Final Report

The progress of the **PD 83071** in the period of **01. 10. 2012 - 30. 09. 2015**.

1. The interaction of proteins with specific ligands is important aspects of pharmaceutical development processes. The whole process of drug discovery is accompanied by analytical methods to monitor and quantify the binding interactions. I have investigated the interactions between a monoclonal antibody (mAb) and its protein antigen human serum albumin (HSA) using CE and isothermal titration calorimetry (ITC). Two different CE methods, a pre-equilibration method capillary zone electrophoresis (CZE) and an equilibration method affinity capillary electrophoresis (ACE) were tested to characterize the binding constants. The CZE revealed the formation of the mAb·HSA and mAb·$(HSA)_2$ complexes and the binding constants determined by plotting the amount of the bound mAb as a function of the concentration of HSA. CZE could determine the binding constant because the interaction was strong and fast enough compared to the time required for the detection. The ACE provided information on the binding strength from the change in effective electrophoretic mobility of the mAb. The ACE did not reveal the formation of the given complexes, the mobility of the single detected peak was the result of the weighted average of the mobilities of three species of the mAb, and the weights are determined by the HSA concentration and the equilibrium constant for interaction. The equilibrium dissociation constant values obtained by CZE and ACE were found to be $2.26 \cdot 10^{-6}$ M for mAb·HSA, $1.22 \cdot 10^{-6}$ M for mAb·$(HSA)_2$ and $4.45 \cdot 10^{-8}$ M for mAb·HSA, $1.08 \cdot 10^{-7}$ M for mAb·$(HSA)_2$, respectively. The dissociation constant data obtained by ACE were in congruence with the values obtained by ITC ($2.74 \cdot 10^{-8}$ M, $1.04 \cdot 10^{-7}$ M). Applying CZE and ITC, we could define the stoichiometry of the interaction (HSA : mAb = 2 : 1). All three methods show that the mAb possesses two binding sites, which exhibit almost the same binding strength. The K_d values obtained by ACE and ITC are close to each other. This can be explained by the similarity of the experimental setups. In both methods, the binding interaction occur during the measurements (electrophoresis and titration). The advantage of CZE was that it revealed the complexation, and it made possible the detection of four different compounds of the interaction. The mAb can bind to HSA with strong affinity because the K_d values are lower than 10^{-7} M. The advantages of CE can be utilized in those

fields where ITC has limitations (sample quantity, solvent, purity). **(Electrophoresis, 36, (11-12) pp. 1274-1281., 2015)**

2. During the investigation into ACE we have to face the problem of the migration time shift. High precision migration time data are needed for the calculation of the binding constant because the binding constant is calculated from the effective electrophoretic mobility of analyte. The variation of the capillary inner surface, which results in changes in EOF and thus the migration times, originates from the steady-state condition of the capillary, the buffer depletion and the wall adsorption. In order to study the effect of the buffer renewal on precision of the migration time and peak area, the sample of temozolomide (TMZ) was analyzed fifty times using a new capillary, while both inlet and outlet buffer solutions were renewed after every tenth injection. Due to the continuously increasing ionic strength and changing pH in the buffer vials (and therefore in the capillary) during the electrophoresis, TMZ migrated slower and slower. In case of a new capillary, the change of the migration times can be particularly significant. Using a new capillary during the first 20 runs, the increases in the migration times between the successive runs gradually decreased, and after around the 20th electrophoretic run the increases became constant. After getting a constant surface of the capillary, the buffer depletion had the highest influence on the precision of the measurements. After each tenth run (using the renewed buffer) a short decline in the migration times could be observed, but after a couple of injections the migration times had increased slowly again up to the next buffer renewal. The use of single or multiple (2-4) internal standards (IS) for the improvement of the repeatability has been studied. The best precision of the migration times could be reached with two ISs applying weighted correction, since it takes into consideration the distance of the internal standards from the analyte. There was no further improvement when more than two internal standards were used. I think that the application of more than two ISs for the migration time correction can be useful when several analytes appearing far from each other in the electropherogram should be corrected. In this case the properly selected ISs should cover the migration time window specified by the slowest and fastest analytes. **(J. Analytical Chemistry, 70, (11), pp. 1360-1367., 2015)**
3. The spread of protein based pharmaceuticals have accelerated in last decade in parallel with the spread of therapeutic antibodies. The electrophoretic techniques have become emphatic for the study of 150 kDA immunoglobuline. The great complexity and variability of biomolecules poses challenge for the analysts. The examination of

physicochemical parameters and biological activity with the help of a variety of techniques have become necessary. I have been studying monoclonal antibodies focusing on CE as a postdoctoral researcher since 2012. A new branch of analytics was developed by protein based biological engineered pharmaceutical products. This new field of science involves several different analytical techniques, hence it is crucial to perceive the methods and the results in a unified system. The role of CE is undeniable in characterizing the macromolecules. The applicability of CZE for the determination of the heterogenity of a therapeutic mAb, rituximab (Rtx) (MabThera, Roche) was studied. A buffer of pH= 5.2 with several additives (800 mM 6-aminocaproic acid, 2 mM triethylenetetramine and 0.05% hydroxypropyl methylcellulose) was used to suppress the interactions between the antibody and the capillary, thus even an uncoated fused silica capillary was applicable for the efficient separations of several charge variants and the main component. In order to support some conclusions obtained by CZE, CGE in sieving matrix separation was used. For instance, the charge variants of the main component that can be separated by CZE provide the same molecular size. It was found that the dilution of the pharmaceutical product, which contains stabilizing components (neutral detergent) caused a slow formation of acidic variants. Incubating the Rtx at higher temperature or basic condition, charge variants and fragments of the Rtx were formed by deamidation and fragmentation. The CZE analysis could reveal the charge variants of Rtx, while the CGE with sieving matrix gave information about the size heterogenity. **(Chromatography Separation Techniques, 6, (1), 2014)**

4. At the present gadolinium chelates are widely used as contrast agents (CAs) in Magnetic Resonance Imaging (MRI) to increase the difference between the contrast of healthy and diseased tissues. Since the constituents of the CAs, both the gadolinium-ion (Gd^{3+}) and the chelate forming ligands are toxic, the Gd^{3+} chelates must have high in vivo stability. However, some in vitro experiments indicated that endogenous metal ions, like Zn^{2+}, Ca^{2+} and Cu^{2+} may displace the Gd^{3+} within the complex, releasing the toxic free Gd^{3+}. Nephrogenic systemic fibrosis (NSF) is a rare and serious syndrome that involves fibrosis of skin, joints, eyes and internal organs and it is associated with the exposure to gadolinium (with gadolinium-based MRI contrast agents) in patients with severe kidney failure. In the development of NSF the first step is presumably the release of Gd^{3+} ions through the dissociation of CAs in transmetallation reactions. So for understanding the onset of the NSF we have to know the kinetics and mechanisms

of the chemical reactions, leading to the release of Gd^{3+} from the CAs. To detect the dissociation of [Gd(DTPA-BMA)] (Omniscan) and the formation of $[Ca(DTPA\text{-}BMA)]^-$, a serum sample spiked with 2mM Omniscan at pH 7.4 and 37ºC was investigated by micellar electrokinetic capillary chromatography (MEKC). The separation of [Gd(DTPA-BMA)] and $[Ca(DTPA\text{-}BMA)]^-$ is possible because these complexes elute before the SDS micelle protein complexes. At 30 h after spiking, the peak due to [Gd(DTPA-BMA)] is smaller, whereas that due to $[Ca(DTPA\text{-}BMA)]^-$ is larger, indicating the dissociation of Omniscan and the formation of $[Ca(DTPA\text{-}BMA)]^-$ complex in the serum. No products of transmetallation reaction of Omniscan with Zn^{2+} or Cu^{2+} in serum could be detected by the MEKC method. **(Chemistry A European Journal, 21, pp. 1-12, 2015)**

5. The therapeutic monoclonal antibody (Rtx) binds to CD20 B-cells. The B-cell suspension was transferred into PDMS (polydimethilsiloxane) based microfluidic chips. The interaction between Rtx and mAb could be followed due to the altered electrophoretic mobilities of the complex. The PDMS chips were prepared using a mold created by soft photolithography. The pattern consisted of a crossing of three 50 μm wide channels and one 150-200 μm wide channel. The separation channel consisted of a straight channel component and an arched segment for a total separation length of 8 cm. The suspension of B-cells was injected by pressure using a peristaltic pump. The injection method follows from the Hagen-Poiseuille law. The principle of the injection technique resembles the split injection often applied in gas chromatography. After the sample was split into the crossing area of the chip, and small volume of the original sample was manipulated into the separation channel, the peristaltic pump was stopped, and the electrophoretic separation initiated by applying 500 V to the ends of the separation channel. The mobilities of the cells are strongly influenced by the EOF formed due to surface charge of the PDMS channel. Due to the fluctuations in EOF and the inhomogenity of cells, poor precision of the CZE was obtained.

Published research papers:

1. Melinda Andrási, Gábor Lehoczki, Zoltán Nagy, Gyöngyi Gyémánt, Attila Gáspár, *A comparative study of capillary electrophoresis and isothermal titration calorimetry for the determination of binding constant of human serum albumin to monoclonal antibody*
Electrophoresis 36, (11-12), pp. 1274-1281., (2015), (IF: 3.161)
2. Melinda Andrási, Gyöngyi Gyémánt, Attila Gáspár, *Analysis of rituximab, a therapeutic monoclonal antibody by capillary zone electrophoresis*
J. Chromatography Separation Techniques, 6:1, 1000259, (2014), (IF: 1.780)
3. Zsolt Baranyai, Ernő Brücher, Fulvio Uggeri, Alessandro Maiocchi, Imre Tóth, Melinda Andrási, Attila Gáspár, László Zékány, Silvio Aime, *The role of equilibrium and kinetic properties in the dissociation of the Gd[DTPA-bis(methylamide)] (Omniscan) at near to physicological condition*
Chemistry A European Journal, 21, pp. 1-12., (2015), (IF: 5.731)
4. Melinda Andrási, László Zékány, Attila Gáspár, *Study on repeatability of the determination of temozolomide by micellar electrokinetic capillary chromatography using internal standards*,
J. Analytical Chemisrty, **70, (11), pp. 1360-1367., (2015), (IF: 0.479)**

Lectures and posters:

1. Melinda Andrási, Gábor Lehoczki, Zoltán Nagy, Gyöngyi Gyémánt, András Pungor, Attila Gáspár
Determination of Binding Constant of Human Serum Albumin to Monoclonal Antibody using Capillary Electrophoresis and Isothermal Titration Calorimetry
10th Balaton Symposium on High-Performance Separation Methods
Siófok, Hungary, 2015 (poster)

2. Melinda Andrási, Álmos Klekner, Attila Gáspár
Application of capillary electrophoresis for human health
15th Symposium and Summer School on Bioanalysis
Tirgu Mures, Romania, 2015 (lecture)

3. Zoltán Nagy, Dávid Nyul, Melinda Andrási, István Bányai, András Pungor
Preparation of antibody coated nanoparticles for high speed proteomics analysis
13th Human Proteome Organization Word Congress
Madrid, Spain, 2014 (poster)

4. Zoltán Nagy, Dávid Nyul, Melinda Andrási, István Bányai, András Pungor
Synthesis and affinity measurements of anti-HSA coated nanoparticles
2014 International Biotechnology, Chemical Engineering and Life Sciences Conference
Okinawa, Japan, 2014 (poster)

5. Melinda Andrási, Attila Gáspár
   Analysis of therapeutic monoclonal antibody heterogenity by capillary electrophoresis
   14th Symposium and Summer School on Bioanalysis
   Smolenice, Slovakia, 2014 (poster)

6. Andrási Melinda, Gáspár Attila
   Monoklonális antitest vizsgálata kapilláris elektroforézissel
   Elválasztástudományi Vándorgyűlés
   Egerszalók, Magyarország, 2014 (előadás)

7. Zoltán Nagy, Dávid Nyul, Melinda Andrási, István Bányai, András Pungor
   Immobilization of monoclonal HSA antibody on TiO_2 nanoparticles
   HISPRA Consortium Meeting
   Vienna, Austria, 2013 (lecture)

8. Melinda Andrási, Brigitta Törzsök, Álmos Klekner, Attila Gáspár
   Analysis of anticancer drug in real sample using capillary electrophoresis
   13th International Symposium and Summer School on Bioanalysis
   Debrecen, Hungary, 2013 (poster)

9. Melinda Andrási, Brigitta Törzsök, Álmos Klekner, Attila Gáspár
   Determination of temozolomide in serum and brain tumor with micellar electrokinetic capillary chromatography
   10th International Interdisciplinary Meeting on Bioanalysis
   Pécs, Hungary, 2013 (poster)