Analytical techniques and formulation strategies for the therapeutic protein alkaline phosphatase

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Chapter 8

Conclusions and perspectives

Pharmaceutical proteins are fundamentally different from the traditional low molecular weight pharmaceuticals in a number of ways. For small drugs a limited number of analytical techniques commonly suffices to reliably characterize them. However, proteins are much more complex, and assessing their characteristics (purity, identity, and conformation) is indeed a challenge. Proteins are constructed from L-amino acids (sometimes conjugated with sugar molecules) forming a sequence called the primary structure. The amino acid chain turns into random coils, alpha helices and beta sheets (secondaty structure) and folds in a three-dimensional shape (tertiary structure). Sometimes protein monomers join to form a quaternary structure. The activity of a protein, but also its potential toxicity/immunogenicity, depends on its structure. Therefore, protein unfolding, aggregation and interactions with formulation excipients are important quality parameters that ideally should be monitored. What also complicates matters is that pharmaceutical proteins may consist of several isoforms. Furthermore, pharmaceutical proteins may comprise (toxic) contaminants, e.g., proteinaceous entities, originating from the cell expression systems. Also, in formulation of biopharmaceuticals components such as buffers, stabilizers and other excipients further complicate the sample. This means that quality control of proteins often poses a mixture analysis problem requiring efficient separation techniques.

In this thesis, particular attention was paid to the potential of capillary electrophoresis (CE) for the analysis of a protein therapeutic. CE offers efficient and fast separations requiring only small sample amounts, and is highly useful for drug impurity analysis. Capillary zone electrophoresis (CZE) is the simplest and most popular form of CE, where separation is carried out using buffers with low ionic strength. Separation is based on differences in mass-to-charge ratios of the analytes being small molecules or large macromolecules such as proteins. The high efficiency of CZE allows separation of molecules with subtle differences in mass-to-charge ratio such as isoforms of glycoproteins. Also changes in protein charge as a result of, e.g., chemical degradation (e.g., oxidation or reduction) are reflected in the observed electrophoretic mobility. CZE was applied to the analysis of the protein alkaline phosphatase (AP), which

potentially is useful for the treatment of sepsis. In this thesis, the utility of capillary electrophoresis, alone and in combination with other techniques, to give information about AP and other protein containing samples was evaluated.

In chapter 2 and 3 the employment of several analytical techniques to monitor the purification and stability of AP is illustrated. It was revealed that the combination of CZE, MALDI-TOF-MS, capillary gel electrophoresis and enzymatic activity assays is a powerful array of techniques that is able to elucidate the progress of an isolation and purification procedure for AP involving ion-exchange chromatography followed by affinity chromatography. In fact, the combination of these analytical techniques showed that ion-exchange chromatography alone was insufficient to achieve a fully purified protein (see chapter 2). The potential of CZE for the monitoring of the stability of placental alkaline phosphatase was also established. AP that was exposed to various harsh conditions, i.e. acidic and basic pH and freeze-drying, lost its enzymatic activity in a manner that was reflected in the appearance of the recorded electropherograms. In fact, after freeze-drying and storage of AP only minor changes in enzymatic activity were observed, while analysis by CE revealed the formation of new entities, probably aggregates (see chapter 3). So exposure of the protein can lead to changes in the observed peak pattern in CE-analysis, although they are not always correlated to changes in activity and vice versa.

The utility of CE for purity and stability analysis of AP was demonstrated. Still, CE offers some additional characteristics for the analysis of pharmaceutical proteins that are worthwhile to be further explored. CE is performed in simple buffers and the high electric field strengths do not affect the protein structure. This means that denaturation or unfolding caused by organic solvents and or high salt concentrations or interaction with stationary phases (like frequently occurs in liquid chromatography) is eliminated. In other words, CE allows detection of proteins in their native state, and thus gives a 'true' picture of the protein in the analyzed sample. In addition, CE in itself can provide information on the structure of the protein. The CE separation mechanism is governed by electrophoretic mobility which also is a function of size of the ionic species. This means that changes in protein size (unfolding/aggregation) in principle could be probed by CE .

To gain more specific information about proteins than what can be retrieved by a UV-detector, combination of CE with mass spectrometry

(MS) is indicated. In chapter 4, it was found that direct coupling of CZE to MS using "normal" CE buffers can be performed. Generally, it has been believed that the buffers commonly employed in CZE, e.g., phosphate buffers and borate buffers, are incompatible with electrospray ionization (ESI) MS and that electrolytes such as acetic and formic acid as well as acetate and formate buffers are the preferred choice. Unfortunately, these often do not yield an optimal performance during CZE. Nevertheless, the findings in this thesis show that borate buffers in particular do not lead to serious ion-suppression effects in the CE-MS of intact proteins. As the influence of only a few electrolyte systems on the performance of CE-ESI-MS was evaluated, the study ought to be expanded to several more buffers over a large pH-interval. This would potentially lead to a tool box of buffer systems to choose from depending on, e.g., the properties of the protein of interest.

It was also shown that MS detection can be used for molecular weight determination and identity confirmation of intact proteins. The work also demonstrated that the loss of the heme group of myoglobin could nicely be followed by CE-MS, thereby touching upon an interesting additional feature of ESI-MS to reveal protein conformational information. Mass spectra of proteins recorded using electrospray ionization (ESI) typically show a number of bands that correspond to differently charged protein molecules. As this charge state distribution reflects the state of the protein in solution and as the conformational state of the protein affects the charge state, ESI-MS could be used to characterize the folding of the protein in solution. An unfolded, denatured protein in solution leads to the formation of higher charge states than the same protein in its native, tightly folded configuration. Clearly, the acquisition of this kind of information on proteins separated by CE, can be very useful for the characterization of protein therapeutics.

Due to the very high resolving power of CE, changes in a protein affecting the mass to charge ratio can be detected. Although, it may be easy to separate degraded protein from non-degraded, it is not possible to determine what part of the protein has changed. To determine the reacted amino acid side-chains, it would potentially be advantageous to perform a protein digestion using trypsine followed by, e.g., CE-ESI-MS. Comparing the peak pattern of the peptides from digests of a fresh protein to that of degraded can give information on what part of the protein has been modified.

The analysis of therapeutic proteins in a formulation, e.g., a solid dosage form, is arguably still a challenge for CE. Therapeutic proteins are usually very potent, so that rather low amounts are present in the samples requiring sensitive detection. Also, the samples usually contain stabilizers (buffers and salts) which could affect the separation efficiency. So, for the effective characterization of formulated protein pharmaceuticals, preconcentration and desalting procedures might be required prior to CE analysis.

In the second part of this thesis (chapter 5-7) investigation into the formulation aspects of AP are described. Proteins are produced as aqueous solutions. A disadvantage is that most proteins are not stable in solution which limits their shelf life. In the dry state, however, proteins are generally much more stable. Therefore, drying a protein solution would give the possibility to store the protein for perhaps years even at room temperature without loss of biological activity. Stabilization of proteins in the dry state can be achieved by freeze-drying of a solution containing the protein and a sugar, e.g., trehalose (in this thesis inulin). For optimal stabilization it is essential that after drying the sugar is in the glassy state. Usually, to keep the pH constant also the presence of a buffer in the solution is required. It has been found that phosphate buffers should be avoided, since during freezing large pH-shifts occur due to the precipitation of one of the buffer salts. Such changes in the pH may lead to degradation, aggregation or denaturation of the protein. However, the properties of high-pH buffers during freeze-drying and their impact on the stability of proteins have not yet been investigated systematically. In this thesis, the high pH buffers, ammediol, borax, CHES, TRIS and Tricine, have been evaluated (chapter 5). It has been found that the pH of the solutions containing these buffers did not change substantially during freezing. However, during freeze-drying ammediol, CHES, TRIS and Tricine were incorporated monomolecularly in the sugar glasses which strongly depressed the Tg of the resulting glasses. Furthermore, borate formed complexes with sugars. Both Tg depression and complex formation could have a detrimental effect on the stability of the incorporated AP.

In the literature it is often claimed that optimal stability is achieved if the protein is freeze-dried from solutions containing disaccharides. In particular, trehalose is recognized as the ultimate stabilizer. Oligo- and polysaccharides are generally considered as poor stabilizers. In

contradiction to this claim, in this thesis it is found that the application of the oligosaccharide inulin as a stabilizer for AP during freeze-drying and storage is preferred over trehalose, especially when the freeze-dried material is exposed to humidified air.

Investigations into the oral administration of proteins are rarely addressed in the literature. Therefore, tablets were made from AP incorporated in sugar glasses of trehalose or inulin. It was found that the compaction properties of the trehalose sugar glasses were poor. Moisture uptake from the air cause severe handling problems. Moreover, during processing, compaction induced crystallization of the sugar occurred which was accompanied with a complete loss of protection of the incorporated AP. In contrast, inulin sugar glasses showed no such behaviour and tablets with adequate tensile strength and friability could be produced without any loss of enzymatic activity (see chapter 6). In vitro experiments revealed that tablets prepared from inulin stabilized AP and provided with an enteric coating showed the required dissolution behaviour, i.e. they did not dissolve at pH 1 (pH of the stomach) but rapidly dissolved at pH 6.8 (pH of the intestines). The tablets were given to rats through oral administration (chapter 7). Evaluation of the intestines revealed that with the inulin glass technology AP can be delivered to the intestinal system via oral administration.

It was found that adequate stability of the therapeutically interesting protein AP can be achieved through freeze-drying with the oligosaccharide inulin. Consequently, it would be interesting to evaluate the versatility of inulin as a stabilizer. That is, to evaluate the stabilization of not only other proteins but also other labile drug substances like liposomes and viruses by inulin during freeze-drying and storage. Spray-drying is another drying technique worthwhile to evaluate because it can yield particles of 1-5 μm. Particles of these sizes are optimal for pulmonary delivery. Drug therapy via inhalation can be highly relevant for pulmonary diseases, e.g., the labile protein DNzse can be used as a mucolytic agent in cystic fibrosis patients.

Another issue is the mechanism of stabilization by inulin. As mentioned earlier, oligosaccharides do generally act as poor stabilizers. Obviously, inulin is an exception. A unique property of inulin is its high chain flexibility in solution. It can be hypothesized that during drying the flexible inulin is smoothly wrapped around the surface of the drug substance. The resulting tight covering of the drug substance may be highly relevant for optimal stabilization. Therefore, evaluation of the stabilizing

capacities and sugar/drug interactions of various oligosaccharides differing in chain flexibility may reveal the validity of the above mentioned hypothesis.