Optimizing Therapeutic Proteins Through PEGylation: Key Parameters and Impacts

Professor João Gonçalves, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
Professor Paolo Caliceti, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

Introductions and Disclosures

Professor João Gonçalves
Welcome. My name is João Gonçalves, and I'm a Professor of Immunology and Biotechnology at the Faculty of Pharmacy at University of Lisbon. I'm thrilled to have you join me, myself and Professor Paolo Caliceti, today to discuss the topic of optimizing therapeutic proteins through PEGylation, the key parameters, and its impacts.

Please note that the content shared today is intended for educational purposes only. This webinar is based on our manuscript titled "Optimizing Pharmacological and Immunological Properties of Therapeutic Proteins Through PEGylation: Investigating Key Parameters and Their Impact," that was recently published in *Drug Design, Development, and Therapy*.

Editorial support for this webinar was funded by Chiesi Global Rare Diseases. All content is disease-state focused and the presentation will not include information on specific products as treatment for any conditions.

These are my disclosures.

Throughout the event, you'll have the opportunity to send in your questions. Simply click the *"Ask a Question"* button located below the video screen to submit your inquiries.

We are excited to hear about your thoughts, but please know that we won't be able to answer your questions live during the session. Your questions will be compiled and addressed in a second part of this video, which will be released in the coming months.

What Is PEGylation and Why Is It Important?

Professor João Gonçalves

What is PEGylation and why PEGylation is important? Therapeutic proteins have diverse origins and applications ranging from treating diabetes to cancer. Basically, we have seen all these approvals by FDA of therapeutic proteins since the '80s where you have the recombinant protein therapeutics like insulin. Then we move to the enzyme replacement therapy. We went to the fusion proteins. We started then with the ADCs in the early 2000 with site-specific PEGylation also with pegfilgrastim. We also moved then to the biospecific antibodies. More recently, we arrived to the 100th unique monoclonal antibodies.

Their molecular complexity and functional diversity make these drugs critical in modern medicine. As you can see, all of these proteins have grown in molecular weight, so the complexity and the structural complexity of these drugs have been really improving over the years.

From enzymes to vaccines, therapeutic proteins serve functions like replacing deficient proteins, augmenting pathways, or delivering cytotoxic payloads. As you can see here from the enzyme and regulatory proteins, in the function to replace all these functions and all these questions related to novel functions can be really useful in clinical applications such as diabetes or Fabry disease.

We also have these target proteins where we can deliver these conjugates that interfere with the molecular organism or deliver these cytotoxic payloads, for example, in clinical indications such as autoimmune disorders or cancer. But we also have the vaccines that can protect against foreign agents that are really key factors for vaccines against hepatitis B, for example, and also cancer. We can also see the protein diagnostics to detect diseases in indications, such as tuberculosis test or pancreatic dysfunction test. Their adaptability underpins their widespread use.

Potential applications of therapeutic proteins are really improving over the years. Despite these benefits, these proteins really face some challenges like immunogenicity, short half-life, or instability issues. But all of these are really due to the fact that we are really dealing with proteins.

The poor in-vitro stability, the low bioavailability, short half-life, the in-vitro stability that really can go on aggregation during storage and during production, that really can have an impact in immunogenicity. These questions limit their efficacy and require innovative solutions like PEGylation.

What Is PEGylation?

In this case, what is important here is to look at the fact that we have this PEGylation as something that is really important, is a key strategy, to increase and address the challenges that I have said before. PEGylation modifies proteins by attaching these PEG molecules, enhancing properties such as pharmacokinetics and reducing immunogenicity. Here, what we can see is that, in many ways, PEGylation modifies the characteristics of the protein. This PEGylation can be really conjugated in different strategies to different parts of the proteins, such as the N-terminus or the C-terminus, or inside the protein by having disulfide bonds. These represent really a significant leap in biotechnology.

PEGylation really addresses key limitations in improving stability, reducing aggregation, or enhancing half-life. Really, you can see that for enhancing, for example, the half-life, since the proteins usually have a short half-life except the antibodies, the potential to increase the pharmacokinetics is really, really high with PEGylation. In this case, the stability is also improved with PEGylation by having then the potential for lowering immunogenicity. These advancements really make it a cornerstone in modern drug design.

In this case, the clinical use of PEGylated proteins are starting to have a widespread in clinics. In cancer, in blood disorders, or immune system-related indications, we are having the opportunity to have these PEGylated proteins that really decrease immunogenicity of the native xenogeneic proteins.

The recently approved drugs that are PEGylated, recently approved by FDA, are really those that are targeting the ocular diseases, rare diseases, such as Fabry disease, pegunigalsidase alpha, and also the blood disorders, like neutropenia with pegfilgrastim. It's really important to see that the pipeline continues to grow, showcasing that this technique is really potential to improve the potential of an optimal drug.

Biopharmaceutical and Immunological Properties of PEGylated Proteins

Professor Paolo Caliceti

Hello, everybody. My name is Paolo Caliceti, and I'm a professor at the University of Pavado in the Department of Pharmaceutical and Pharmacological Sciences, where I teach pharmaceutical technology and drug delivery. I work since several years in PEGylation of proteins. In the next few minutes, and with the next slides, I will provide few information about the biopharmaceutical and immunological properties of PEGylated proteins.

Please remember, if you wanted to ask questions, to click on the bottom below, as was already mentioned by Professor Gonçalves before.

What is PEGylation? We heard by Professor Gonçalves the general introduction of PEGylation. PEGylation is a mature and unique technique to produce a new protein conjugate, new protein derivatives.

Since about 50 years ago, when it was proposed by **[inaudible 00:09:05]** and Davis who worked at the Rutgers University in the USA. PEGylation of protein became a mature technology that provided for a great solution for the delivery of proteins. The new class of drugs there, which were developed at the end of the past century, have had a lot of progress in formulation because they are big molecules, very fragile, they undergo degradation, denaturation, and whatever. So PEGylation provided a good solution for their formulation and delivery.

PEGylation is a technology which produces new drug entities by de facto because it is the chemical conjugation of a polymer, polyethylene glycol, to proteins. Since there is a covenant conjugation, the result is a new molecule, where the polymer and the proteins are covalently bonded to each other. So there is the possibility to obtain a number of different bioconjugates, which are not similar to each other because their structure and their performance in-vivo and the biological activity and biopharmaceutical properties depend on the polymer, depend on the proteins, depend on the number of polymer chains, shape, and the length of the polymer, the conjugation site, and the chemistry of conjugation. So they are not the properties of one

PEGylated protein that can be extrapolated to another. But, of course, we can have a general indication about the effect of polymers of PEGylation on the final bioconjugation.

What we have in terms of characteristic of PEGylated proteins and their properties are affected by the conjugation of the polymers. By the protein standard view, there are several modifications of the performance of the proteins after polymer conjugation.

First of all, it is the permanence in circulation, that was the first main problem, which was, in some way, overcome by PEGylation. Actually, proteins are rapidly cleared when their size is below 60,000 Dalton, which is the threshold for glomerular filtration. In the case of PEGylated proteins, the proteins have a prolonged permanence in circulation. This is due to a number of different effect, but the main effect is related to the increased size of the bioconjugate compared to the native protein. This is due to the fact that the PEG is a very hydrophilic polymer, each monomer coordinates 2 or 3 water molecules. And this has a result that the fact that the polymer is hydrated and the hydrodynamic size is much larger than the physical size and the duration or radius.

Accordingly, the PEGylated protein has a larger size and larger hydrodynamic radius, and this prevented the ultrafiltration. This is, of course, not the only mechanism. Accordingly, each of the circulation is prolonged. Also, the ultrafiltration by kidney is reduced because we usually think that ultrafiltration is just a simple filtration, but after, there are many other mechanisms, which include the structure of the protein, the uptake, re-uptake, and interaction of the protein with the glomerulus tissue. In this case, it is also masking.

In terms of biodistribution PEG and elimination PEG has a strong effect on the protein profile. The kidney is the main way still, the main way for elimination of the protein, and that depends on the mass of the polymers, which is conjugated to the protein.

The mass of the polymer depend on the size of the polymer chain and the number of polymer attached to the protein structure. When the polymer is very small, and we can say that the PEG with a molecular weight lower than 20 kiloDalton, the elimination is quite rapid, and it

decreases as the PEG increases in size and in ultrafiltration. So the higher is the mass of the polymer on the proteins are placed, the slower is the kidney filtration.

High molecule with PEG dictates also the accumulation of the conjugate, even in low amount in liver and in the lymphatic system. Also, PEG has an effect in what is called the cellular vacuolisation, where there is the formation of vacuoles into the cells, which is, however, not a permanent effect and that they disappear throughout the time.

PEG has also a great relevance in protein stability, either for what concerns the physical stability of the proteins, and the stability towards proteases. In terms of physical stability, PEG, which is a very hydrophilic material, prevented the association, aggregation, and the absorptions on the surfaces of primary packaging. In terms of protein stability towards proteases, the polymers cloud around the protein, prevent the approaching of enzymes and the recognition of the sequences, which are prone to degradation. So there is a protection from degradation.

Finally, the PEG on the surface, also reduces the immunogenicity of proteins, especially normal molecules proteins, and xenogeneic proteins, which are often used in therapy. So the immunogenicity is decreased because the protein is not recognized by the immune system. There is also a reduction in antigenicity because there is a lower interaction of preformed antibodies, which are recognized in the protein because the protein is protected into the core of this conjugate. As a result, we have an increased stability and prolonged permanence in the body.

The benefit of PEGylation is well-represented by the example of interferon. Interferon is a drug with high effect and therapeutic activity against the hepatitis C virus. The traditional therapy was frequent administration of this cytokine, which was, at least, 3 administrations per week. That was due to the fact that this protein, which has more or less a molecular weight around 20 kiloDalton, is rapidly cleared from the body, so it was necessary to boost the administration. Unfortunately, the frequent administration of this interferon had a very, very high side effects, adverse effects, in terms of pain, in terms of headache, high temperature, that was very highly debilitating and prevented the therapy in some way.

The result that was obtained with PEGylation, it was to obtain new interferons which have much lower side effect and that could allow for a single administration per week. We can see here the pharmacokinetics of the native interferon in green, and in color red, we can see 2 different PEGylated interferons.

With the pink, we can see interferon PEGylated with a 40-kiloDalton branched PEG, and we can see that the permanence in the bloodstream of the conjugate, and in light pink, we can see the activity of this conjugate, which has slowly decreased throughout the time. In lighter yellow, we can see pharmacokinetics of interferon modified with a linear 12-kiloDalton PEG. In orange, we can see the activity of this conjugate, which decreases over throughout the time.

The PEG structure affected the properties of PEGylated proteins, and we have different PEGs that can be used for the protein conjugation, and that they are different by molecular weight and shape. Also, we can have different conjugation chemistries, and also we can obtain derivatives with a different number of PEG chains attached to the proteins.

As a result, we obtain derivatives with a variable mass of PEG conjugated to the protein, and the result is extremely different, 1 by 1 to each other. We can have a single linear PEG with different molecular weight, which can be attached to a single site of the protein. We can add also a branched the PEG, and that was used, for example, for interferon, and in such a case, it was a 40 kiloDalton PEG, which is targeted to one side of the protein.

We can also have a single-branched PEG with three arms. It is a different-branched PEG. We can have several PEG chains attached to the protein, and also in a very few cases, we can have also cross-linked dimeric PEGylated proteins, which are obtained by using PEG, which is functionalized to both at the end of the polymer chains.

The conjugation of this PEG have different effects in terms of permanence in the blood streams and in terms of stability of the protein, in the immunogenic profile of the proteins, and also in terms of activity. For example, as reported here, the attachment of 40 kiloDalton PEG branched to interferon produced derivatives, which maintains only 7% of the activity of the native proteins. This is a very high decrease of activity, which is compensated anyway by the excellent

performance in terms of permanence in the bloodstream and in terms also of reduced side effects.

Clinical Advantages of PEGylation

We have several clinical advantages of PEGylation. As I mentioned before, PEGylation can increase the half-life of the protein by up to 20-fold in humans. We can see here the case of pegunigalsidase alpha. We have in these derivatives, we have 4 PEGs with a molecular weight of 2 kiloDaltons, which are conjugated to the proteins, which is used in the Fabry disease. There are two molecules of proteins attached to these polymers. The result is an increase of the permanence of the protease from 3 hours to several hours, between 53–121 hours.

There is the protection from proteolysis, which has been observed in mice. There is an increase in thermal stability and slow down diffusion. Also, this is used to have a prolonged permanence in the bloodstream when it is administered by intramuscular or subcutaneous administration.

And then PEGylation decreases the immune reactivity of the protein, and this allows for the administration also of non-human proteins, such as the case of asparaginase and adenosine deaminase, which are not human proteins. Their use was limited by their high immunogenicity, and the use of polyethylene glycol reduced the immunogenicity of the protein, and it allowed multiple administration.

The potential drawbacks of PEGylation, as I mentioned before, is a decrease of bioactivity that depends on the protein and the protein activity, and also on the mechanism of activity and the PEGylation process, the number, the mass of the protein, the polymer attached to the protein, the dramatic effect of conjugation of 40 kiloDalton PEG to interferon, which reduces the activity of 93%. It's not so high in many cases, and there are techniques to prevent this high decrease of activity.

One reason of the reduction of activity is the conjugation of PEG to amino acids, which are involved in the biological activity of the proteins. One strategy to reduce this decrease of bioactivity is to conjugate PEG to amino acids which are far from the active site of the protein.

Another drawback is the anti-PEG antibodies, which in a few cases represent a limitation for the long permanence of the proteins. Anti-PEG antibodies can be generated by administration of the related proteins, but in many cases, they are already present in the organism because they are produced by the use of cosmetics or other products.

Then there is the immunoreaction to alter the protein formation because PEGylation is a chemical conjugation to protein, and this chemical conjugation to proteins can result in alteration of the protein structure, and the protein could move to be more immunogenic.

What are the technologies used for PEG conjugation by a chemical point of view? One of the first method used for protein conjugation was the reaction of activated PEG to the amino groups of the protein. This is due to the fact that the amino groups of the proteins are very abundant in the protein structures, and they are exposing on the surface, and they are rather well reactive. So it is rather easy to conjugate polymers out to these amino groups.

However, throughout the years, several other methods have been developed to obtain more selective conjugation and to exploit also different functional groups of the proteins. For example, the conjugation to disulfide bonds, and we will see later an example, the conjugation of specific amino acids, and also the conjugation to thiol groups.

Chemical Conjugation of PEG

We can see here the conjugation to the PEG and several different derivatives. For example, the aldehyde groups is well-used to conjugate the terminal amino groups of proteins. This is rather selective by working at specific pHs, which make these amino groups more reactive than the lysine amino groups of the proteins.

Then there is the isocyanate, the phenylglyoxal, and then there are several derivatives which have been used for conjugation to the thiol groups. Conjugation of thiol groups is very interesting because thiol groups are very subtle in the protein structure, and usually they are engaged in disulfide bonds because they are exploited inspired by protease to stabilize the protein.

But it is very easy to obtain derivatives of proteins by genetic engineering, introducing cysteine in the sites of the proteins, which are far from the active sites of the proteins. And then it is possible to conjugate selectively these thiol groups by using specifically activated polyethylene glycols. It is possible to have a site-directed conjugation, and this allows for the obtaining of derivatives with a very well-defined structure.

Finally, it is reported, this very interesting method for conjugation of PEG to the disulfide bond, which was developed by Steve Brocchini a few years ago.

The other methods to obtain selective conjugation is the use of enzymatic conjugation, and there are two methods which have been particularly investigated. The first one is based on transglutaminases. Transglutaminase allows for the conjugation to amino groups, to glutamine groups. It is possible to use a PEG terminating with an amino group to conjugate to the glutamine functional groups of proteins or vice versa. This is very interesting because enzymes are very specific in the substrate of conjugation.

Another method was developed a few years ago in 2006 by DeFrees, and this is glycoPEGylation, and this is a multistep conjugation which exploit different enzymes. The first step is the glycosylation of sites in which have not been engaged in glycosylation naturally, and, for example, serine or threonine. And then there is the conjugation of PEG to a salinated PEG to a sialic transfer. Also, in this case, it's possible to obtain derivatives with a selected site of PEGylation.

Differences in PEGylation Methods: Interferon

In this slide, I will show you the example of interferon that I mentioned before. This is very interesting because there are two different strategies to obtain derivatives with a very high therapeutic activity, interferon Alpha 2b and interferon Alpha 2a.

In the first case, in the case of interferon Alpha 2b, we have the conjugation of PEG 12 kiloDalton to the amino groups of the protein. One of the conjugation was obtained at the histidine 34 of the sequence of this cytokine. This derivative is very interesting because the bond is cleavable, and the results is that the PEG is released throughout the time after

administration, and the genetic protein is released. There is not the problem of high influence of the polymer with a reduction of the activity by masking of the active site.

Indeed, these derivatives maintain more or less 35 percent of the activity of the native protein, and this is due to the fact that the molecular size of the polymer is only 12 kiloDaltons. It's a linear polymer. Only one polymer chain is attached to the protein, even though in a random way, but only one polymer per protein molecule. Nearly 50% of the polymer is attached to histidine 34 by obtaining a releasable PEG.

On the other side, we have interferon Alpha 2a. In such a case, a 40 kiloDalton PEG was conjugated to the protein. The bond was stable so that we didn't have a releasable PEG. The high molecular weight and the branch shape of the polymers produce a derivative with only 7% of activity as I said before. The two derivatives are both excellent therapeutic agents. They have been used for, and they are still used, in therapy with high activity and excellent performance. Even though they are different in terms of activity, but they have also differences in terms of permanence in the bloodstream.

In the conjugation method that affect the protein properties, and in particular, they affect the permanence in the bloodstream and the immunogenicity of the protein and also the stability. Usually only PEG is activated on one side to obtain mono PEGylation. That means that one PEG chain is attached to the protein. When the PEG is activated to both sides of the chain, we obtain a functional PEG with the possibility to obtain cross-linked bioconjugate, like in the pegunigalsidase alpha.

Then we have the different conjugation method, and we can obtain a single, one only polymer pair proteins, such as in the case of the interferons, or we can have several polymer chains attached to the proteins, like in PEG-asparaginase or PEG-uricase or PEG-adenosine deaminase.

In both cases, the conjugation is random, so the PEG is conjugated to different sites of the proteins, and the activity of the protein is an average of the activity of all the isomers which are obtained. Alternatively, there is the site-directed conjugation by using different strategies like the cysteine conjugation or the conjugation to the terminal amino group or by using enzymes.

In this slide, we can see the effect of the PEGMAs of the conjugator on the permanence in the bloodstream, and as an example of avidine, which was conjugated with a PEG 5 kiloDalton, PEG 10 kiloDalton, PEG 20 kiloDalton. All these PEGs are linear chains. We can see that by increasing the molecular weight of PEG, there is a significant remarkable increase of the permanency in the bloodstream.

On the other side, there is an example of the use of PEG in comparison with different polymers, which were used throughout the years as alternatives of PEG, such as polyvinylpyrrolidone or polyacrylonitrile morpholine, and also the results obtained with a linear PEG or a branched PEG.

Here is reported the immunogenicity of uricase, which is a heterologous protein with high immunological properties, immunogenicity. What we can see here is that, actually, polyvinylpyrrolidone and polyacrylamine are not very effective in reducing the production of anti-uricase IgM and anti-uricase IgG.

Both of them are not very protective, and they do not reduce very much the immunogenicity of this protein. On the other side, PEG, linear PEG, is much more effective in reducing both IgM and IgG, but the best performance was obtained with a branch of the PEG because branch of PEG has the ability to cover the protein surface like an umbrella, for protecting the protein inside from the recognition of the immune system from antibodies, anti-protein, and also from enzymes and from degradation.

So, I hope my presentation here, was useful for people who wanted to approach PEGylation and wanted to know something more, and, be useful for anyone who is interested in these topics.

I'm, of course, available to answer any question that you want to pose and, as it was said at the beginning, please, use the button and click and, post your questions, and I will be more than happy to answer your questions and to discuss with you any issue that you find interesting and, you need the clarification. Thank you very much.

Immunogenicity

Professor João Gonçalves

Going now to immunogenicity and the impact of PEGylation on immunogenicity. Immunogenicity refers to the immune system response to therapeutic proteins, which can negatively impact the clinical benefit risk ratio. Immune response may neutralize the drugs biological activity, accelerate its clearance, or even cause adverse events such as systemic allergic reactions.

So when we look at immunogenicity, we have to look at immunological aspects of the therapeutic proteins. So when you have immunogenicity, we have antidrug antibodies that can be a humoral mediated by b-cells or, cell mediated involving, t-cell responses.

What is important here is that these antibodies may neutralize the drug, cross-react with endogenous proteins that are similar to the drug, or increase the risk of, adverse events. This underscores the need for strategic strategies to mitigate these risks during the drug development. So in order to avoid this negative impact of immunogenicity on pharmacokinetics and pharmacodynamics.

Clinical Consequences of Immunogenicity and Strategies to Overcome It

Immunogenicity can lead to serious consequences in many cases, but what is important here is that when you look at the clinical sequela, we can see that the impact of the clinical symptoms and signs usually is really in a low frequency. We have more binding antibodies or PK-altering antibodies, then we can have neutralizing antibodies, but really, the most impact is really when you have allergic ADAs across reactive neutralizing ADAs.

So we have examples of immunogenicity issues with therapeutic proteins such as, in anemia where we have this study where we can have, immunogenicity against recombinant EPO when they were caused by neutralizing anti EPO, antibodies, but also we can have that in IBD for antibodies, for therapeutic antibodies such as infliximab.

So to mitigate, these problems to have mitigation strategies, we can modulate the patient's immune system, the immune response of the patients. Usually, it's not so well appropriate, but

we can have immune suppression or immune tolerance induction, but we also can modify protein characteristics such as reducing administration frequency, removing this TB cell epitopes, and shield the epitopes with extend off life, strategies such as PEGylation.

So in this case is really what is the important point here is really to mitigate these factors that really can alter the the the protein characteristics in terms of a drug. So PEGylation can mask antigenic epitopes and reduce immune recognition by creating this hydrated shield around the protein.

This really can potentially decrease immunogenicity and prolong drug action by reducing administration frequency and stabilizing the protein. So, basically, when you have a longer ALF life, you have this potential to reduce drug administration frequency. We might have this opportunity here to reduce the immunogenic responses.

PEGylation can sometimes trigger an immune response against PG, or PEG related protein. So, usually, we what we can have in many cases is anti-PEG response that can be weak or may not cause adverse reactions.

Usually, this is against these small stretches of PEG, but usually when they have a long PEG stretch, a long PEG polymer, we might have a longer and more reactive immune response. So what is important is that the anti-PEG antibody response may be increased when the protein is or a human protein that is deficient in the recipient or a high immunogenic nonhuman protein.

Neutralizing ADAs against these PEGylated proteins may sometimes occasionally cause serious conditions, like, for example, in a PEGylated thrombopoietin, and this, the PEGylated thrombopoietin was discontinued after neutralizing anti TPO ADAs that really cause severe thrombocytopenia in 30 patients out of 325 healthy volunteers and also in patients treated with the drug.

So this might occur in certain aspects of PEGylated proteins that really can have developed this decreased efficacy due to neutralization, decreased bioavailability to the accelerated clearance, and cause these hypersensitivity allergic reactions.

Impact of Pre-Existing Anti-PEG or Anti-Drug Antibodies

Another point which is quite important here is the impact of preexisting anti-PEG or antidrug antibodies. So this is something that we came to know in the last 5 to 6 years is that, basically, some patients around, for example, 70% of the general US population have anti-PEG antibodies. This might occur due to the exposure of PEG that happens through food, cosmetics, and medicines, and patients treated with PEGylated drugs can have this contact to these preexisting, and induced anti-PEG, antibodies.

So this presence of anti-PEG antibodies may not always be clinical relevant, and this is really important to see in many studies where these anti-PEG antibodies that the preexisting in patient that started therapy with PEGylated proteins could not see an effect of an adverse reaction of a reduced activity of the drug.

The exposure to preexisting ADAs is something that should be really, analyzed and evaluated in these clinical studies with PEGylated drugs. So the risk of immune response is really influenced by the unique properties of the PEGylated protein.

What we have seen from Professor Paolo Caliceti is that usually PEGs, they have different molecular weights. Some proteins have a high molecular weight and size that usually are associated with a higher reactivity. The site of attachment, and linker properties is also important. Usually, we know that the conjugation to a cystine residues or terminal immunogroups can cause less immunogenicity.

We also have this branched versus linear PEGS that are really associated with the NAS immune shielding. The site specific PEGylation also minimizes the exposure of new epitopes and ADA formation, and really PEG by itself is weekly immunogenic. The problem is that sometimes in due to the technology, when you do conjugation with PEG, sometimes we can expose, epitopes that usually are not present in the original protein.

Besides this, we also have protein and patient-related factors that also impact the risk of immunogenicity. We know that immunogenicity can be triggered by therapeutic protein-related factors, as I said before, due to the origin, the post-translation modification, stability of

the protein and aggregation, but also patient related factors. We know that age and sex is one really important factor that might trigger patient related immunogenicity.

The haplotype of the patient due to these genetics of HLA, the previous exposure to PEGylated proteins might trigger the development of anti-drug, antibodies against PEG, but also the fact that the disease, the concomitant treatments of the disease, whether the disease is chronic, or the disease is autoimmune, we may also have here the possibility to induce these immunogenic responses.

But the fact that we also have another characteristic, which is the drug administration and target factors, like dosing, the frequency of and duration of treatment. We know that probably subcutaneous administration is more immunogenic than IV administration, and also the target nature of the target or to the drug, whether it is at the cellular membrane or if it is soluble. All these factors might implicate immunogenicity, so we cannot say that immunogenicity comes from one factor, but it's a multifactorial way of developing antidrug antibodies.

Monitoring for Immunogenicity

Now we have to monitor immunogenicity, and monitoring immunogenicity is essential, especially in groups or patients that are at risk, such as atopic patients or those which are pretreated non-PEGylated proteins. So these ADA, levels can be measured using various assays helping to detect or re to reduce efficacy, or adverse reactions very early.

In this case, really, what is important is to assess these ADAs in a regular way. It's not possible here to understand the development of immunogenicity if you just measure once a year. You really have to maintain the monitoring, during 3 or 4 times a year really to assess the kinetics of development of, immunogenicity against any biological drug.

The measurement of ADA, we have screening assays that usually are done by direct or indirect, ELISA. Usually, we have we need to have a method that is really not implicated with the development of false positives or false negative results. Usually, these tests must be tolerant to the drug, so it's important here that you don't have a high concentration of the drug when you measure anti-drug antibodies, and then you need to have a confirmation, assay.

It's a screening assay, a confirmation assay, and then you can characterize the types of antibodies. To characterize is really to do the titration of these antidrug antibodies, titrate the immunogenic response, but also the neutralization, to see whether these, antidrug antibodies neutralize, not only the activity of the enzyme, but also the entrance of the enzyme in the cells, for example.

It's important really to assess the best ADA assays. So they should be tolerant, as I said to the therapeutic drug. They should have susceptibility to interference by serum components. They should be really aware of that. The variable specificity of ADA should also be done and assessed during the monitoring.

The consistency of the ADA testing will allow us then to do comparable, evaluation of the different results that the clinician is having over the years. So it's very important here that when you measure ADAs, you should measure several times a year, but it's also very important that the clinician can really maintain the consistency of the method that is being used in order to compare the results over the years.

Further research is really important here and should address the remaining challenges to enhance this therapeutic potential of, PEGylated proteins. So more research is really essential to further enhance this potential of PEGylated proteins such as understanding immunogenicity. We really need to see the mechanisms why some patients have more immunogenicity than others.

The factor of the patient is really important to be a target of research. Also, other factors that influence long term safety, should be evaluated and research on these long term studies and understanding the impact of preexisting ADAs is very important because we need to understand whether the titer of antidrug antibodies are maintained over time or are reducing over time in order to evaluate whether you are creating immunogenicity or if you are creating tolerance for that drug.

We need also to do research on optimizing PEGylation, develop new PEGylation techniques. There are several, as Professor Caliceti was saying, but we need really to invest more on maximizing the therapeutic efficacy and minimizing the adverse immune reactions in order

really to have this personalized medicine that really tailored PEGylate design to individual patients.

Conclusions

Professor Paolo Caliceti
Well, just to draw some conclusions for what concern the strategies for production of PEGylated proteins. What we can conclude is that there is not one single specific strategy that can be used for all proteins. The conjugation proteins must be designed and set up according to the protein and the structure of the protein, the therapeutic performance of the protein and its biopharmaceutical, physicochemical, immunological properties.

Today, there is a large number of possibilities to obtain different bioconjugates, and each conjugation strategies must be designed according to what is the expected result that we all wanted to obtain and which kind of derivatives we wanted to obtain.

We can choose a branched or linear PEG. For example, monometoxy-PEG, which is mono activated in order to skip the possibility to have a cross-linked product. We can also use alternative to hydrophobic metoxy-PEG such as hydroxy-PEG, and these derivatives have been found to produce less buckles or to be less immunogenic than monometoxy-PEG.

There is the possibility to use PEG oral MERS administered before regulated drugs. We can use high molecular weight or low molecular weight polyethylene glycol. We can also design conjugation in order to have a random conjugation. When it is possible it is simpler, but it is not the best way because the the the derivatives are not well very characterized.

Much better is to have site specific conjugation with respect to random conjugation. Random conjugation is possible, but it entails the risk to have a high decrease of activity in because there is the contribution of the polymers to the protein site, which could be very close to the active site of the proteins.

There is the possibility now to have selective conjugation by attaching, for example, polymers to the terminal amino groups or to the cysteine, which are genetically introduced in the

proteins to obtain mutants or also by enzymatic conjugation. There is also the possibility to control the reaction conditions in order to favor the conjugation of the polymers to a specific site of the proteins.

One important thing is also the chemistry, which is used for the conjugation because also the link used for the conjugation of the polymers to the protein has an effect in the performance of the proteins, the biological performance, the physical-chemical properties, and also the immunological profile of the proteins.

Professor João Gonçalves
Basically, the conclusion here is that both drug and patient characteristics should inform PEGylation strategies. We know that PEGylation design should consider these two factors. For example, smaller PEG chains may be better suited for patients with preexisting anti-PEG antibodies while site specific conjugation really reduced risk of immune reactions.

We know that patients have characteristics such as the genetics, the previous exposure to PEG, healthy conditions, pre-existing ADAs. So the risk of developing immunogenicity in some patients is much higher, and we also know that a large majority of US patients, they have anti-PEG antibodies pre-existing before the therapies.

It's really important to have a drug which is really tailored to these characteristics. So a short stretch smaller back chains are really important in this case of patients that are at risk of developing immunogenicity. As a conclusion, regulation really offers clinical benefits to protein based drugs. Potentially lowering immunogenicity and improving stability, decreasing aggregation, reducing the capacity of developing immunogenicity, improving bioavailability and half life.

PEGylation is most beneficial for therapeutic proteins that they have this high risk of developing the immunogenicity and suboptimal pharmacokinetic features. This is really important because pharmacokinetic features of some proteins, which is really the short half life and poor stability really are a negative side of certain proteins. Improving that with PEGylation is very important.

Certain properties of PEGylated proteins affect immunogenicity in different ways and really can either reduce or increase, immune reactions. Further research to conclude is really needed to optimize speculation design strategies, really, to overcome the risk of triggering an immune response.

Thank you so much for joining us to listen our presentation on optimizing therapeutic proteins through PEGulation. We hope you found the session valuable, and this is providing to you, and we have provided you with useful insights. We'd love to hear from you. If you have any questions or thoughts about the webinar, please don't hesitate to send them in. Please simply click the ask question button below, and we'll make sure to address them in the second part of this video, which will be released in the coming month.

Note that when you ask a question, you'll have the opportunity to answer three quick questions about your experience with this webinar. We'd love to hear your thoughts. Thank you again for your participation, and we look forward to connecting to you soon.

Professor Paolo Caliceti
Thank you.

Professor João Gonçalves
Thank you.