NEW METHODS FOR QUALITY CONTROL OF PROTEIN PHARMACEUTICALS

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To our knowledge mass spectrometry as well as flow cytometry are not widely used as methods for routine lot-to-lot testing of protein pharmaceuticals. Versatility of both methods in a quality control environment is shown and advantages in comparison to other methods are discussed.

Recent developments in mass spectrometry have pushed characterization of proteins to new frontiers. We see considerable potential of this method for routine protein identity testing and heterogeneity profiling. MALDI-MS was used over several years for rapid identity confirmation of incoming materials. For proteins below 20 kD mass accuracy of ± 1 D and long-term precision of 0.007 % was readily obtained using standard commercial equipment. Employment of MS/MS techniques with or without prior protein cleavage extends this application to the identification of monoclonal antibodies.

Fluorescence activated cell sorting (FACS) can be used to discriminate labelled from unlabelled cells as well as vital from apoptotic or dead cells.

We have developed and validated a method to monitor binding activity of antibodies to target cells expressing antigen by means of competition with fluorescent dye labelled antibodies. Assay design is straightforward, no washing steps are required and total assay time (starting from cultured cells with suitable density) is about 4 hours. Using automated incubation and plate handling, the FACS system can be run overnight improving overall output to about 50 samples per week. Very nicely S-shaped binding curves are obtained for standard and sample which allow for easy curve fitting and relatively precise IC50 determination. A typical intermediate precision in the range of 10-15 % was shown during method validation.

In addition we have developed the FACS method to determine the potency of antibodies to induce apoptosis in suitable target cells using the nuclear dye Yo-Pro. Again well-shaped activity curves are obtained that allow for easy IC 50 determination. Method validation aspects are discussed.

SHIFTING PARADIGMS: BIOPHARMACEUTICALS VERSUS LOW MOLECULAR WEIGHT DRUGS

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Biopharmaceuticals are pharmaceutical products consisting of (glyco)proteins. Nowadays, a substantial number of the FDA approved drugs belong to this class of drugs. The first generation of biopharmaceuticals (the name biopharmaceuticals was given much later) replaced animal or human cadaver derived pharmaceutical proteins such as insulin and human growth hormone; these drugs entered the market around 1985. Later, not-earlier-marketed, endogenous or almost endogenous biopharmaceuticals were successfully launched, among those interferons, interleukins and different growth factors. The clinical and economic importance of the group of monoclonal antibodies is illustrated by the observation that at present time well over 60 billion euros are annually spent on biopharmaceuticals and this number is growing fast.

Biopharmaceuticals deserve special attention as they have a number of characteristics that set them aside from low molecular weight drugs. Their activity depends on their complicated geometry based on secondary, tertiary and (sometimes) quaternary structures. As the conformational structure of a protein is easily disturbed, formulation and handling of biopharmaceuticals needs special attention in order to optimize the therapeutic effect and to minimize adverse reactions, among which immune responses.

The pharmaceutical community had to get accustomed to the idea that these structures can not be fully defined with our present set of analytical techniques and approaches for potency testing. They often are the same as (or closely resemble) endogenous proteins. This means that in safety testing and clinical test programs questions have to be addressed regarding species specific responses, selection of dosing schedules and the possible occurrence of immunogenicity.

Finally, with a few exceptions, all biopharmaceuticals are administered via the parenteral route: ‘through the needle’. As the biopharmaceuticals grew in number and importance, the need for alternative (needle-free) routes of administration increased as well. Therefore, alternative routes of administration for biopharmaceuticals were studied for years. But, at present the needle is still the administration tool of choice.

In conclusion, biopharmaceuticals are complex molecules that can not be characterized fully in terms of their structure like low molecular weight drugs. The performance of biopharmaceuticals relies on strict production protocols and close monitoring of their activity in the clinical situation. And, …….. the needle is still around…..

Biologicals:
Cold Chain and Temperature Control
from Product Design to Finished Product

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Many biological products need to stay within a well controlled temperature range during storage, transport and handling to assure their efficacy and safety are not impacted by inadequate conditions.

Already at the stage of product design the first considerations need to be given to the later temperature requirements of a product for optimal stability all through shelf life. This refers as well to the containers as to excipients to be employed.

The data established during development and in stability studies will then determine what kind of temperature range is finally acceptable.

Any finished product needs to stay within its defined conditions not only during storage at the production facility, but also during shipment from the production site through the distribution channel until it reaches its point of use. There are different ways how to assure, assess and evaluate the actual transport conditions for this purpose.

SURFACE DISPLAY AS A TOOL FOR THE ANALYTICS AND THE EVOLUTIVE DESIGN OF BIOLOGICS

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Display of heterologous proteins on biological surfaces has become a common and powerful tool for probing the interactions of macromolecules with interesting chemical or pharmaceutical properties. The application of surface display technologies has led to significant advances in protein engineering, drug discovery, vaccine development, molecular evolution and ligand optimization. As an alternative to the most commonly employed phage display systems, a bacterial display system has the advantage of being self-replicative, thus enabling simple multiplication of cells and stringent selection for a desired binding phenotype by HTS methods, as e.g. flow cytometry.

We use autodisplay [1], an efficient surface display system in *E. coli*, for setting up new assays for the detection and the quantification of biologics. Far beyond from being a standard lab method, autodisplay was applied for the development of ELISAs to screen patient sera, for signal amplification in SPR biosensor detection of antibodies and for the surface display of functional antibodies fragments for diagnostic purposes. Moreover, autodisplay of functional antibody fragments laid the track for developing combinatorial libraries, that could subsequently screened by a new tumor antigen. A specific antibody fragment was identified and cells displaying this fragment could be used for tumor cell targeting. A similar strategy was applied for the evolutive design of small peptide ligands, e.g. enzyme inhibitors. Taking together, autodisplay could be an interesting tool for the development and for the detection of biologics or small protein drugs.

[1] Jose, J., Meyer, T.F. (2007) *Microbiol Mol Biol R*, 71:600-619.

THE PITFALLS OF FILL & FINISH - INTERACTIONS OF BIOLOGICS WITH PACKAGING MATERIALS

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It must be assumed that aseptically pre-filled injection systems i.e. syringes, cartridges and vials will remain – at least for a certain time – the predominant application systems for sensitive Biologicals like monoclonal antibodies, growth hormones, and proteins.

Alternative approaches like nasal or inhalative applications, “painless” needle free systems or transdermal application with micro-needles have not yet proved to be robust and nearly all fell short of the initial expectations.

The application systems mentioned above consisting of a more or less siliconised glass barrel, and rubber or other elastomer closing parts are frequently and incorrectly considered to be inert. This may be the case in the majority of applications, but there a numerous examples of incompatibilities and interactions of biological APIs with primary packaging materials and/or with components used in the manufacturing process that induced degradation or side reactions or that introduced unwanted impurities into the products. The impact of and strategies how to control extractables and leachables intake from elastomers have long been discussed and are common knowledge today. However, from every now and than there are some spectacular examples of interactions between Biologicals and surfaces of primary packaging materials or process equipment popping up.

Some of these examples are presented here, including interactions between the API and siliconization, elastomers, but also the “naked” glass.

PROTEIN ANALYSIS USING GEL ELECTROPHORESIS: PRECISION AND PERFORMANCE

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Gel electrophoresis (GE) is known for its often unsatisfactory precision. In quantitative analyses percental relative standard deviations (RSD%) up to 60% have been reported. Therefore, an improvement of precision is a primary concern using GE, especially for the quality control of biological pharmaceuticals. Potential major sources of variability for this technique include staining or rather detection of separated proteins, the transfer between first and second dimension (2-DE) and the analyst's expertise. In our works, the remarkable and completely irregular changes of the background signal from gel to gel were identified as one of the governing error sources. These background changes can be strongly reduced by using a signal detection in the near-infrared range. This detection method provides the most sensitive approach for conventional Coomassie stained gels which is reflected in a total error of just 5 - 10% RSD% with a quite good signal-to-noise ratio for simple one-dimensional separations. A further alternative is the direct detection of separated proteins by utilising their native fluorescence. More than a threefold better signal-to-noise ratio was found compared to Ruthenium-(II)-tris-(bathophenanthroline disulfonat) (RuBPS) and Coomassie staining, although the sample was used in an 800-fold lower concentration. This improvement together with well-defined peaks resulted in a better quantitative spot reproducibility of approximately 12 – 16% RSD%. Possibly the variabilities due to detection and evaluation are already reduced to minor error components here. According to the law of error propagation, the major error sources dominate the total error. Other sources of variability such as sample preparation, strip rehydration, protein loading, transfer between dimensions, interactions between gel and proteins, gel scanning and spot integration have to be reduced next.

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF PROTEINS BY MASS SPECTROMETRIC METHODS

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Pharmaceutical research is being driven to improve the rate of new drug discovery. Using the three-dimensional structures of proteins and protein complexes as a template for drug design presents an important component of such improved discovery methods. High-resolution structural analysis of proteins is currently accomplished by NMR spectroscopy and X-ray crystallography. These techniques, however, are time- and material-consuming methods and moreover, they are not applicable to all proteins or protein complexes. A promising strategy with the potential to obtain low-resolution structural information on minute amounts of proteins within a few days is based on a combination of chemical cross-linking and mass spectrometry [1,2]. Using this approach, we determine low-resolution three-dimensional structures of proteins and protein-ligand complexes. The general procedure involves conjugation of functional groups in proteins and drugs with various cross-linkers followed by enzymatic proteolysis of the cross-linking products. The highly complex peptide mixtures are subsequently analyzed by high-resolution mass spectrometric techniques (MALDI-TOF/TOF-MS, ESI-LTQ-Orbitrap-MS). Based on the distance constraints derived from the chemical cross-linking data in combination with computational methods, we are able to generate three-dimensional structure models of the proteins. This integrated approach is likely to have wide ranging implications for structural studies of protein-ligand interactions.

[1] Sinz, A., *Mass Spectrom. Rev.* 25, 663-682 (2006)
[2] Sinz, A., *Angew. Chem.* 119, 670–673 (2007)