties of Langmuir films (L-films) results in a very efficient biomolecule separation process based on a ligand technique [2], the "Affinity Separation by Langmuir–Blodgett (LB) Deposition", originally introduced in a previous paper [3]. In this case, the specific ligand is an amphiphilic molecule such as a carboxylic acid that forms an L-film at the air–water interface,

*Abbreviations:* BSA, bovine serum albumin; AA, arachidic acid; AFM, Atomic Force Microscopy; TR, transfer ratio; L, Langmuir; LB, Langmuir-Blodgett.

* Corresponding author. Tel.: +34 923294479; fax: +34 923294574.
*E-mail address:* e.diaz@usal.es (M.E. Díaz).

http://dx.doi.org/10.1016/j.seppur.2015.01.036
1383-5866/

serving as bait to "fish" from the water subphase a biomolecule (protein) for which this ligand has specific affinity. Once the ligand–protein L-film is formed, the protein can be separated from the subphase by transferring the L-film onto a solid substrate using the LB deposition technique.

In the experiments described here, a biofilm is formed by bonding a carboxylic acid (arachidic acid (AA) = ligand), which conforms the L-film, and bovine serum albumin (BSA), soluble in the aqueous subphase. Few proteins besides albumin can bind fatty acids, and none can bind them as tightly or in such large amounts [4]. This fact ensures the specificity of the separation. In a previous paper [3], we analyzed the formation of the biofilm at the air–water interface with the purpose of establishing the optimal conditions to undergo the separation of the BSA by affinity separation using LB deposition. According to the results obtained, prior to the formation of the biofilm, BSA migrates from the water subphase towards the air–water interface following a two-step scheme: diffusion from the bulk to the interface and rearrangement. The biofilm is formed by the interactions between the BSA contained in the subphase with the polar acid group as well as the non-polar hydrocarbon chain of the arachidic acid in the L-film at the air–water interphase. This process is greatly affected by the subphase pH due to the changes in solubility and conformational structure of the protein. At pH = 8.2, the protein is at its B-form and shows poor adsorption/association characteristics (high solubility). In contrast, at pH = 3.8 and 5.1 (F and N-forms of the protein, respectively), the protein-fatty acid interaction is significant. Compression isotherms of the L-film, which are also affected by pH, suggest that BSA molecules penetrate the monolayer at low pH, being this penetration inversely related to the solubility of the protein. The L-biofilm collapses at approximately the same area per molecule than a pure AA L-film, indicating that, at the point of collapsing, BSA molecules have been ejected from the monolayer. In short, pH is a critical variable for biofilm stability. Even the small amount of BSA assimilated with the L-film at pH = 8.2, makes the film highly stable while at the lower, more acidic pH values of 5.1 and 3.8, the loss of material by dissolution is higher.

Once the biofilm is formed at the air–water interphase, the transfer of such film onto a solid surface using the LB process points to a full list of new variables that must be optimize, where the type of counterion and the pH of the subphase solution, are the most important. The incorporation of metal ions (counterions) to the LB films gives special properties to the resulting multilayers, which are of great importance in a wide range of scientific fields [5], as well as in the affinity separation by LB deposition. Counterions are added to the water subphase in the form of a chloride or sulfate salt of the metal cation. In the subphase and in contact with the L-film at the interphase, the ionized metal cations form salts with the head groups of the amphiphilic molecule. This phenomenon is more pronounced at the $pK_A$, that is the point at which half of the molecules containing the hydrophilic group are ionized. Moreover, when the monolayer is ionized, direct electrostatic repulsions or attractions occur both within the film and between the film and the subphase ions. Additionally, the presence of counterions in the aqueous subphase sets up ionic double layer effects at the interphase. These counterions can promote or inhibit phase transitions which, in turn, have a strong effect on the shape of the surface pressure versus area isotherms [6]. Since AA, the monolayer forming material is a weak acid, depending on pH, the polar head groups dissociate to form hydrogen ions in the subphase and carboxylate ions in the film. The intrinsic $pK_A$ of a typical long chain carboxylic acid over a subphase of pure water has been estimated to be 5.4 [7]. However, the presence of counterions in the subphase may alter this value and the particular range at which the acid to organic salt conversion takes place is specific for each counterion. Divalent counterions such as $Cd^{2+}$ [8–10], $Ca^{2+}$ [8,10] and $Pb^{2+}$ [8,10]) are often added to the subphase to improve the deposition characteristics of the monolayer.

Since the objective of our study is to prove the concept of the use of LB films as a method for purifying proteins by using the model system AA-BSA, the deposition of multilayers instead of just a single layer is desirable, as it improves the global efficiency of the separation process. Typically, LB multilayer deposition types are classified as Y, X and Z [11]. Once the first monolayer has been deposited, subsequent movements in and out of the LB trough often lead to the deposition of an additional layer on each passage. Such a deposition mode called Y-type leads to a stack in head-to-head and tail-to-tail configuration. Although the Y-type may be the desirable configuration under certain conditions, under different conditions the floating monolayers are transferred only during immersion (X-type) or only during emersion (Z-type) of the substrate [11]. It is important to point out that the different types of depositions refer to the behavior during the deposition, not to the final structure of the formed film, since re-organization of the LB film structure may occur after deposition as described by Takamoto et al. [11].

There are previous reports of LB transfer of biofilms on solid substrates with variable results. Chovelon et al. [12,13] reports the formation of a mixed film of octadecylamine and butyrylcholinesterase, which is latter transferred as a LB film thanks to the mechanical stability provided by the crosslinking of the amine terminal moieties and glutaraldehyde molecules. Kamilya et al. [14] show an non-defined interaction between stearic acid and ovalbumin that leads to a consistent LB film. Girard-Egrot et al. [15] assembled a systematic review of pairs enzyme-amphiphilic molecule based on different interactions mainly used for biosensing purposes. Nylander [16] suggests that there are other parameters affecting the protein-amphiphilic molecule interaction besides the electrostatic interactions. Finally, Yin et al. [17] produced an electrode modified with protein by the LB technique.

In this article, a study of the multilayer deposition of AA-cadmium arachidate (CdA)-BSA biofilm by means of the LB technique is presented. Based on previous [3] and recent studies of our research group, the experimental conditions for deposition are established. The quality of the deposited film was analyzed by AFM and TR characterization. The amount of BSA deposited (separated from the bulk) is measured by UV–Vis spectroscopy. Results indicate the remarkable potential of the LB technique as a novel method for protein purification.

## 2. Experimental

### 2.1. Materials

Crystallized and lyophilized BSA (BSA $\geqslant$ 96%, CAS No. 9048-46-8; Mw = 66400 Da) was purchased from Sigma–Aldrich Chemical Co.; it was used as received without further purification. AA (eicosanoic acid, 99%, CAS No. 506-30-9, Mw = 312.5) was purchased from Acros Organic. Reagent grade chloroform, acetone and 2-propanol supplied by Sigma–Aldrich Chemical Co. were used for the cleaning of the trough. Ultrapure water (resistivity of 18.2 MΩ cm) was obtained from a Wasserlab Ultramatic Purifier system. The pH of the subphase was adjusted by addition of dilute solutions of hydrochloric acid (ACS reagent 37%, Sigma–Aldrich) and sodium hydroxide (ACS reagent 97%, Sigma–Aldrich) and measured by means of a pH-Meter BASIC $20^+$ (Crison Instruments, S.A.). Cadmium chloride (CAS number 10108-64-2) (ACS reagent, anhydrous) was purchased from Acros Organics. Tricosaethylene glycol mono-n-dodecyl ether (Brij 35®, CAS 9002-92-0) was supplied by Scharlau and used for preparing in the UV–Vis spectroscopy solutions. Eurotube® microslides (76 mm × 26 mm × 1 mm)