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High-performance liquid chromatography as a technique to determine protein adsorption onto hydrophilic/hydrophobic surfaces

Tongtong Huang[a,b], Karine Anselme[a], Segolene Sarrailh[b,c], Arnaud Ponche[a,*]

[a] Institut de Science des Matériaux de Mulhouse (IS2M)—UMR 7361 CNRS-UHA, 15 rue de Jean Starcky B.P. 2488, 68057 Mulhouse Cedex, France
[b] Aptar Pharma, Route Des Falaises, 27100 Le Vaudreuil, France
[c] Aptar Stelmi, 22 Avenue des Nations, BP 59415 Villepinte, 95944 Roissy CDG Cedex, France

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ABSTRACT

The purpose of this study is to evaluate the potential of simple high performance liquid chromatography (HPLC) setup for quantification of adsorbed proteins on various type of plane substrates with limited area ($<3\ cm^2$). Protein quantification was investigated with a liquid chromatography chain equipped with a size exclusion column or a reversed-phase column. By evaluating the validation of the method according to guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), all the results obtained by HPLC were reliable. By simple adsorption test at the contact of hydrophilic (glass) and hydrophobic (polydimethylsiloxane: PDMS) surfaces, kinetics of adsorption were determined and amounts of adsorbed bovine serum albumin, myoglobin and lysozyme were obtained: as expected for each protein, the amount adsorbed at the plateau on glass (between 0.15 $\mu g/cm^2$ and 0.4 $\mu g/cm^2$) is lower than for hydrophobic PDMS surfaces (between 0.45 $\mu g/cm^2$ and 0.8 $\mu g/cm^2$). These results were consistent with bicinchoninic acid protein determination.

According to ICH guidelines, both Reversed Phase and Size Exclusion HPLC can be validated for quantification of adsorbed protein. However, we consider the size exclusion approach more interesting in this field because additional informations can be obtained for aggregative proteins. Indeed, monomer, dimer and oligomer of bovine serum albumin (BSA) were observed in the chromatogram. On increasing the temperature, we found a decrease of peak intensity of bovine serum albumin as well as the fraction of dimer and oligomer after contact with PDMS and glass surface. As the surface can act as a denaturation parameter, these informations can have a huge impact on the elucidation of the interfacial behavior of protein and in particular for aggregation processes in pharmaceutical applications.

1. Introduction

Proteins are widely used in formulation in the pharmaceutical field and play a major role in biological functions. It is well known that protein adsorption on solid surface is always observed for a long-term storage, which will result in a reduced dose of active compound or a loss of biological activity (Burke et al., 1992; Nault et al., 2013b). In some cases, only short time of contact are sufficient to drastically modify the protein conformation: for instance, insulin losses 52% of its biological activity after 5 min contacting with glass surface (Petty and Cunningham, 1974), as well as a loss of 30% of cetrorelix is observed after 2 h (Grohganz et al., 2004). Among all parameters, the time frame of the denaturation process is strongly related to the protein stability and surface properties. The understanding of protein adsorption has therefore become a crucial issue in the pharmaceutical industry.

Since usually the amount of adsorbed protein is extremely low, the measurement requires a high sensitivity. Some conventional and historical techniques are currently in use, such as the optical-sensitive method: ellipsometry, which is based on the modification of polarization angles of the light reflected by the surface of the sample (Elwing, 1998; Seitz et al., 2005). It is yet required to have reflecting metal surfaces or ceramic surfaces. Quartz Cristal Microbalance with Dissipation (QCM-D) technique is also involved in the direct measurement of amount of protein adsorbed on the surfaces. According to the change in the oscillating frequency, one can obtain the effective mass loaded (Dolatshahi-Pirouz et al., 2008; Rechendorff et al., 2006). Protein adsorption measurement can be also implemented by using radiolabeling technique, iodine ^{125}I for instance (Chan and Brash, 1981), according to the decrease

* Corresponding author.
E-mail address: arnaud.ponche@uha.fr (A. Ponche).

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in radioactivity in the solution in the function of time of adsorption. However, the labeling technique can induce artificial protein aggregation or degradation (Holmberg et al., 2007).

The ultraviolet adsorption method is considered to be the simplest direct method for the measurement of protein concentration in solution without any standard for every assay (Deutscher, 1990). It can be performed very fast and the relationship between protein concentration and absorbance is linear. However, it can only be employed to a small range of protein concentration and the limit of detection is rather high in solution (Deutscher, 1990). Compared to the UV detection at 280 nm, chemical methods based on the reduction of copper II ions in copper I by proteins and formation of a colored complex with bicinchoninic carboxylic acid (BCA) are more precise and sensitive (Eberlein, 1995; Noble and Bailey, 2009), which is required by the low amount of proteins adsorbed onto hydrophilic surface, in particular when the surface of the sample is not extensive. However the detection range is quite narrow (from 0.5 to 20 μg/mL) for micro BCA assays (Deutscher, 1990), which limits the quantification of the solution at high concentration. To maximize the effect of the surface on protein adsorption and continue to use quantitiation techniques operating in liquid conditions, several authors deal with modified glass beads, inorganic nanoparticles or grinded substrate to maximize the surface/volume ratio. This approach is hardly useable for polymeric samples as the risk of surface modification during the grinding is considerably increased.

Over the last 20 years, as a straightforward and convenient approach the high performance liquid chromatography (HPLC) has become a central technique in the characterizations of peptides and proteins (Hartmann et al., 2004; Lensen et al., 1984; Sviridov et al., 2006; Umrethia et al., 2010). It can be attributed to a number of advantages including wide range detection, good reproducibility, high recoverie (Garcia et al., 2008), excellent resolution and sensibility (Aguilar, 2004). As proteins possess secondary and tertiary structures, they contact the chromatographic surface in an orientation-specific manner. This method is therefore based on the specific interaction between protein and chromatographic column (Aguilar, 2004).

In this study, we propose to use HPLC to estimate the amount of protein adsorbed on a flat surface by determining the depletion of the proteins in solution. The classical HPLC system is equipped with a diode array UV detector combined with two different kinds of column: size-exclusion and reversed-phase. The earlier separates biomolecules in order of decreasing molecular size. Size exclusion chromatographic columns are prepared with wide-pore, small-diameter silica particles densely packed: large molecules are excluded from pores and have the shortest path through the column, they are therefore eluted earlier. The small molecules permeate freely into the pores, which retards their movement through the chromatographic bed. By contrast, the reversed-phase chromatography is based on the hydrophobic binding of solute molecule from the mobile phase to the stationary phase. Proteins adsorb on the hydrophobic surface while entering the column and desorb when the organic modifier concentration reaches the critical value (Aguilar, 2004). The concentration of the supernatant protein solution will be determined by HPLC before and after contact with a flat PDMS surface or glass vials. Knowing the contact area of the solution with the surface, the adsorbed amount of protein will be calculated.

Many materials enter in the fabrication of pharmaceutical containers including glass of course, but also elastomers for joints, metals for seals or different polymers of pharmaceutical grade for wall of newly developed dispensers. The goal of this article is to evaluate the potency of size exclusion liquid chromatography to quantify amount of adsorbed proteins on these various substrates (flat and limited in size) and to evaluate informations given on the denaturation process at the vicinity of the surface leading to aggregation in long term storage.

2. Materials and methods

2.1. Proteins and chemicals

Albumin, from bovine serum (BSA, M_w ~67,000 g/mol), myoglobin, from equine skeletal muscle (MGB, Mw ~17,800 g/mol) were purchased from SIGMA life science. Lysozyme (LSZ, 14,600 g/mol) from chicken egg white was obtained from Fluka Analytical. Some properties of the proteins that may be relevant to the adsorption behavior are summarized in Table 1.

Sodium dodecyl sulfate (SDS) (M_w ~288.38 g/mol) and phosphate buffered saline (PBS) were provided by SIGMA life science. Acetonitrile (ACN) (CHROMASOLV® Plus, for HPLC, ≥99.9%) (41.05 g/mol) and trifluoroacetic acid (TFA) (CHROMASOLV® Plus, for HPLC, ≥99.0%) (114.02 g/mol) were provided by Sigma–Aldrich.

Flat PDMS surfaces were prepared by mixing the silicone elastomer (Sylgard 184, Dow Corning Corporation) and the curing agent in a 10:1 ratio. After pouring the prepolymer in a flat polystyrene box and 12 h degassing at ambient pressure, the silicone sheet has been thermally treated at 70 °C during 4 h.

Both glass surface and PDMS were contacted with protein solution at 20 μg/mL. All protein solutions were prepared in phosphate buffered saline (PBS) at concentration of 137 mM sodium chloride, 10 mM sodium phosphate and 2.7 mM potassium chloride (by dissolution of 1 PBS tablet with 200 mL Milli-Q water).

Protein adsorption process was carried out as a function of time at room temperature at pH 7.4. The supernatant was then analyzed by both HPLC and mBCA approach.

2.2. Size exclusion chromatography (SE-HPLC)

Chromatographic analyses were carried out using an Agilent 1100 Series (Agilent Technologies GmbH) equipped with a quaternary pump and coupled with a UV detector.

A Zorbax Bio Series GF-250 column with dimensions of 9.4 (i.d.) × 250 mm (Agilent Technologies) was chosen for protein quantifications. 100 μL protein solution was injected on a size-exclusion column at 25 °C, with detection at λ = 215 nm. Analyses used PBS (137 mM NaCl, 10 mM Na_3PO_4 and 2.7 mM KCl; pH 7.4) for eluting bovine serum albumin and myoglobin. The mobile phase for LSZ was PBS with 0.1% Sodium dodecylsulfate (wt%). The elution was performed during 15 min with an appropriate flow rate of 1.0 mL/min.

Table 1
Properties of three proteins (Arai and Norde, 1990; Norde and Favier, 1992).

Protein	Molar mass (g/mol)	Size (nm^3)	Isoelectric point (pH unit)	Conformational stability
Lysozyme	14600	4.5 × 3.0 × 3.0	11.1	High
Myoglobin	17800	4.5 × 3.5 × 2.5	7.0	Low
Bovine serum albumin	67000	11.6 × 2.7 × 2.7	4.7	Low