INTERACTION OF CIBACRON BLUE WITH HUMAN SERUM ALBUMIN A PROMISING CANDIDATE FOR REGENERATION OF ALBUMIN DEPLETION COLUMNS

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Abstract: Proteomic analysis of human plasma has an increasing interest on the field of life sciences, biomedical and diagnostic applications. To get a full protein map of the sample investigatedprior separation is usually requiredto get information of low molecular weight or less abundant proteins such as biomarkers. These steps are usually time consuming processes, and have a chance to eliminate important fragments from the sample. Depletion columns with immobilized antibodies are the best units for these tasks; however, the strong antibody-antigen interaction allows only a single use of these columns. Cibacron Blue dye might be a promising candidate to break this strong complex upon binding selectively to the albumin. In this work we focused on the albumin-cibacroninteraction and describe it with various techniques.

Keywords: Proteomics, HSA, antibody, antigen, dye, NMR, affinity, depletion.

I. INTRODUCTION

The human plasma contains large number of proteins with different size and functions. The peptidome is thought a rich source of biomarkers that can be used to examine organ functions, get information about the status of diseases or analyze clinical samples such as blood samples during the IVF procedures.[1] Usually these biomarkers have low abundance or small molecular weight that makes detectiondifficult especially at the presence of high abundance proteins such as serum albumin (HSA) and immunoglobulins. The concentration range of the plasma proteins can be a few orders of magnitude.[2] As one of the main techniques for proteomics is mass spectrometry (MS) or MS/MS, the large difference in protein concentrations makes the low abundant proteins practically undetectable.[3,4] Thus prior separation or depletion is required for these complex samples. The most common commercially available depletion units are affinity kits, which can remove the most abundant proteins selectively from the injected sample. Some of these kits can be coupled with mostly microflow HPLC systems; however, the chromatographic matrix has to be chosencarefully because of the limitation of the back pressure or the flow rate that is crucial for coupled measuring techniques. The depletion columns usually containimmobilized molecules with high affinity to the selected component, such as antibodies.

In our previous study we investigated the interaction of human serum albumin (HSA) with monoclonal antibody (mAb) with electrophoretic methods (Capillary Zone Electrophoresis (CZE), Capillary Gel Electrophoresis (CGE) and Affinity Capillary Electrophoresis (ACE)) and Isothermal Titration Calorimetry (ITC). It was found, that the monoclonal antibody can bind two equivalents of albumin in a cooperative way. The calculated dissociation constants (2.26×10^{-6} and 1.22×10^{6}, respectively) indicated the strong interaction, as well as the similarity of the two binding sites. From these measurements it also turned out, that the antibody has a high selectivity toward HSA, no interaction could be detected with BSA even at higher excess of the ligand.[5]

When the antibody was immobilized onto a TiO_2 nanoparticle the mAb partially lost its albumin binding capability probably because some of the functional groups of the binding sites had been biotinylated during immobilization.However, it still remained active, and it was possible to incorporate into monolithic columns. This column than had been tested with IVF samples. It was shown, that the column is capable for albumin depletion.[6]

One of the main disadvantages of these depletion units, that they cannot be regenerated after saturation. In our current work we tried to find a specific molecule that can bind selectively to the binding moiety of the HSA, therefore this reaction can compete with the formation of antibody-antigen complex under the appropriate circumstances.

Cibacron BlueF3GA is a sulfonatedpolyaromatic dyethat can bind to many proteins and enzymes. The molecule can interact specifically to proteins having dinucleotide binding domain.[7-9]HSA has this moiety and cibacron can be used to isolate it.It has also been reported that albumin can be removed from the plasma using this anthracene based dye.[10]Thus it might be a good candidate for the previously mentioned regeneration process. In this paper we present our latest research on the HSA-cibacron interaction, and the studies on the ternary HSA-mAb-cibacron systems. This work also includes the characterization of the solution of cibacron in order to find out the proper conditions for the competing reaction.

EXPERIMENTAL DETAILS

2.1. Materials

Human serum albumin (HSA) was purchased from Sigma Aldrich and used without further purification. The monoclonal antibody (mAb) was synthesized by Biosystem International. The purchased phosphate buffered solution (2mg/ml) was delivered in frozen form without preservatives. Cibacron Blue F3GA was a production of Abcam and used after purification (dissolved in ultrapure water, membrane filtered and recrystallized). Samples for the UV/Vis spectrophotometric measurements were made with phosphate buffer (Sigma Aldrich) to keep the pH at constant value.
For the ^{1}H NMR measurements all the solution was made with D_2O (99.9% deuterium content, Eurisotop) under buffered media. The solid, water free NaH_2PO_4 and Na_2HPO_4 was purchased from Merck.

2.2. Methods

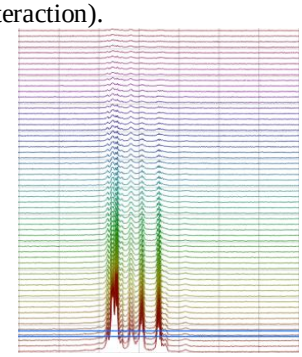
2.2.1 ^{1}H NMR measurements

The NMR experiments were done on a 400 MHz Bruker Avance II instrument equipped with a z-gradient(50G/cm) probehead. All the measurementswere carried out at 298±0.2K. For the diffusion studies the parameters (diffusion time, □ and gradient amplitude, □) was optimized to detect both large and small molecules.TopSpin2.1 pl8 software was used to operate the instruments, while MestreNova 8.1 package was used to evaluate the obtained NMR data. The intensity series from the diffusion measurements (DOSY, Diffusion Ordered SpectroscopY) were fitted with a single or a multiexponentialcurve, and the diffusion coefficient were calculated according to the following equation:

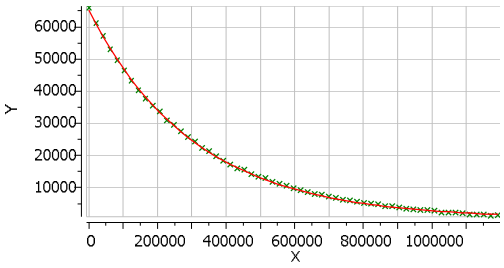
$$S(2\tau) = \sum_n \left(\frac{S(0)^n}{2} \exp\left(-\frac{2\tau_1}{T_2^n} - \frac{\tau_2}{T_1^n} \right) exp\left(-\gamma^2 g^2 D^n \delta^2 \left(\Delta - \frac{\delta}{3} \right) \right) \right)$$

where S(2□) is the intensity of given peak after the diffusion, n is the number of components belong to the given signal(s), $S(0)^n$ is the signal intensity of the component „n" without the gradient, □□□□

which is quite common for aromatic systems (stacking interaction).



a,



b,

Figure 2.
DOSY experiment on a 5 mMcibacron solution spectra series (a)
and the exponential fitting of intensities(b)

Table 1.
Diffusion coefficient of Cibacron Blue at different concentrations

concentration (mM)	diffusion coefficient (cm^2s^{-1})
0.5	3.80×10^{-6}
5.0	3.25×10^{-6}
50	2.52×10^{-6}

3.2. Titration of cibacron with albumin

Albumin solution was prepared in buffered deuterated media and added stepwise to the buffered solution of cibacron. The recorded ^{1}H NMR spectra are shown on Figure 3. The aromatic region is being followed where both cibacron and albumin have signals. As we previously seen, at low concentration the cibacron has narrow peaks in this chemical shift region, while the albumin provides wide, overlapping signals as HSA is a macromolecule. It can be seen, that at low albumin concentration the narrow peaks of the dye molecules appear in the spectrum. The presence of the free dye is also proven by the DOSY experiments. At low albumin concentration the signal decay can be fitted with a double exponential curve. The two diffusion coefficients are calculated to 8.6×10^{7} cm^2s^{-1} and 6.6×10^{6} cm^2s^{-1} where the higher value belongs to the free cibacronwhile the lower one to the albumin and the albumin-cibacron complexes. Increasing the albumin concentration causes the disappearance of the free dyes, that represented by the lower addition of the fast diffusion to the exponential decay in the DOSY experiments.

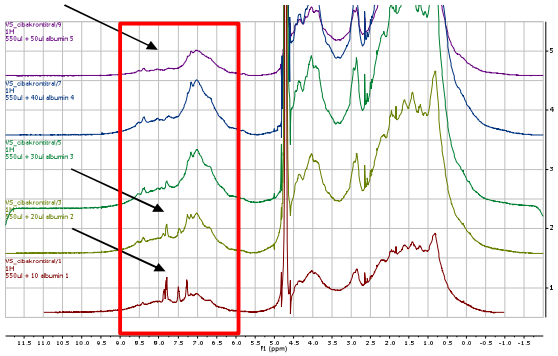


Figure 3.
^{1}H NMR titration of cibacron solution with HSA (increasing concentration from bottom to top)

Further increase of the HSA in the sample resulted in the loss of free cibacron signals from the ^{1}H NMR spectrum, while the DOSY spectra series can be fitted with a single exponential curve, and the calculated diffuison coefficient refers to the presence of a macromolecule. Itmeans,that practically all the cibacron content is bound to the albumin.

3.3. Equilibrium studies on the albumin-cibacron system

The interaction of the albumin and the dye molecule was followed by UV/VIS spectrophotometric method. In order to calculate the amount of bound cibacron as a function of concentration samples with constant HSA and increasing dye concentration were made at constant pH. The unbound cibacron was separated by a 30 kDa membrane filtration, and its concentration was determined by spectrophotometric measurements. (Previously it was found, that the absorbance values follow the Lambert-Beer law up to a 0.1 mM concentration, no association were detected under these conditions). Figure 4.shows the amount of bound cibacron as a function of its initial concentration. From this curve we can conclude, that at low cibacron concentration all the dye molecules are bound to the protein, while at higher concentration the cibacron partially remained free in the solutions. These results are in good agreement with the NMR findings. The complete “saturation” was not reached, because of technical issues, the absorbance values were too high at more concentrated solutions to get reliable data, and we wanted to avoid the formation of J-aggregates.[13]

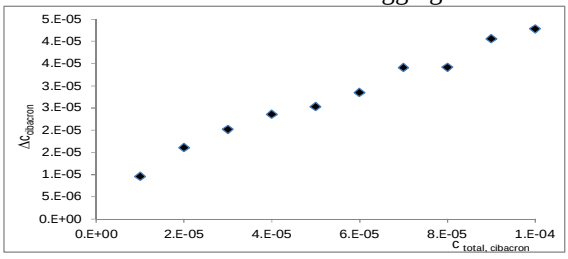


Figure 4.
Formation of HSA-cibacron complexes upon increasing cibacron concentration

3.4. Effect of cibacron to the HSA-mAb complexes

In this experiment cibacron was added to the freshly prepared HSA-mAb 2:1 complex in increasing concentration (up to 40 equivalents compared to the albumin). The complexes of antibody were removed by a 100 kDa membrane filtration, and the solution passing through was analyzed by UV/Vis method. As the antibody has a MW ~250kDa, all the antibody and its complexes can be removed, the filtrate does not contain any of these particles. The UV/Vis obtained spectra are shown in Figure 5.

The absorbance values in the visible region show that more and more cibacronis present in the filtrate, but comparing the full spectrum with the spectra of the dye (Figure 6.a) we can see a significant difference in the UV region.

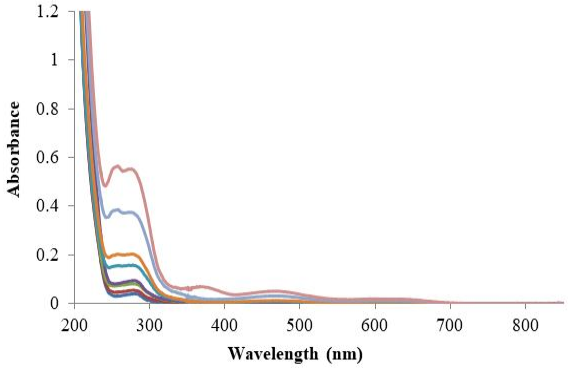
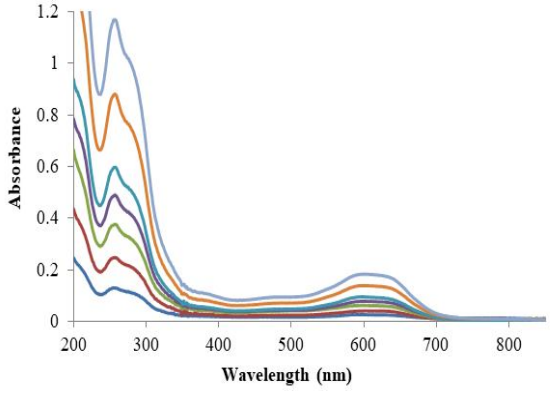
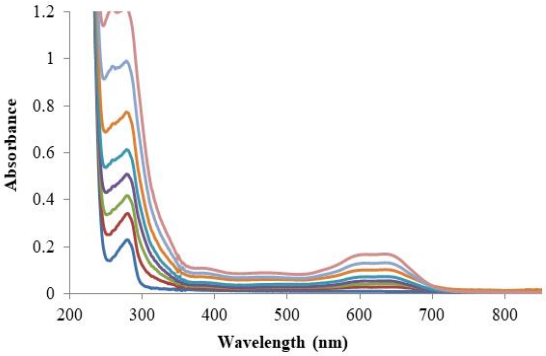


Figure 5.
UV/Vis spectra of 30kDa filtrate from ternary HSA-mAb-cibacron systems at increasing cibacron concentration

The absorbance values at 275 nm cannot be interpreted by the presence of only cibacron in the filtrate. However, comparing the spectra with the ones obtained for binary HSA-cibacron systems (Figure 6.b) we can see that the peak at 275 nm probably belongs to albumin-cibacron complexes. This findings support that the dye in large excess can compete to the binding sites of the HSA in the ternary system, thus cibacron is able to release the albumin from the stable HSA-mAb complexes. In order to calculate the released albumin concentration further techniques have to be used, and the reversibility should also be tested.



a,



b,

Figure 6.
UV/Vis spectra of cibacron (a) and cibacron-HSA solutions

CONCLUSION

The sulfonated polyaromatic dye, Cibacron Blue was tested if it can compete with the monoclonal antibody for the binding sites of human serum albumin. NMR investigation of cibacronblue solution revealed an association process based on stacking interaction at higher concentration. This equilibrium might be important in parameter optimization and has to be considered in other spectroscopic methods.

The interaction of albumin and cibacron was investigated with NMR and UV/Vis technique at different ratios. It was found, that at low cibacron to HSA ratio all the dye molecules are bound to the protein (probably to the most strong binding sites) while at larger concentration the free dye can be detected in the systems.

The combined ultrafiltration and UV/Vis spectrophotometric technique on the ternary systems showed that the increased cibacron concentration can cause the appearance of albumin in the filtrate in increased amount. This means the dye can break the strong interaction between the HSA andmAb.Further tests are required to determine the released amount of the protein.

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