

The weakest link: looking at the toxicity challenges of biologic drug linkers

Marie Rock, Ph.D., Vice President - Protein Bioanalysis, Midwest BioResearch

Over nine times more biologics are under development now than in 2002 and biologics are more likely to successfully move through preclinical and clinical testing versus small molecule, new chemical entities¹. However, bringing biological drugs to market is still difficult because the cost of development and commercialisation is a major hurdle.

One way to lower costs is to provide a drug that has a relatively long plasma half-life, which benefits both patients and manufacturers by requiring less frequent administration of the therapeutic agent. Typically, the human body is able to eliminate a drug protein from the bloodstream within hours using a variety of clearance mechanisms. It is possible, however, to modify chemically a protein to slow clearance and thus prolong the plasma half-life. These types of protein modifications are produced through chemical modifications that require the use of small molecules.

“Typically, the human body is able to eliminate a drug protein from the bloodstream within hours using a variety of clearance mechanisms”

The need for linkers

Binding the protein to an inert polymer like polyethylene glycol (PEG) confers a shielding effect that prolongs circulation at therapeutic concentrations and thus enhances efficacy. Because PEG is a highly inert molecule, chemists use small organic chains called

linkers to join PEG with proteins (see Figure 1). While these chimeric compounds are usually non-toxic, the linker molecules are reactive by nature and thus have potential to bind and damage DNA. Linkers (either whole or in part) can be present in drug substance or product as either impurities or degradation products. Carefully designing linkers, removing these from drug substance or product, and preventing their degradation require the diligent use of the latest testing technologies.

PEG is often used in bio-therapeutics, cosmetics, toothpastes, and even in food because of its ability to dramatically increase solubility and mobility of proteins^{2,3,4,5}. PEG itself is also non-toxic, generally known to be non-immunogenic and easily cleared from the body without structural modification.

PEGylation, especially using long chains, confers the properties of the PEG polymer to the protein conjugate: sending previously insoluble proteins swimming into solution without

registering a blip on the body's immune system “radar”³. These properties enhance a drug's pharmacokinetics and thus its ability to perform its intended therapeutic function.

Interferon-alpha, a treatment for chronic viral hepatitis C, has poor pharmacokinetics despite subcutaneous injections^{6,7}. Hoffman-La Roche has improved therapeutic levels of the drug by attaching a 40 kD, branched PEG^{8,9}. In another example, PEGylating filgrastim, the injectable therapeutic for a condition characterised by the lack of certain white blood cells, increased the serum half-life of filgrastim from 3.8 hours to 42 hours¹⁰.

While PEGylation can produce significantly better drug half-lives, PEG-protein conjugates can also degrade during



long-term storage, springing free potentially mutagenic unconjugated linkers.

Hazards of organic contaminants

Exposed linkers can pose a series of hazards: they can form toxic dimers, associate with undesired sites on the therapeutic protein, bond with a multitude of human proteins, and exert carcinogenic effects by binding or damaging DNA.

Between 1998 and 2004, nearly 200 anemic patients receiving epoetin-alpha injections developed pure red cell aplasia, a specific type of anaemia characterised by the exclusive loss of red blood cells. Nine-tenths of the cases were found in patient's receiving one commercial formulation: Eprex. Intensive studies determined that Eprex's activation of the immune response was potentially due to the organic compound polysorbate 80

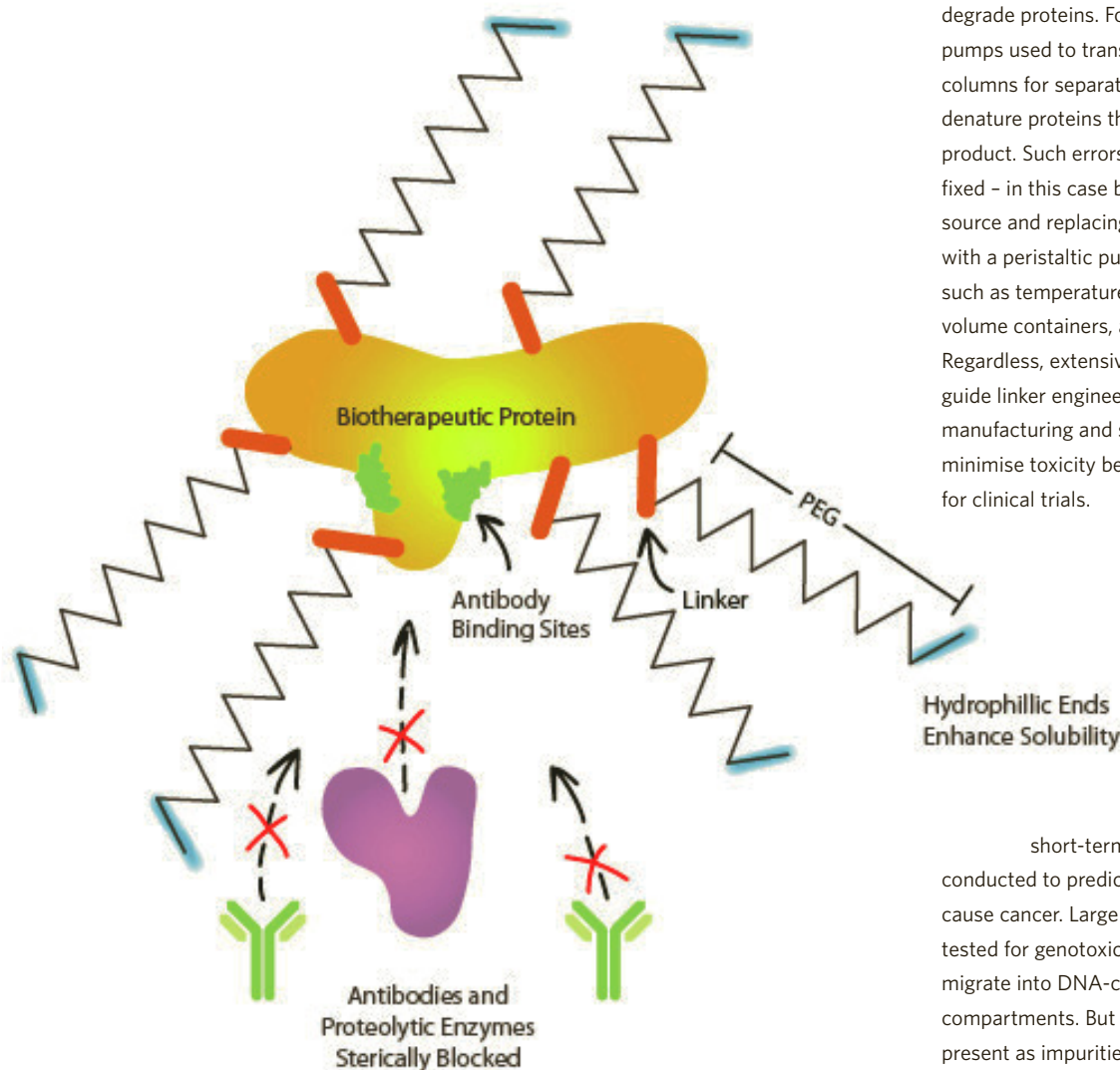
leaching other organic compounds from rubber plungers in prefilled syringes or leakage of the syringes' silicone oil lubricant. Typical handling procedures such as freeze-drying can also facilitate the oxidation of protein and trigger the immune response. After modifications to the storage, handling and administration of Eprex, incidence of pure red cell aplasia decreased 83 percent worldwide.¹¹ Most commonly in other examples, linker bonds can break or change to create a spot for an antibody to be generated against a piece of that drug and thus trigger a deleterious immune response. There are examples when this process continues to occur with time and there can be batches of the drug that may have greater amounts of the antigenic form of the molecule than others.

Great care must be taken when designing linkers to avoid undesired degradation.

Significant problems can also arise during manufacturing scale-up in preparation for longer duration efficacy and toxicology studies. Small-scale, bench top synthesis can

“Several commercial tests exist for genetic toxicology screening, but regulatory agencies only accept results from the GLP regulatory genetic toxicity tests”

produce minute, undetectable (and possibly benign) amounts of byproduct that is magnified as massive vats replace small flasks. Even if small-scale synthesis does not produce a single byproduct, the shear forces exerted by hardware used during scale-up can create uneven reaction conditions and degrade proteins. For instance, mechanical pumps used to transport solutions down large columns for separation chromatography can denature proteins that end up in the final product. Such errors must be diagnosed and fixed - in this case by carefully tracing the source and replacing the mechanical pump with a peristaltic pump. Many other problems, such as temperature uniformity in large volume containers, are more difficult to solve. Regardless, extensive testing is required to guide linker engineering, optimise manufacturing and storage protocols and minimise toxicity before the drug is released for clinical trials.



Predicting linker toxicity

Before clinical trials, researchers assess a drug's toxic effect, including the potential for carcinogenicity. In

terms of carcinogenicity,

short-term genetic toxicity testing is conducted to predict which compounds may cause cancer. Large molecules are not always tested for genotoxicity because they cannot migrate into DNA-containing sub-cellular compartments. But since linkers can be present as impurities or degradation products,

Figure 1: PEGylation's can enhance a drug's half-life by protecting it from degradation by endogenous enzymes, enhancing solubility with PEG's hydrophillic ends, and preventing glomerular filtration by enlarging its apparent molecular size. It can also prevent adverse immune responses by sterically hindering antibodies from accessing the protein surface.

genetic toxicology testing may be important to demonstrate safety.

Several commercial tests exist for genetic toxicology screening, but regulatory agencies only accept results from the GLP regulatory genetic toxicity tests. The Ames test is one such test developed in the 1970's by Bruce Ames and his colleagues at the University of California at Berkeley. This test uses several strains of bacteria to determine mutagenicity. The bacteria are engineered with mutations that disable the bacteria's histidine production genes so that it cannot grow without external histidine. These mutated bacteria are incubated, along with the drug, on a plate of agar that contains just enough histidine to support the bacteria while the drug exerts any possible mutagenic effects. After the histidine supply is depleted, bacteria will not grow unless their mutated histidine genes have been again mutated back to wild type. A genotoxic drug will re-mutate Ames bacterial strains so that they synthesise histidine, thus enabling bacterial growth in the histidine-free environment. Other compounds, such as liver extracts, are added to mimic metabolism of the drug compound in case the drug itself is not toxic, but the metabolites are^{12,13}.

“Typical handling procedures such as freeze-drying can also facilitate the oxidation of protein and trigger the immune response”

The GLP regulatory Ames test can evaluate the genetic mutagenesis potential of a drug, but it requires a gram or more of drug compound. Production of such a large amount prior to the setup of large-scale production infrastructure requires laborious and expensive efforts that can span months. Through structure-toxicity knowledge, the molecular attributes of the organic linker can be evaluated for their potential to pose a genetic toxicology risk. If the linker may be genotoxic, researchers should consider whether it is warranted to pre-test the entire drug conjugate, impurities, and/or degradation products prior to GLP regulatory testing. Several commercialised options exist to predict the GLP Ames test while requiring only a fraction of compound necessary for regulatory testing. Almost all measure only certain facets of the GLP regulatory Ames test

and use incomplete or non-ICH-compliant protocols or bacterial cell lines. Incomplete testing can leave toxic compounds undetected and thus result in a costly failure of the full Ames test. Thus it's critical to identify screens that use five ICH-compliant *Salmonella/E. coli* tester strains, liver S9 activation systems and agar format to mirror and fully predict accurately the Ames test.

Ideally, PEG and its associated linker would attach properly to a specific site on the protein's 3-D surface without interacting at other sites or creating toxic byproducts during synthesis. Or better yet, biologics would not need PEGylation to boost efficacy by preventing clearance or masking it from the human immune system and increasing plasma half-life. In reality, however, every one of the multitudes of reactions during drug synthesis produces an array of byproducts that must be controlled and tested. Furthermore, the complexity that these large proteins present and the changing environments during production scale up never offers such Elysian conditions. As better, more accurate tests like the microAmes screen emerge, the development of conjugated biologics should become less expensive and safer for human administration.

References

1. *CMR International. CMR Industry Success Rates. (2002).*
2. *Harris, J. Milton and Zalipsky, S., Editors, Poly(ethyleneglycol): Chemistry and Biological Applications, ACS Books, Washington, DC (1997).*
3. *Harris, J. Milton. Editor. Polyethylene Glycol Chemistry. Biotechnical and Biomedical Applications. Plenum, New York (1992).*
4. *Nucci, M.L et al. The therapeutic value of poly(ethylene glycol)-modified proteins. Advanced Drug Delivery Reviews. (1991).*
5. *Delgado, E., et al. The uses and properties of PEG-linked proteins. Critical Reviews in Therapeutic Drug Carrier Systems. (1992).*
6. *Liang, J.T. et al. Pathogenesis, natural history, treatment and prevention of hepatitis C. Annals of Intern. Medicine (2000).*
7. *McHutchison, J.G. Interferon α -2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. New England Journal of Medicine (1998).*
8. *Bailon, P. et al. Pharmacological properties of five polyethylene glycol conjugates of*

interferon α -2a. International Conference on Therapies for Viral Hepatitis: December 6th, Maui, Hawaii. (1999).

9. *Shiffman, M. et al. A controlled, randomized, multicenter ascending dose phase II trial of pegylated interferon α -2a (PEG) vs. standard interferon α -2a (IFN α) for treatment of chronic hepatitis C. Gastroenterology (1999).*
10. *Molineux, G. The Design and Development of Pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). Current Pharmaceutical Design. 2004.*
11. *Bennett, Charles et al. Pure Red-Cell Aplasia and Epoetin Therapy. The New England Journal of Medicine. (2004).*
12. *Ames, Bruce N., et al. Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. Proceedings of the National Academy of Sciences. (1973).*
13. *McCann, Joyce, et al. "Detection of Carcinogens as Mutagens: Bacterial Tester Strains with R Factor Plasmids". Proceedings of the National Academy of Sciences. (1975).*



Marie Rock, Ph.D.

Marie Rock is the Vice President of MBR's Protein Bioanalysis group. Her responsibilities include directing and designing method development and validation experiments, interpreting and reporting results, and preparing summary regulatory documents.

Marie has more than 25 years of experience in the pharmaceutical industry. At Pfizer/Pharmacia, she was Director of Analytical Methodologies for Experimental Medicine. At Searle/Monsanto, she was Senior Research Advisor for the Clinical Pathology/Immunoassay Laboratory in Metabolism and Safety Evaluation. Marie was also the Head of Drug Metabolism at Nova Pharmaceuticals. Prior to that, she was Director of Immunoassays at Ortho (J&J) Pharmaceuticals. Marie also served as the Vice President of R&D for Diagon and Director of Clinical Pathology and Assistant Professor at Johns Hopkins.

Marie holds a Ph.D. in clinical chemistry from the University of Windsor.