Polysorbates, peroxides, protein aggregation, and immunogenicity – a growing concern.

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Review Article

ABSTRACT

Aggregation can have a number of deleterious effects on biotherapeutics including the loss of efficacy, the induction of unwanted immunogenicity, altered pharmacokinetics, and reduced shelf life. Aggregation is ameliorated by the inclusion of surfactants in biotherapeutics formulations, typically non-ionic polymeric ether surfactants. The most commonly used examples are Tween® 20 (Polysorbate 20) and Tween® 80 (Polysorbate 80). Others include Triton™ X-100, Pluronic® F-68, Pluronic® F-88, Pluronic® F-127 (poloxamers), and Brij 35 (polyoxyethylene alkyl ether). The usefulness of polysorbates, in particular in preventing protein aggregation in biotherapeutic formulations, is well accepted. However, polysorbates contain ether linkages and unsaturated alkyl chains that have been shown to auto-oxidize in aqueous solution to protein-damaging peroxides and reactive aldehydes including formaldehyde and acetaldehyde. The peroxides principally affect methionine and tryptophan moieties. The aldehydes react with primary amino groups on proteins and are known to induce immunogenicity of proteins in the absence of aggregation or adjuvants. Detection of protein aggregation and prevention of aggregation using polysorbates is relatively straightforward using light scattering or size exclusion chromatography methods. Detection of oxidative damage to amino acyl moieties or increased immunogenicity resulting from the reaction of biotherapeutics with the degradation products of polysorbates is considerably more difficult and has generally been ignored in the scientific literature. As an increasing number of biotherapeutic agents come into use in common clinical practice, including both as innovator and as biosimilar products, these latter issues will come under increased scrutiny. Substitution of non-ionic, non-ether-based surfactants, could offer significant improvements in stability, reduced immunogenicity, and shelf life, and represents a significant unmet need in the field of biotherapeutics formulation.

KEY WORDS: Polysorbate, alkylsaccharide, peroxides, oxidation, immunogenicity, biotherapeutics, biosimilars

INTRODUCTION

Non-ionic surfactants are widely used in the formulation of protein pharmaceuticals to prevent aggregation and minimize surface absorption of proteins, both during the manufacturing process and in the final product formulation. Manufacturing processes such as filtration, pumping, lyophilization, agitation, and concentration may lead to aggregation. Since aggregation is concentration-dependent, frequently encountered concentration proces-

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ses, such as ultrafiltration, affinity chromatography, selective absorption chromatography, ion exchange chromatography, lyophilization, dialysis, and salting-out are all potential sources for unwanted aggregation during manufacturing. In addition to reducing manufacturing yield and increasing production costs, for example as a result of membrane fouling caused by protein aggregates, aggregation can compromise product performance by altering the pharmacokinetic or pharmacodynamic profile of the drug by reducing bioavailability, thus reducing the overall efficacy.

Among the most serious problems resulting from protein aggregation is the induction of unwanted immunogenicity (1). Such immunogenic responses may result in a decrease in the therapeutic efficacy of the polypeptide or worse. Neutralizing antibodies to interferon beta used to treat multiple sclerosis, for example, result in a higher relapse rate and more disease activity as measured by brain MRI scans (2, 3). Antibodies developed against recombinant erythropoietin (EPO) have been shown to produce a life-threatening condition known as 'pure red cell aplasia' in some patients (4) which is a potentially fatal side effect. In yet another example, 15%-30% of hemophilic patients treated with recombinant human Factor VIII (rFVIII) developed inhibitory antibodies toward this essential clotting factor. In the case of hemophilia A, neutralizing antibodies to Factor VIII can cause life-threatening bleeding episodes resulting in significant morbidity and necessitating treatment with a prolonged course of a tolerance-inducing therapy to reverse immunity (5-7).

As a result of the serious nature and unique problems associated with the development of unwanted immunogenic responses to protein therapeutics, the concerns of the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) and other regulatory agencies about protein aggregation have significantly increased. For obvious reasons, biopharmaceutical companies have greatly increased efforts to understand and control or eliminate aggregation since, by preventing protein aggregation, surfactants can reduce or potentially eliminate unwanted aggregation-associated immunogenicity.

Among the most widely used non-ionic surfactants in protein formulation are the polysorbates (PS20) and (PS80) also known as Tween® 20 and Tween® 80 respectively. For example, it is estimated that more than 70% of the marketed formulations of monoclonal antibody products contain one or the other of these two surfactants. PS20 and PS80 are comprised of mixtures of structurally-related fatty acid esters of polyoxyethylene sorbitan and lauric acid or oleic acid, respectively. In the case of PS20, the monolaurate fraction comprises 40% to 60% of the alkyl chains, with alkyl groups of different chain length making up the remainder of the molecules. In the case of PS80, approximately 60% of the alkyl chains are derived from oleic acid with the remainder of the esters derived from other fatty acids (8). In addition, the commercial products contain measurable amounts of polyoxyethylene, sorbitan polyoxyethylene, and isosorbide polyoxyethylene fatty acid esters (9-11).

It has long been known that polysorbates undergo intrinsic degradation via auto oxidation yielding reactive peroxides, as well as by hydrolysis (12-17). Less attention has been paid to the formation of reactive aldehydes such as formaldehyde and acetaldehyde (18) known to induce immunogenicity of soluble proteins as discussed in greater detail below.

SURFACTANTS ARE ESSENTIAL

Surfactants are essential components of many protein formulations. They provide for the development of manufacturable and stable dosage forms and allow the management of a broad range of physical instability phenomena such as protein-protein association, aggregation, precipitation, and interaction with container surfaces. Monoclonal antibody therapeutics provide a particularly stringent

challenge because they usually require a relatively high dose (1 to 2 mg/kg) and so need to be concentrated sufficiently to allow for reasonable administration volumes in a variety of settings such as in hospitals through intravenous injection or infusion or at home through subcutaneous injection. Monoclonal antibody therapeutics have been shown in some cases to become inactive as a result of protein aggregation.

The types of aggregates that can be encountered in biotherapeutic formulations can vary in size and can include soluble, insoluble, covalent, noncovalent, reversible or irreversible aggregates. They may be visible or may be present as subvisible particles. The principal concern with unwanted aggregation in protein formulations is the induction of unwanted immunogenicity. As already stated, two of the most commonly used surfactants to prevent aggregation in biotherapeutic formulations are PS20 and PS80 (19). For example, these particular nonionic surfactants are used to stabilize certain monoclonal antibodies including Rituxan®, Remicade®, ReoPro®, and Humira® as well as the dimeric Fc fusion protein Enbrel®. Pluronic® F127 and F68 have been used to stabilize recombinant human growth hormone (rhGH) against aggregation both in the daily injectable, as well as, in the extended action formulation (20, 21). Other nonionic surfactants used in pharmaceutical formulations include Triton™ X-100, Pluronic® F-68, F-88, and F-127 (poloxamers), Brij 35 (polyoxy-ethylene alkyl ether), polyoxyl stearate 40, Cremophor® EL, and alpha-tocopherol TPGS. Each of these surfactants have in common the fact that they all contain polyoxyethylene moieties and thus to a greater or lesser extent, exhibit a similar problem, in that the polyoxyethylene moiety auto oxidizes to produce reactive peroxides along with other chemically reactive species discussed later. This creates somewhat of a conundrum since both aggregation, which is prevented by the addition of surfactants, and peroxide damage, which is caused by surfactant-generated peroxides cause an increase in unwanted protein immunogenicity. The recently issued "FDA Guidance for Industry" on biotherapeutics/biosimilars (22) has highlighted the serious concern over immunogenicity and the need to quantitate relative immunogenicity of biosimilars in comparison to the innovator biotherapeutic in order to ensure safety and efficacy, thus elevating the visibility of the polysorbate peroxide damage issue across the industry.

IMMUNOGENICITY OF PROTEINS

Unmodified native proteins in their natural environment are most often non-immunogenic. Any disruption of the native structure however, for example by chemical modification or denaturation due to mechanical, chemical, or temperature-induced physical insult, results in a partially or significantly denatured structure which can induce an unwanted immune response. The degree and chemical type of glycosylation of a biotherapeutic as well as the site or method of administration may contribute to unwanted immunogenicity as well, but the major sources of unwanted immunogenicity remain aggregation and denaturation due to oxidative damage and chemical modification by other reactive species such as aldehydes. Proteins that are administered on a regular basis, such as insulin, frequently give rise to an antibody response in patients. Fortunately, antibodies developed against insulin do not generally neutralize insulin's biological effectiveness. For beta interferon, which must be administered regularly for the treatment of multiple sclerosis, neutralizing antibodies typically develop slowly over a period of years incrementally diminishing the effectiveness of this very important drug (3). At the extreme end of the spectrum, the development of antibodies against the hematopoietic protein erythropoietin can result in very serious adverse effects including the death of the patient.

AUTOXIDATION OF POLYETHER SURFACTANTS

The accumulation of peroxides in polyether–containing surfactants has been known since the 1970's (13). The generation of peroxides, hydroperoxides, and carbonyl compounds such as formaldehyde and acetaldehyde (23, 24) from the autoxidative degradation of the polysorbate surfactants appears to result from reactive radical formation at both the polyoxyethylene and the olefin sites within the molecules. PS80 is more prone to the generation of oxidative species compared with PS20 as a result of the greater content of unsaturated alkyl side chains in PS80 (25). In aqueous formulations polysorbates can also hydrolyze with an apparent half-life of five months at 40°C (12, 26, 27). The degradative changes are accelerated by light and increased temperatures (17). There is even evidence of degradation in previously unopened commercial samples of polysorbates (12). Literature studies report that the oxidation of polysorbates is greatly accelerated once placed into aqueous solution (15).

In practice, efforts to control the level of residual peroxides in polysorbate-containing solutions, including degassing and filling under nitrogen or inclusion of antioxidants, such as cysteine, glutathione, tryptophan, or methionine (28) may be technically effective, but perhaps inconvenient, if not impractical. It has been reported that, in some instances, interactions with the protein can cause further degradation or precipitation. Reactive carbonyl containing compounds such as formaldehyde and acetaldehyde can cause contact allergies as has been reported for technical grade PS80 (29). Physical methods for removing peroxides such as vacuum drying have been described (30). However, since autoxidation is spontaneous and may subsequently be detected after several weeks, these are short-term solutions (31).

POLYSORBATE INDUCED OXIDATIVE DAMAGE TO PROTEINS

Oxidative damage caused by polysorbates in biotherapeutic formulations resulting from peroxides, as well as other reactive chemical species, as described above can occur, in the presence of air, in both the liquid and solid states(15). The principal sites of oxidative damage within proteins are the methionine and tryptophan moieties (32-34). While most attention has been focused on protein damage resulting from polysorbates in aqueous solution, oxidation has been shown to occur in the solid state during the annealing process in lyophilization suggesting that the impact of an annealing step for formulations containing polysorbates needs to be carefully assessed during process development. The resulting damage is mediated through aggregation and denaturation. While aggregation alone has been found to be sufficient to increase immunogenicity, oxidation has been shown to exacerbate this problem (35). Oxidation can be a serious problem since proteins that are generally sensitive to oxidative degradation are often formulated at relatively low concentrations. As an example, the dual effect of Tween® 80 on the stability of IL-2 has been described by Wang *et al.* (36), in which the authors demonstrated that the initial prevention of aggregation induced by shaking with Tween® 80, was followed by the subsequent oxidation and generation of aggregates during storage for over 2 months at 40°C. Other examples include oxidative degradation of recombinant human ciliary neurotrophic factor (rhCNTF) in solution (37) and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in solution during storage (38) by the residual peroxides in Tween® 80. While there has been considerable focus on aggregation as a source of increased immunogenicity, chemical modification of proteins through the reaction with aldehydes is a well-known and highly potent source of increased immunogenicity (39-42) Reactivity with formaldehyde and acetaldehyde can occur at the epsilon amino groups of lysine or at the N-terminus and such chemical modifications have been shown to be potent inducers of protein immunogenicity even in the absence of aggregation or the addition of an exogenous adjuvant.

IMMUNOGENICITY CONCERNS AND FDA GUIDANCE TO INDUSTRY

The development of anti-drug antibodies following the administration of protein therapeutics is a very serious and growing concern among manufacturers, physicians, informed patients, and regulatory bodies alike (43). Anti-drug antibodies may have a neutralizing capacity through steric or allosteric interference at the site of biological action thus interfering with the efficacy and safety of the drug. Anti-drug antibodies may also accelerate clearance from the systemic circulation (44). Of equal, or potentially greater concern, is the increasing identification of auto-antibodies (i.e., antibodies developed against naturally occurring proteins in naïve patients) (45,46). Monoclonal antibody therapeutics pose a particular challenge since they typically contain from 2 to 8 aggregation-prone structural motifs (47).

For hormone-related biotherapeutics, for example, insulin, GLP-1 analogs, human growth hormone, the interferons, hematopoietic proteins, and the like, development of auto-antibodies may further compromise the ability of the patient's own critical residual biological effector proteins to function. While acquired pure red cell aplasia (4, 48) resulting from the administration of certain formulations of recombinant erythropoietin is perhaps the best known problem, one of the most studied examples, is the development of immunogenicity of beta interferon resulting from protein aggregation and the reduction in immunogenicity by prevention or reversal of aggregation (33,49,50).

In 2009 the Committee for Medicinal Products for Human Use (CHMP) at the EMEA issued marketing authorization guide-lines for general recommendations on how to assess an unwanted immune response following the administration of a biotherapeutic drug (51). The recently issued FDA guidance to industry document titled "Guidance for Industry - Scientific Considerations in Demonstrating Biosimilarity to a Reference Product" (22) further highlights the importance of, and requirement for, actual clinical immunogenicity assessments to be made to evaluate potential differences between a new biotherapeutic and the corresponding reference product. It is essential to establish that there are no clinically meaningful differences in the immune response between a biotherapeutic and the established reference product because immune responses may affect its safety and efficacy, for example by altering the pharmacokinetics and pharmacodynamics, thus causing anaphylaxis, or by developing neutralizing antibodies, as well as, associated native or endogenous proteins. In some cases both pre-market, as well as, post-market studies, may be required to detect what may be subtle, but very important differences, in the immunogenicity of new biotherapeutics. Thus, the selection of an appropriate non-damaging surfactant for a biotherapeutic formulation can have a great economic impact on the development process and ultimate success of new biotherapeutic products.

Concepts surrounding regulatory treatment of biosimilars are still in the formative stage both at the FDA and at the EMEA. The assessment of the equivalence of different excipients in the stabilization of biotherapeutics has not been well defined but will necessarily be an integral part of the regulatory landscape. For example, in the case of polysorbates, most studies carried out to date, have looked at the prevention of aggregation as the definitive endpoint. However, it is becoming clear that the prevention of aggregation, while an important indicator of likely reduced immunogenicity and, a necessary performance requirement, other indicators will likely be included in future testing requirements, such as measuring direct damage of the protein due to oxidation upon storage, for example by examining the destruction of methionine and tryptophan, or measuring increased immuno-genicity caused by aldehydic haptenization which will require in vivo assessment.

According to the recently issued FDA Guidance For Industry (22), the FDA indicated that it shall consider the "totality of the evidence" in determining bioequivalence of subsequently developed biosimilars. Since factors such as biological activity and immunogenicity (or lack thereof) will be assessed during the course of preclinical and clinical development, the inclusion of a novel excipient, in particular one that imparts superiority to the final product with respect to immunogenicity and potential stability or extended shelf life, would seem to fall under the umbrella of "totality of the evidence". It is an open question as to how the FDA or the EMEA will view the continued use and, possible replacement, of polysorbates with alternative non-ether surfactants in clinical practice, as they become available in the future.

ALTERNATE EXCIPIENTS

The potential problems associated with the use of polysorbates and other polyoxyethylene containing surfactants are clear, as is the need for alternative surfactants that can meet the need for the prevention of aggregation, while not inadvertently introducing to the biotherapeutic new unintended problems. A new class of non-ionic, non-ether-based surfactants that offers significant improvements in stability and reduced immunogenicity, has recently been reported (49, 52). Alkylsaccharides are non-ionic surfactants comprising a sugar moiety coupled to an alkyl chain. A broad range of such molecules have been studied extensively as transmucosal absorption enhancers (53-55). More recently, it was discovered that some of these molecules are highly effective in preventing aggregation for an increasing number of protein molecules.

There are a variety of options in creating the chemical linkage between the sugar moiety and the alkyl chain. Glycosides, esters, thioesters, or amides are among the choices that have been examined. For pharmaceutical applications esters and glycosides offer the advantage of containing no hetero atoms such as nitrogen or sulfur thus metabolizing quickly and cleanly to the free sugar and the corresponding long chain fatty acid, or corresponding alcohol. Specific examples include sucrose esters such as sucrose mono-dodecanoate, and the glycosides formed between maltose and C8-C16 long chain alcohols (alkyl maltosides). Both sucrose esters and alkylmaltosides are highly stable at physiologically acceptable pH values below pH 7. The glycosides are also stable above pH 7 while the sucrose esters undergo very slow hydrolysis above pH 7 at increased temperatures. The alkylmaltosides have a further advantage in that they may be prepared in a pure isomeric form either in the alpha or beta anomeric configuration whereas in the case of sucrose esters preparation of a single species is more difficult. Sucrose esters contain three primary hydroxyl groups, sites where esterification can take place in a facile manner, the C-6, C-6' and C-1' positions, along with five secondary hydroxyl groups that can also react, but to a much lesser extent, to form esters. Thus, while the structure of sucrose monoesters is often represented as a single species formed at the C-6 position, in actual fact, commercial samples of sucrose mono- dodecanoate contain a mixture of esters formed at the three primary hydroxyls. This is not necessarily problematic from a regulatory perspective, especially when one considers that the polysorbates contain a mixture of saturated, as well as, unsaturated alkyl chains of varying lengths and yet, have widely been accepted as pharmaceutical excipients. However, different reaction conditions can lead to different populations of the three dominant ester species.

In a study using dodecyl maltoside, it was demonstrated that the administration of beta interferon does not elicit an immune response in the Experimental Allergic Encephalomyelitis (EAE) animal model of multiple sclerosis whether administered by injection or intranasally. In contrast, beta interferon administered either by injection or intranasally in the same animal model resulted in a substantial immune response over the course the 30-day study (49). Both nasal and injected

interferons 1a and 1b were effective in reducing neuronal damage. Interestingly, nasally administered beta interferon appeared to exert a substantially increased pharmacodynamic effect (five-fold) compared to the injected interferon. Studies on stabilization of other proteins under various solution conditions using simple right angle light scatter have also been reported for recombinant human insulin, parathyroid hormone 1-34 (cyclic), beta interferon 1a, beta interferon 1b, monoclonal antibodies, and pramlintide (36).

CONCLUSION

The alkylsaccharides represent just one class of non-ionic non-ether-based surfactants that offer a potential alternative to the use of polyoxyethylene-containing surfactants in biotherapeutics formulations. The chemical diversity of potential alternatives is essentially unlimited, and it can reasonably be expected that ongoing and future research will result in additional alternatives to address the need for aggregation prevention without concomitant oxidative damage.

REFERENCES

1 Sauerborn M, Brinks V, Jiskoot W, Schellenens H., Immunological mechanism underlying the immune response to recombinant human protein therapeutics. Trends Pharmacol Sci 31(2): 53-9, 2010.

2 Kappos L, Clanet M, Sandberg-Wollheim M, Radue E.W., Hartung H.P., Hohlfeld R, Xu J, Bennett D, Sandrock A, Goelz S., Neutralizing antibodies and efficacy of interferon β-1a: A 4-year controlled study Neurology, 65: 40-47, 2005.

3 Vartanian T, Sorensen PS, Rice G., Impact of Neutralizing Antibodies on the Clinical Efficacy of Interferon Beta in Multiple Sclerosis. J Neurology, 251: II25–II30, 2004.

4 Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian J, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Bruno Varet, and Patrick Mayeux., Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N Engl J Med, 346(7):469-75, 2002

5 Purohit V.S., Middaugh C.R., Balasubramanian S.V., Influence of aggregation on immunogenicity of recombinant human Factor VIII in hemophilia A mice. J Pharm Sci, 95(2):358-71, 2006.

6 Hooks W.K., Urgent inhibitor issues: Targets for expanded research., Haemophilia, 12:107–113, 2006.

7 Reipert B.M., van den Helden P.M., Schwartz H-P., Hausl C., Mechanisms of action of immune tolerance induction against factor VIII in patients with cogenital haemophilia A and factor VIII inhibitors, Br. J. Haemophilia, 136:12–25, 2007.

8 Kerwin B.A., Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways, J. Pharm. Sci., 97: 2924–2935, 2008.

9 Ayorinde F.O., Gelain S.V., Johnson J.H Jr., Wan L.W., Analysis of some commercial polysorbate formulations using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom., 14(22):2116–2124, 2000.

10 Brandner J.D., The composition of NF-defined emulsifiers: Sorbitan monolaurate, monopalmitate, monostearate, monooleate, polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80. Drug. Dev. Ind. Pharm., 24(11):1049–1054, 1998.

11 Frison-Norrie S., Sporns P., Investigating the molecular heterogeneity of polysorbate emulsifiers by MALDI-TOF MS, J. Agric. Food Chem., 49(7):3335–3340, 2001.

12 Donbrow M., Azaz E., Pillersdorf A. Autoxidation of polysorbates, J. Pharm. Sci., 67(12):1676–1681, 1978.

13 Donbrow M., Hamburger R., Azaz E., Surface tension and cloud point changes of polyoxyethylenic nonionic surfactants during autoxidation, J. Pharm. Pharmacol., 27(3):160–166, 1975.

14 Donbrow M., Hamburger R., Azaz E., Pillersdorf A., Development of acidity in nonionic surfactants: Formic and acetic acid, Analyst (Lond) 103(1225):400–402, 1978.

15 Ha E., Wang W., Wang Y.J., Peroxide formation in polysorbate 80 and protein stability, J. Pharm. Sci., 91(10):2252–2264, 2002.

16 Wasylaschuk W.R., Harmon P.A., Wagner G., Harman AB., Templeton A.C., Xu H., Reed R.A., Evaluation of hydroperoxides in common pharmaceutical excipients, J. Pharm. Sci., 96(1):106-16, 2007.

17 Liu J.Y., Li Y.Z., Chang W.B., Measurement of the peroxidation of Brji-35 in aqueous solution by hemin and horseradish peroxidase catalyzed fluorogenic reaction, Fresenius J. of Analytical Chem., 365(5): 448-51, 1999.

18 Erlandsson B., Stability-indicating changes in poloxamers: the degradation of ethylene oxide-propylene

oxide block copolymers at 25 and 40°C., Polymer Degradation and Stability, 78(3):571–575, 2002.

19 Bondos S.E., Bicknell A., Detection and prevention of protein aggregation before, during and after purification, Anal. Biochem., 316: 223-231, 2003.

20 Katakam M., Bell L.N., Banga A.K., Effect of surfactants on the physical stability of recombinant human growth hormone, J. Pharm. Sci., 84(6): 713-6, 1995.

21 Wei G., Lu L.F., Lu W.Y., Stabilization of recombinant human growth hormone against emulsification-induced aggregation by Pluronic® surfactants during microencapsulation, Int. J. Pharmaceutics, 338(1-2): 125-132, 2007.

22 Guidance for Industry, February 2012, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product. Source: Center for Drug Evaluation and Research and the Center for Biologics Evaluation and Research at the Food and Drug Administration. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

23 Nassar M.N., Nesarikar V.N., Lozano R., Parker W.L., Huang Y., Palaniswamy V., Xu W., Khaselev N. Influence of formaldehyde impurity in polysorbate 80 and PEG-300 on the stability of a parenteral formulation of BMS-204352: identification and control of the degradation product, Pharm. Dev. Technol., 9(2):189-95,2004.

24 Ding S., Quantitation of hydroperoxides in the aqueous solutions of non-ionic surfactants using polysorbate 80 as the model surfactant, J. Pharm. Biomed. Anal., 11(2):95-101, 1993.

25 Yao J., Dokuru DK., Noestheden M., Park S.S., Kerwin B.A., Jona J., Ostovic D., Reid D.L., A quantitative kinetic study of polysorbate auto-oxidation: the role of unsaturated fatty acid substituents, Pharm. Res., 26(10): 2303-2313, 2009

26 Chafetz L., Hong W-H., Tsilifonis D.C., Taylor A.K., Philip J., Decrease in the rate of capsule dissolution due to formaldehyde from Polysorbate 80 autoxidation, J. Pharm. Sci., 73:1186-1187, 1984.

27 Kishore R.S., Kiese S., Fischer S., Pappenberger A., Grauschopf U., Mahler H.C., The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics, Pharm. Res., 28(5): 1194-210, 2011.

28 Ji JA., Zhang B., Cheng W., Wang Y.J., Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization, J. Pharm. Sci., 98(12): 4485-500, 2009.

29 Bergh M., Magnusson K., Nilsson J.L., Karlberg A.T., Contact allergenic activity of Tween® 80 before and after air exposure, Contact Dermatitis, 37: 9–18, 1997.

30 Kumar V., Kalonia D.S., Removal of peroxides in polyethylene glycols by vacuum drying: Implications in the stability of biotech and pharmaceutical formulations, AAPS PharmTechSci 7(3): E47-E53, 2006.

31 Ray W.J. Jr., Puvathingal J.M., A simple procedure for removing contaminating aldehydes and peroxides from aqueous solutions of polyethylene glycols and of nonionic detergents that are based on the polyoxyethylene linkage, Anal. Biochem., 146(2): 307-12, 1985.

32 Simat T.J., Steinhart H, Oxidation of Free Tryptophan and Tryptophan Residues in Peptides and Proteins, J. Agric. Food Chem., 46 (2): 490–498, 1998.

33 Chu J.W., Jin Yin J., Wang D.I.C., Trout B.L,. Understanding Oxidative Instability of Protein Pharmaceuticals, http://hdl.handle.net/1721.1/3955

34. Milzani A., Rossi R., Di Simplicio P., Giustarini D., Colombo R., Dalledonne I., The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin., Protein Science, 9:1774–1782, 2001.

35 van Beers M.M.C., Sauerborn M., Gilli F., Brinks V., Schellekens H., Jiskoot W., Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice, Pharm. Res., 28(10): 2393-402, 2011.

36 Wang W., Wang Y.J., Wang D.Q., Duel effects of Tween® 80 on protein stability, Intl. J. Pharmaceut., 347:31-38, 2008.

37 Knepp V.M., Whatley J.L., Muchnik A., Calderwood T.S., Identification of antioxidants for prevention of peroxide-mediated oxidation of recombination human ciliary neurotrophic factor and recombinant human nerve growth factor, J. Pharm. Sci. Technol., 50:163–171,1996

38 Herman A.C., Boone T.C., Lu H.S., Characterization, Formulation, and Stability of Neupogen (Filgrastim) a Recombinant Human Granulocyte-colony Stimulating Factor, Plenum Press, New York, 1996.

39 Thiele G.M., Tuma D.J., Willis M.S., Miller J.A., McDonald T.L., Sorrell M.F., Klassen L.W., Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant, Alcohol. Clin. Exp. Res., 22(8):1731-9, 1998.

40 Moghaddam A.E., Gartlan K.H., Kong L., Sattentau Q.J., Reactive Carbonyls Are a Major Th2-Inducing Damage-Associated Molecular Pattern Generated by Oxidative Stress, J. Immunol., 187(4): 1626-1633 ,2011.

41 Allison M.E. Fearon D.T., Enhanced immunogenicity of aldehyde-bearing antigens: a possible link between innate and adaptive immunity, Eur. J. Immunol., 30(10):2881-7, 2000.

42 Lagergård T., Lundqvist A., Wising C., Gabrielsson V., Ahlman K., Formaldehyde treatment increases the immunogenicity and decreases the toxicity of Haemophilus ducreyi cytolethal distending toxin, Vaccine 25(18):3606–3614, 2007.

43 Sharma B., Immunogenicity of therapeutic proteins. Part 1: impact of product handling, Biotechnol. Adv., 25(3): 310-7, 2007.

44 Baker M.P., Jones T.D., Identification and removal of immunogenicity in therapeutic proteins, Curr. Opin. Drug Discov. Devel., 10(2): 219-27, 2007.

45 Sauerborn M., Schellekens H., B-1 cells and naturally occurring antibodies: influencing the immunogenicity of recombinant human therapeutic proteins? Curr. Opin. Biotechnol., 20(6): 715-21., 2009.

46 Wang X., Sing S.K., Kumar S., Potential Aggregation-Prone Regions in Complementarity-Determining Regions of Antibodies and Their Contribution Towards Antigen Recognition: A Computational Analysis, Pharmaceutical Research, 27(8): 1512-1529, 2010.

47 Wang X., Singh S.K., Kumar S., Potential aggregation-prone regions in complementarity-determining regions of antibodies and their contribution towards antigen recognition: a computational analysis, mAbs, 1(3): 254-267, 2009

48 Rossert J., Casadevall N., Eckardt K., Anti-Erythropoietin Antibodies and Pure Red Cell Aplasia, J. Am. Soc. Nephrol., 15: 398–406, 2004.

49 Rifkin R.A., Maggio E.T., Dike S., Kerr D.A., Levy M., n-Dodecyl-β-d-Maltoside Inhibits Aggregation of Human Interferon-β-1b and Reduces Its Immunogenicity, J. Neuroimmune Pharmacology, 6(1): 158-6, 2011.

50 van Beers M.M.C., Sauerborn M., Gilli F., Brinks V., Schellekens H., Jiskoot W., Aggregated recombinant human interferon Beta induces antibodies but no memory in immune-tolerant transgenic mice, Pharm. Res., 27(9): 1812-24, 2010.

51 Jahn E.M., Schneider C.K., How to systematically evaluate immunogenicity of therapeutic proteins - regulatory considerations, N. Biotechnol., 25(5): 280-6, 2009.

52 Maggio E.T., Use of excipients to control aggregation in peptide and protein formulations, J. Excip. Food. Chem., 1(2): 40-49, 2010.

53 Pillion D.J., Atchison J.A., Gargiulo C., Wang R.X., Wang P., Meezan E. Insulin Delivery in Nosedrops: New Formulations Containing Alkylglycosides, Endocrinology, 135(6): 2386-2391, 1994.

54 Ahsan F., Arnold J., Meezan E., Pillion D.J., Enhanced Bioavailability of Calcitonin Formulated with Alkylglycosides Following Nasal and Ocular Administration in Rats, Pharm. Res., 18(12): 1742–1746, 2001.

55 Pillion D.J., Ahsan F., Arnold J.J., Balusubramanian B.M., Piraner O., Meezan E. Synthetic long-chain alkyl maltosides and alkyl sucrose esters as enhancers of nasal insulin absorption, J. Pharm. Sci., 91(6): 1456-1462, 2002.