Endotoxin Test Concerns of Biologics

Kevin L. Williams

Hospira Inc.

Limulus Amoebocyte Lysate (LAL) users are exploring regimens to study the effects of adding endotoxin to undiluted biologics in reaction to Chen's studies (Genentech) on Low Endotoxin Recovery (LER) [1] and, moreover, in response to the addition of verbiage to the FDA Q&A Guidance [2] on establishing the "stability of assayable endotoxins content"[1] in biologics. Chen's discovery that low levels of endotoxin added to undiluted biologics are sometimes unrecoverable by any means, leaves us to demonstrate that we do not have an endotoxin spike recovery problem with a given drug. After so many years of using Blumenthal's in-plate spike method [3], users have grown accustomed to overlooking the potential for adverse effects from *undiluted* solutions. To clarify, the worry is that undiluted solutions can modify added spike (a proxy for product contamination) such that it is undetectable via LAL yet remains able to bring about fever responses *in vivo*. This contrasts with the recovery of in-plate spikes that are only exposed to dilute solutions of product. Chen states that LER can occur in placebo containing polysorbate with either citrate or phosphate buffer as well, thus the facts of LER are complex and still being investigated.[2] Regardless of how the LER issue turns out,[3] it is causing users to examine a range of spike recovery from their products.

Two broad areas of concern in the recovery of endotoxin spikes from complex protein solutions will be discussed. Both areas contain a large amount of information that must be sorted out in terms of how screening tests will be performed and the potential effects product heterogeneity may have on such testing:

1. Chen et al. spiked low levels of CSE into Biologics and could not recover them either soon after spiking (hours) or after several days (up to a week). Therefore, the converse should also be true: if low level CSE spikes are successfully recovered from undiluted drug solutions over time then we do not have either a protein binding or LER issue. Spike recovery development data is shown here for two monoclonal antibody (mAb) solutions.
2. The performance of BET (Table 5) is not conducive to preserving mAb native protein conformation and is often at odds with package insert precautions (Table 4) that center around preventing protein aggregation. Such changes to proteins may exacerbate endotoxin spike recovery efforts. Since protein heterogeneity, especially aggregation, is a variable factor (an inherent instability in the interaction of the molecule with minute environmental changes), preclusion by a one-time test may prove too simplistic a proposal if there are a plethora of aggregate types[4] induced by a variety of stressors – some avoidable and others unavoidable[5] [4, 5].

1. The full statement in Q&A question 3 is "The ability to detect endotoxins can be affected by storage and handling. Firms should establish procedures for storing and handling (which includes product mixing) samples for bacterial endotoxins analysis using laboratory data that demonstrates the stability of assayable endotoxins content. Protocols should consider the source of endotoxins used in the study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins."
2. Personal communication, LER will be the subject of a break-out session at Oct's PDA Global Micro Conference.
3. LAL users remain uninformed of many of the details of LER. Verbiage intended to preclude LER occurrence was added to the Q&A Guideline published in June of 2012.
4. See Table 1 for a list of 20 classification types [5].
5. "Aggregation can occur at any stage during manufacture, storage, distribution, or handling of products, and it results from various kinds of stress such as agitation and exposure to extremes of pH, temperature, ionic strength, or various interfaces (e.g., air–liquid interface). High protein concentrations (as in the case of some monoclonal antibody formulations) can further increase the likelihood of aggregation" [5].

***Kevin Williams**, currently at Hospira Inc., has 30 years' experience in the Pharmaceutical industry specializing in endotoxin testing and control. He has written extensively on the subject of LAL technology including authoring/editing the book "Endotoxins" (Informa Healthcare, 2007).*

1. Screening for BET Spike Recovery from Undiluted Protein Solutions

Development tests were performed on two different mAb solutions to answer the question: "Is there a spike recovery problem with this drug solution?" As purified endotoxin has long been considered worst case in terms of clinging and binding to container/closure surfaces due to its hydrophobic lipid A/fatty acid end, likewise its recovery from undiluted products has historically represented a worst case recovery scenario as the hydrophobic end is not associated with cellular debris as is naturally occurring, non-purified endotoxin (NOE). The screens below involved adding CSE at low levels (<10 EU/mL) to *undiluted* drug product and testing for recovery over time. Results are shown using a lyophilized (reconstituted) mAb (Table 1) and a liquid mAb (Tables 2/3).

mAb A (Table 1)

The data for mAb A showed no difficulty in recovering low level endotoxin spikes over the test series using the addition of and continued dilution in dilute surfactant (Table 1).

Table 1. Reconstituted (lyophilized) mAb endotoxin spike into undiluted product. Formulation includes polysorbate. Tested via Kinetic Turbidimetric.

Test Parameter	Day 0	Day 1	Day 3	Day 4	Day 7 (week later)
Recovery in EU/mL	0.265	0.256	0.257	0.259	0.221
%PPC (in-plate spike)	112	78	78	68	63
% of spike recovered (from undiluted product)	106	107.6	107.9	108.8	92.8
Reconstitution method	Spike Theory		Dilution	Storage	
20 mL vial recon - 20 mL Sterile Water for Injection (SWFI). Added 50 microliters undiluted surfactant post spike and reconstitution.	1.0 mL 50 EU/mL CSE = 50EU / 21mL 1:1 = 2.38 EU/mL 1:10 = 0.238 EU/mL		Tested 1:10 in 0.5% surfactant	Samples refrigerated at 5˚C between runs and held in a box (dark). All vortexing is 1 minute.	

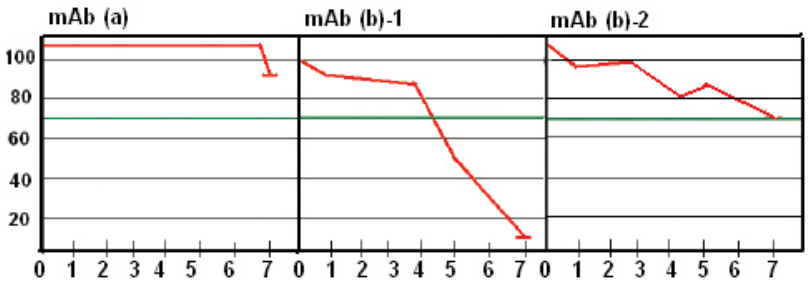
Table 2. Liquid mAb endotoxin spike into undiluted product. Formulation includes Polysorbate + Citrate.

Tested via Kinetic Turbidimetric					Sample Reconstitution / Spike / Diluents / Dilution Scheme
Day 0					
Series	Dilution	EU/mL recovery	%PPC	% spike recovery	Liquid sample No surfactant added. 10 mL in original vial. CSE Spike Theory 1mL of 100 EU/mL CSE into 10 mL = 100 EU per 11mL= 9 EU/mL 1:10 = 0.9 EUmL 1:50 = 0.18 EU/mL 1:100 = 0.09 EU/mL Test Iterations
1	1:50	0.144	98	80	
2	1:100	0.0686	109	76.2	
3	1:50	0.146	90	81.1	
4	1:100	0.0694	110	77.1	
Day 1					Tests 1, 2 Test 1:50 and 1:100 diluted in 0.5% surfactant.
1	1:50	0.121	91	67.2	
2	1:100	0.0625	109	69.4	Tests 3, 4 Test 1:50 and 1:100 diluted in 1% surfactant
3	1:50	0.122	88	67.8	
4	1:100	0.0625	107	69.4	Dilution Scheme 1:10 is 0.25 mL sample into 2.25 mL diluent 1:50 is 0.5 of 1:10 into 2.0 mL diluent 1:100 is 1 mL of 1:50 into 1.0 mL diluent Storage - Samples refrigerated at 5˚C between runs and held in a box (dark). Vortexing is 1 minute between dilutions and for undiluted solutions.
Day 4					
1	1:50	0.113	92	62.8	
2	1:100	0.098	94	108.8	
3	1:50	0.0855	88	47.5	
4	1:100	0.0556	107	61.8	
Day 5					
1	1:50	0.102	100	56.6	
2	1:100	0.0914	104	101.6	
3	1:50	0.0576	111	27.7	
4	1:100	0.0527	110	58.55	
Day 7 (week later)					Note that by day 7 dwindling recovery tested at 1:10 and 1:50 in 0.5% surfactant (3 and 4 failed at days 4 and 5).
1	1:10	0.0755	113	8.3	
2	1:50	0.0146	106	8.1	

Table 3. Recovery for Liquid mAb B Vial --formulation includes Polysorbate + Citrate.

Tested by Kinetic Turbidimetric					Sample Reconstitution / Spike / Diluents / Dilution Scheme
Day 0					
Series	Dilution	EU/mL Recovery	%PPC	% spike recovery	Liquid Sample 20 mL in original vials. Prepared separate vials for sets1/2, 3/4, and 5/6. Vial for 1/2 added 50 microliters of surfactant and others did not (3/4 and 5/6). Spike 1.2 mL of 100 EU/mL CSE = 120 EU /21.2mL = 5.66 EU/mL Spike Theory 1:1 = 5.66 EU/mL 1:10 = 0.566 EU/mL 1:50 = 0.1132 EU/mL 1:100 = 0.0566 EU/mL Test Variations
1	1:50	0.110	71	97.1	
2	1:100	0.0448	90	79.2	
3	1:50	0.125	98	110.4	
4	1:100	0.0565	115	99.8	
5	1:50	0.111	100	98.0	
6	1:100	0.0526	106	92.9	
Day 1					
1	1:50	0.108	65	95.6	
2	1:100	0.0488	88	86.2	
3	1:50	0.117	91	103.4	Tests 1, 2 -50 microliters surfactant added to sample vial only at Day 0. Tested 1:50 and 1:100 as diluted in 1% surfactant
4	1:100	0.0537	110	94.9	
5	1:50	0.114	89	100.7	3, 4 -No Surfactant added to vial - Diluted in 1% surfactant cut to 0.5% using equal volume 100 mM Tris buffer
6	1:100	0.0555	113	98.1	5, 6 -No surfactant added to vial - Diluted in 1% surfactant prepared in 10 mM $MgCl_2$
Day 4					Storage All samples refrigerated at 5˚C between runs and contained in a box (dark). Vortexing is 1 minute between dilutions and for undiluted solutions. Dilution Scheme 1:10 is 0.25 mL sample into 2.25 mL diluent 1:50 is 0.5 of 1:10 into 2.0 mL diluent 1:100 is 1 mL of 1:50 into 1.0 mL diluent
1	1:50	0.0845	83	74.6	
2	1:100	0.0461	77	81.4	
3	1:50	0.0845	107	74.6	
4	1:100	0.0452	100	79.9	
5	1:50	0.0921	101	81.4	
6	1:100	0.0497	109	87.8	
Day 5					
1	1:50	0.0991	65	87.5	
2	1:100	0.0391	88	69.1	
3	1:50	0.0939	97	83.0	
4	1:100	0.0435	113	76.9	
5	1:50	0.0977	67	86.3	
6	1:100	0.0479	124	84.6	
Day 7 (week later)					
1	1:50	0.0737	68	65.1	
2	1:100	0.0341	86	60.2	
3	1:50	0.0852	95	75.3	
4	1:100	0.0391	108	69.1	
5	1:50	0.0835	92	73.8	
6	1:100	0.0393	110	69.4	

Snapshot of % recoveries obtained from Tables 1, 2, and 3 over 7 day test period.



mAb B-Test 1 (Table 2)

Difficulties with both logistics and spike recovery were experienced in MAb B-Test 1 (Table 2). In terms of logistics, early tests to establish the amount of spike to add and the amount of subsequent dilution to use relative to MVD showed poor and inconsistent recovery (0-60%). One wants to add a low but assayable amount of spike, while allowing enough dilution to overcome product interference. The interference properties of some drugs may require dilution that necessitates very low recovery of spike. Logistics should be worked out to ensure that recovery achieved is a metric of protein binding rather than due to interference from an under-diluted test. Results shown for mAb B were improved from earlier recovery efforts, yet the recovery still dropped off moderately at day 5 and drastically at day 7. The reduction in spike over time may be due to protein aggregation and/or the generation of surfactant-generated peroxides [6].

mAb B-Test 2 (Table 3)

The final set of study results show improved recoveries due to changes in diluent combinations while using almost half the previous spike

level: consistent recovery of a very low amount, ~0.05 EU/mL, after dilution. Because the adjustment of traditional development parameters (addition of buffer to diluent) resulted in improved recovery, it suggests a conventional interference mechanism (reversible binding) at work rather than an irreversible event as associated with LER of biologics as previously encountered [1]. There remains some diminishment of spike recovery, but consider that the low levels recovered represent approximately 0.1 (1:50) to 0.05 (1:100) EU/mL, which is 0.02 to 0.01 nanograms (10 to 20 picograms) of endotoxin (by definition relative to EC-2). Thus the difference in recovery of 100% versus 70% of 10 picograms represents only a 3 picogram difference! Spike recovery variability may sometimes seem large in CSE recovery studies, but the standard recovery metric of 50 – 200% is relative to the sensitive levels detected. Whether 1 nanogram is called a ½ nanogram or 2 nanograms should lend credence that a two-fold error is not a large amount.[6] The state of the art is such that an average of >70% is not an unreasonable expectation for these kind of spike recoveries.

The success of using a dilute surfactant points to a reversible aggregation mechanism while the necessity of using an additional buffer for mAb B may suggest that degradation byproducts over time may be a secondary issue.

NOE

Some advocate the use of "natural endotoxin" (non-purified) for such studies, yet NOE is likely to give both more variability and conflicting results depending upon the organism(s) chosen. The values shown by Bower and Tran differed widely among cultured bacteria (they recovered <50 to >58,000 EU/mL) [8]. NOE is not really an entity that can be readily produced in the lab as it would, if natural, consist of a mixture of water-borne bacteria and cannot be ordered from ATCC[7] other than as bacterial isolates (often enterics) grown on enriched media. Many users have seen solutions of killed Gram negative bacteria increase in potency over time, presumably from the continued release of bound LPS from cellular membrane debris as solutions are vortexed over time. This could have the effect of "padding" the recovery of NOE spike results. Employing user-prepared NOE would make standardizing such testing very difficult.

2. BET at Odds with Package Insert Intent to Preserve the Native State of mAb Products

MAb Package Inserts (Table 4)

The delicate nature of mAbs as far as the prevention of protein aggregate formation is concerned is an important issue [9]. The package inserts of most mAbs detail the very specific conditions required to avoid aggregate formation during administration. Protein aggregate formation is an ever-present concern with mAbs during manufacture, packaging and right up to the point of injection or infusion into the patient. Formulations and containers, even container-closure coatings (i.e. silicone), have all been carefully selected to prevent aggregation [10]. Protein aggregation diminishes protein potency, can bring with it patient immunogenicity, the potential for hyperreactivity, and may be a factor in endotoxin binding of added spikes. After Herculean efforts by manufacturers to produce and preserve the specific conformation of these complex molecules, the first thing BET users do is disrupt the native structure. Granted, users need not adhere to package insert recommendations for reconstituting and testing products for BET (the samples are not going into a patient), yet if one is trying to recover endotoxin bound to protein then a method likely to cause protein aggregation may not be the best approach. Similarly, generating a form of the drug that differs from that used to generate other supporting analytical data (such as particulate and potency testing) seems undesirable [11]. How users could maintain the natural state of the protein while performing BET activities presents an interesting issue for future consideration.

Table 4. Commercial mAb package insert quotes on product reconstitution and dilution for administration.

(----------®) WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED... **DO NOT SHAKE.**
...the vials after reconstitution should be used immediately, not re-entered or stored. The diluent to be used for reconstitution is 10 mL of Sterile Water for Injection, USP. The total dose of the reconstituted product must be further diluted to 250 mL with 0.9% Sodium Chloride Injection, USP. Infusion should begin within 3 hours of preparation... **DO NOT SHAKE.**
Carefully inspect the solution in the (----------®) Pen or prefilled syringe for particulate matter and discoloration prior to subcutaneous administration. If particulates and discolorations are noted, do not use the product. (----------®) does not contain preservatives; therefore, discard unused portions of drug remaining from the syringe.
Multiple use vials: (----------®) should be reconstituted aseptically with 1 mL of supplied Sterile Bacteriostatic Water for Injection, USP (0.9% benzyl alcohol) giving a solution of 1.0 mL containing 25 mg of (----------®).
(----------®) a humanized monoclonal antibody... Do not shake the content of the vial. A vigorous shaking could denaturalize the protein and affect the biological activity of the product.
Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Withdraw necessary amount of (----------®) and dilute in a total volume of 100 mL of 0.9% Sodium Chloride Injection, USP. Discard any unused portion left in a vial, as the product contains no preservatives. **DO NOT ADMINISTER OR MIX WITH DEXTROSE SOLUTION.**
• **DO NOT DILUTE THE PRODUCT.** • **DO NOT SHAKE OR VIGOROUSLY AGITATE THE VIAL.** • Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. Do not use any vials exhibiting particulate matter or discoloration.
(----------®) may be sensitive to shear-induced stress, e.g. agitation or rapid expulsion from a syringe. **DO NOT SHAKE.**
Product names redacted

Unknown Effects of Aggregates on BET (Table 5)

MAbs are a special class of molecule that have FDA-mandated testing for immunogenicity [12, 13]. If mAb protein aggregation is a factor in endotoxin-protein binding, and if mAbs are as prone to protein aggregation due to individual sample treatment as inserts suggest, then a successful study today may not preclude subsequent batches from encountering protein aggregation and thus changing the spike recovery matrix [14]. Arguably, the presence of various aggregate population types batch to batch could call into question the ability of a one-time test to preclude all spike loss scenarios. On the other hand, successful spike recovery via a simple screen may be the best precaution currently available. Simply stated, adding complexity around such studies adds no further guarantee

6. EU is defined as 0.2 nanogram of EC-2 (1/5 the 1 ng/kg established as the threshold pyrogenic response) [7]

7. American Type Culture Collection

Table 5. BET activities known to provoke protein aggregation; advised against in package inserts		
1	Shaking / vortexing	Vigorous mixing is necessary to recover endotoxin but is expressly advised against in many mAbs package inserts [22].
2	Addition of contaminants	Adding endotoxin may provoke protein aggregation via a so-called "seeding" effect: "One plausible mechanism is that aggregation is driven or catalyzed by the presence of a small amount of a contaminant" [23].
3	Immediate use	Some mAbs are not intended to be stored after reconstitution or after opening (require immediate use).
4	Use of diluents	Inserts specify diluents to use for administration. BET can be performed with any diluent(s) that aid endotoxin recovery and/or aid in overcoming interfering factors yet the use of some diluents may promote protein aggregation, especially in prolonged storage (itself often contraindicated).

of their value. The difficulty of maintaining the natural state of biologics and the unpredictability of non-native states is hinted at below:

> *Recombinant human protein (rP) and glycoprotein (rGP) therapeutics are established in the clinic. However, a variety of adverse reactions are reported that may differ between individual therapeutics, between the same therapeutic produced by different companies or different lots produced by the same company. Even when these parameters are controlled, there still remains the final "black box": the patient and individual disease manifestations. A feature of all rP/rGP is a potential to be immunogenic i.e., cause the generation of anti-therapeutic antibodies (ATA). Such antibodies may neutralize the therapeutic, result in enhanced clearance or precipitate severe adverse reactions. To limit the generation of ATA, an rP/rGP should, ideally, have exactly the same structure as the natural product since departure from such structural fidelity constitutes "altered-self", and the potential to be immunogenic [15].*

Summary

1. Screening for BET Spike Recovery from Undiluted Protein Solutions

A simple screen performed using low levels of CSE spikes into undiluted biologics (mAbs) has been described. In summary, the routine application of such a test involves only a few, easily reproducible steps:

1. Establish the logistics of testing a specific sample based upon the (a) MVD, (b) known drug interference properties, (c) sample volume and (d) spike starting concentration.
2. Assign acceptance criteria such that a pass/fail distinction is not ambiguous.
3. Inoculate undiluted product samples with low level CSE spike (<10 EU/mL). The need for the use of >10 EU/mL solutions may arise if sample interference necessitates large dilutions.
4. Test over the course of a week (or more) for percent spike recovery. Include an in-plate PPC to gauge the on-going recovery.
5. Repeat the test to demonstrate reproducibility and consistency.
6. Ensure the key parameters of the routine test matches the test used for screening.
7. The inability to recover undiluted spike will require additional tools to address the issue (i.e. risk assessment).

Whereas LER is a new issue, the masking phenomenon for BET spikes into protein solutions is not new. Petsch et al. [16] used proteinase K digestion of several proteins (including human IgG) to recover endotoxin in 1998. The fact that IgG bound endotoxin shows this is not a hypothetical issue. If a screen using CSE in low levels succeeds in spike recovery from undiluted protein solutions over time, then assurance has been gained that a given drug does not have a protein binding or low endotoxin recovery (LER) issue.

2. BET at Odds with Package Insert Intent to Preserve the Native State of mAb Products

It would be a simple proposal to spike and recover endotoxin from protein solutions if mAbs were monolithic forms, however, conformations are easily changed by various BET activities that act as stressors and remain invisible to users.[8,9] Package insert prohibitions safeguarding the conformation of the proteins tested are not currently adhered to for BET because it is not clear how BET could be performed while adhering to such precautions. Neither is it clear if, when, and what aggregate states may exacerbate protein-endotoxin binding for various products. There seems to be a keen awareness of aggregation issues at the two ends of the spectrum (manufacturing[10] and patient administration) but less so in individual analytical test performance. For now, adding contaminants (endotoxin spikes), storing after opening, using various diluents not specified in inserts,[11] and vigorous shaking is unavoidable for mAb BET testing.

BET disruption of the natural protein structure is acceptable if it does not affect endotoxin-protein binding (most visible here at day 7 - Table 2). This is an area that has not been a subject of study. As shown in Figure 1 there are many more variables than commonly recognized. It is not known if the disruption of the native states produces consistent aggregate forms (population types and proportions of various types, see Figure 2) from one BET test to the next or how these forms interact with endotoxin in its various aggregation states [20, 21].[12,13] These activities (Table 5) add a potentially confounding layer to spike studies and seem to present an opportunity to develop alternative methods of endotoxin detection that do not alter the natural state of complex proteins. Given these various

Figure 1 - Derived from Hirayama and M. Sakata's diagram of endotoxin attachment mechanisms in protein solutions as commonly used in the removal of endotoxins via adsorbents [24].

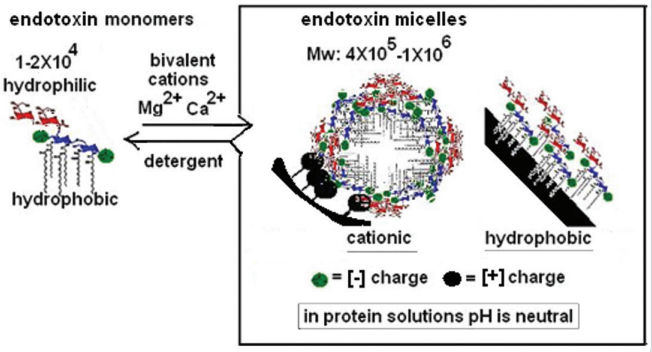
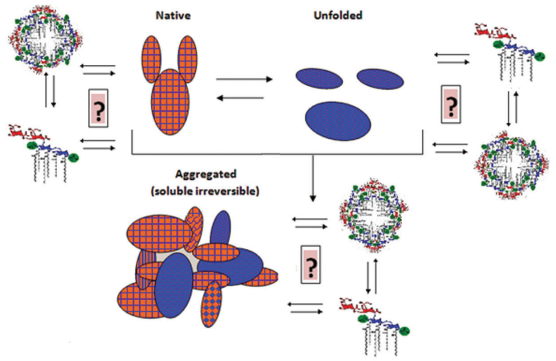


Figure 2 - Various conformational states of protein with various aggregation states of endotoxin. Question marks indicate unknown protein-endotoxin binding / interaction characteristics of various forms. Each of three (or more) protein conformation states shown may be present in various proportions for a given compound as well as various endotoxin aggregation properties. Micelles shown as cross section. MAb figures based upon [25].



dilemmas, it is important to remember that the best assurance of very clean drug solutions (endotoxin-free) is to ensure endotoxin removal at relevant process steps and the prevention of bioburden in manufacturing processes, the existing expectation for all cGMP drugs. Yet these same concerns apply to aid in better understanding BET testing between processing points as different phases of production may have different protein aggregation and thus endotoxin binding propensities.

References

1. *J. Chen, "Low Endotoxin Recovery in Common Biologics Products." Presented at the 2013 PDA Annual Meeting, Orlando, FL, April 2013.*
2. *FDA-Guidance for Industry, Pyrogen and Endotoxins Testing: Questions and Answers, Jun 2012.*
3. *Robert Blumenthal, BioWhittaker 1990 -Personal communication*
4. *Rajesh Krishnamurthy et al., Emerging Analytical Technologies for Biotherapeutics Development, BioProcess International May 2008, pg. 32-42.*
5. *Mire-Sluis et al., Analysis and Immunogenic Potential of Aggregates and Particles, BioProcess International 9(10) November 2011.*
6. *Kerwin, B.A., Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways, Journal of Pharmaceutical Sciences 97 (2008): 2924-2935.*
7. *Endotoxins, Informa Healthcare, 2007, page. 169.*
8. *Creation of an In-house Naturally Occurring Endotoxin Preparation for Use in Endotoxin Spiking Studies and LAL Sample Hold Time Analysis, Bowers and Tran, September/ October 2011 issue of American Pharmaceutical Review - Volume 14, Issue 6.*
9. *Mire-Sluis et al., Analysis and Immunogenic Potential of Aggregates and Particles, BioProcess International 9(10) November 2011*
10. *Esfandiary et al., Characterization of Protein Aggregation & Adsorption on Prefillable Syringe Surfaces, West Pharmaceutical Services, 2010.*
11. *Chirino and Mire-Sluis, Characterizing Biological Products and assessing comparability following manufacturing changes, Nature Biotechnology Vol. 22, No. 11, Nov. 2004.*
12. *FDA: Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1997).*
13. *Draft guidance: Immunogenicity Assessment for Therapeutic Protein Products, Feb. 2013.*
14. *Aggregates in Monoclonal Antibody Manufacturing Processes, Mari´a Va´zquez-Rey and Dietmar A. Lang, Biotechnology and Bioengineering, Vol. 108, No. 7, July, 2011.*
15. *Human immunoglobulin allotypes, Possible implications for immunogenicity, by Roy Jefferis and Marie-Paule Lefranc,, July-Aug, MABS 2009; 1(4): 332-338.*
16. *D. Petsch, W.-D. Deckwer, and F.B. Anspach, Proteinase K Digestion of Proteins Improves Detection of Bacterial Endotoxins by the Limulus Amebocyte Lysate Assay: Application for Endotoxin Removal from Cationic Proteins, Analytical Biochemistry 259, 42-47 (1998), pg. 42-48.*
17. *Protein Aggregation and Bioprocessing, Cromwell et al., The AAPS Journal, 2006; 8 (3) Article 66.*
18. *V. Kayser et al., Conformational stability and aggregation of therapeutic monoclonal antibodies studied with ANS and thioflavin T binding, mAbs 3:4, 408-411; July/August 2011; © 2011 Landes Bioscience.*
19. *Demeule et al., New methods allowing the detection of protein aggregates, mAbs 1:2, 142-150; March/April 2009; ©2009 Landes Bioscience.*
20. *Jourbert, et al., Classification and Characterization of Therapeutic Antibody Aggregates, 2011, Jour. Biological Chemistry, 286, 25118-25133.*
21. *Luo et al., Chemical Modifications in Therapeutic Protein Aggregates Generated under Different Stress Conditions, 2011, Jour. Biological Chemistry, 286, 25134-25144.*
22. *Lahlou A, et al. Mechanically-induced aggregation of the monoclonal antibody cetuximAb. Ann Pharm Fr (2009), doi:10.1016/j.pharma.2009.05.008*
23. *Aggregation Analysis of Therapeutic Proteins, Part 1, General Aspects and Techniques for Assessment Tsutomu Arakawa,\ et al., BioProcess International 4(10):42-43 (November 2006).*
24. *Chromatographic removal of endotoxin from protein solutions by polymer particles, C. Hirayama and M. Sakata, Journal of Chromatography B, 781 (2002) 419–432.*
25. *V. Kayser et al., Conformational stability and aggregation of therapeutic monoclonal antibodies studied with ANS and thioflavin T binding, mAbs 3:4, 408-411; July/August 2011; © 2011 Landes Bioscience.*

8. "...some of the most severe adverse events associated with immunogenicity occur at frequencies low enough to require very large studies." pg. 1386 [11].
9. "The effect of the presence of self-associated species is not always known" [17]
10. "...the potential interaction of aggregates with surfaces, e.g., needle, tubing, column, will lead to the loss of sample and thus an inaccurate analysis" [18].
11. "Both the excipients used during the lyophilization process and the excipients used during the reconstitution process should ensure the stability of the protein" [19].
12. "Different stress treatments can generate aggregates with widely varying properties. Stressed samples can potentially have different particle size distributions, particle morphology, chemical modifications, reversibility, percent aggregation, conformations (native-like versus unfolded), and particle surface hydrophobicity" [20].
13. "The chemical modifications (Met oxidation) seen with mild stirring are similar to those seen with H_2O_2 treatment, which suggests that mild stirring stress increases interactions of the protein with air…" [21].