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Coimbra_____ de_____ de 2015.

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Coimbra,___ de Setembro de 2015

Acknowledgments

I would like to express my gratitude to Vera Moura for letting me know more about PEGASEMP™ and for being so helpful and available from the beginning. I wish her all the best for her project and for TREATU.

My sincere thanks also goes to Professor João Nuno Moreira for proposing me this challenge and for showing his support and guidance.

To my family for their love and encouragement through my study and to my friends for the great times.

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Abbreviations

ADME - Absorption, Distribution, Metabolism, and Excretion

ASTM - American Society for Testing and Materials

CHMP - Committee for Medicinal Products for Human Use

CTA – Clinical Trial Application

CMC - Chemistry, Manufacturing and Controls

CTD – Common Technical Document

DLS - Dynamic light scattering

EMA - European Medicines Agency

EPR - Enhanced Permeability and Retention

EU – European Union

FDA - Food and Drug Administration

GLP – Good Laboratory Practices

GMP – Good Manufacturing Practices

HPLC – High Performance Liquid Chromatography

IMPD - Investigational Medicinal Product Dossier

IND – Investigational New Drug

ISO – International Standard Organization

NCL – National Characterization Laboratory

NDA - New Drug Application

PEG - Polyethylene glycol

PK – Pharmacokinetics

RP – Reflection Paper

Abstract

The nanotechnology products' approval for clinical trials appears to be a highly regulated and costly process. The regulatory framework applicable to innovative products, in particular, is still unclear on the characterization parameters required for clinical trials authorization. This monograph efforts to specify which physicochemical parameters would be needed so that an innovative liposomal nanoproduct, PEGASEMP™, could successfully initiate its clinical trials in the European Economic Area. PEGASEMP™ is the first platform of the spin-off TREAT U, and it is notorious for having certain features such as its size, drug release mechanism and coating.

The strategy used for the monograph was to review existing legislation and guidance in order to draw up a list of PEGASEMP™'s critical parameters that should be submitted on the clinical trial application. The description of the techniques used to determine these parameters are also mentioned in the monograph. The monograph will be part of the investigational work that is being carried out by TREAT U to prepare for the clinical trial authorization.

Resumo

A entrada de um produto nanotecnológico em ensaios clínicos afigura-se um processo altamente regulado e de investimento elevado. No caso específico dos produtos inovadores, a regulamentação é ainda muito generalista no que refere aos parâmetros específicos de caracterização para entrada em ensaios clínicos. Esta monografia procura especificar os parâmetros de caracterização físico-química necessários para que um nanoproduto inovador liposomal, PEGASEMP™, seja aprovado com sucesso para a realização de ensaios clínicos no Espaço Económico Europeu. O PEGASEMP™ é a primeira plataforma da spin-off TREAT U e é dotado de características muito particulares como o seu tamanho, drug realease mechanism e coating.

A estratégia utilizada nesta monografia foi a revisão da legislação e normativas existentes, tendo em conta a categoria e as características da molécula referida, para elaborar uma lista de parâmetros críticos que devem constar na caracterização do PEGASEMP™ para pedido de entrada em ensaios clínicos. Foi feita também uma descrição das técnicas de caracterização correntes para avaliação dos parâmetros referidos. A monografia será incorporada no trabalho de investigação da empresa TREAT U para preparação do pedido de entrada em ensaios clínicos.

1. Introduction and background

1.1. Nanomedicine and nanoparticle drug-delivery systems

The field of nanotechnology has been expanding steadily over the past few decades. At this present time its applications range from the food industry to electronics and space engineering. Naturally, Nanomedicine has also been evolving as a multidisciplinary science that seeks to maintain and improve human health at a molecular scale. According to the definition used by European Technology Platform on nanomedicine we can also add that "it exploits the improved and often novel physical, chemical, and biological properties of materials at the nanometric scale". The resulting products have presented unique solutions on the diagnosis, monitoring, control, imaging, prevention and/or treatment of diseases by reason of their novel profile. They have proven to be more advantageous on many levels, especially toxicologically and on effectiveness, comparing with their bulk or small-molecule analogs.

Patenting activities of these products have shoot up since the beginning of the past decade and, as a matter of fact, the European Patent Office reveals 2000 patent filings in the nanomedicine sector in the year 2003, up from 220 in 1993. It also discloses that the United States were the leading researcher on this field, accounting for 32% of the publications and 54% of the patent filings in 2006. As for the topic of research, it showed that "Drug delivery" was the dominant one with a share of 76% of the scientific papers and 59% of the patents. [1] This trend is likely to continue in the years to come , being cancer targeting nanoparticles the medical products with the biggest boost on the number of patents and overall value [2].

The result of the massive investment put into nanodrug delivery systems can be witnessed by the number of nanoproducts either in development or on the market , such as Doxil/Caelyx®, Abraxane®, Amphotec® , Genexol® [3]. .The main idea behind these therapeutics is to make drug fate dependent on the carrier in which it is encapsulated. These drug-load carriers come in different sizes, architectures, materials, coatings among other traits that modulate their biological behaviour and hence the distribution of the drug they foster. Liposomes, dendrimers, gold nanoshells, quantum dots and fullerenes are the most frequent examples of nanodelivery systems. They have received great attention from the scientific world as they represent a revolutionizing medical therapy, particularly in cancer therapy, with efficient and smart therapeutics[4]. Besides being capable of addressing several drug delivery problems, which could not be effectively solved in the past (e.g. targeting, cell barriers, cell resistance) these nanoparticle carriers can also reduce systemic side effects of the carrying

drug and have a better ADME, safety and biodistribution profiles than their conventional counterparts. However, it is undeniable that nanomedicine is still at its early stage and success stories are few and isolated [5, 6]

1.2. Background information on TREAT U and PEGASEMP™ early development

TREAT U, S.A. was set-up as a spin off from the University of Coimbra, on January 2010. This spin-off was born out of sheer necessity after a series of promising results made by a doctoral program on a new lipid-based nanoparticle loaded with doxorubicin (PEGASEMP™). The project headed by Vera Moura, dates back to 2006 and it developed a nanoparticle that circumvents some of the limitations of conventional chemotherapeutic drugs. PEGASEMP™ owes much of its success, as potential drug against breast cancer, to its stealth specific and triggered drug delivery. The nanoparticle itself and most of the features that enable a safe and effective delivery of the drug into the cancer cells and tumour blood vessels will be further discusses on the next topics and along the monograph.

TREAT U has grown into a company with a vast experience and knowledge in the field of Biotechnology and Pharmaceutical Technology. In addition, the TREAT U team has grown in collaborators as well, adding more value to the company with their expertise. Other partners, such as the CNC from the University of Coimbra, Bluepharma and Biocant Park among others have also stepped in, providing financial and intellectual support.

PEGASEMP™ which is TREAT U's first platform has now two patents granted in the US and has already filled a Patent Cooperation Treaty (PCT) in Europe. After some major breakthroughs, such as the validation of the primary pharmacology and the proof-of-concept, PEGASEMP™ has reached preclinical studies for advanced breast cancer. It is now facing a critical stage before entering into the clinical trials, which TREAT U team expect it to happen by the end year 2015. The "leap" into the clinical phase, which most new drugs do not make, requires further insight into PEGASEMP™ in order to meet the tight regulatory requirements.

2. PEGASEMP™ - an innovative nanoproduct

2.1PEGASEMP™'s description and mechanism of action

PEGASEMP™ is a new nanoformulation of doxorubicin. It is a liposomal product and it is comprised of three main components:

a) A carrier which is composed by five synthetic lipid components cholesterol (CHOL), cholesterol hemisuccinate (CHEMS), *dioleoylphosphatidylethanolamine*

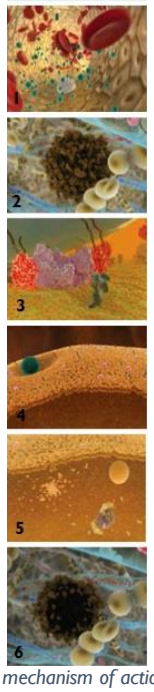
(DOPE),*dioleoylphosphatidydistearoylphosphatidylcholine*(DSPC), *distearoylphosphatidylethanolamine methoxy (polyethyleneglycol)*(2000) (DSPE-PEG). DOPE and CHEMS are yet to be approved for human use by FDA.

b) A targeting ligand, which is covalently conjugated to DSPE-PEG of the carrier through a 5-carbon spacer. The synthetic ligand is a 31 aminoacid F3 peptide which binds specifically to a membrane receptor, nucleolin. This receptor is overexpressed in cancer cells and endothelial cells from tumour blood vessels.

c) Doxorubicin, an anthracycline with cytotoxic and anti-angiogenic properties.

PEGASEMP™ is a lipid-based nanocarrier that delivers effectively its doxorubicin payload into specific cell populations in the tumour microenvironment, namely tumour cells and endothelial cells from tumour blood vessels. The mechanisms of action can be briefly described as the following:

1. The nanoparticles flow through the bloodstream demonstrating biological compatibility and immune stealth ability.
2. PEGASEMP™ escapes from bloodstream into the tumour mass, through the tumour blood vessels nanopores.
3. Targeting ligands bond specifically with receptors on the tumour cells.
4. The nanocarrier is internalised into the cell.
5. The nanoparticle releases the doxorrubicin into the tumour cell by a pH triggered system.
6. Doxorubicin displays its cytotoxic action leading to the cells death and tumour recession.



Picture 1 - Proposed mechanism of action

Source: TREAT U

A number of properties change when a material enters the nanoscale. PEGASEMP™ (100-200nm) as all nanoparticles is no exception to that, taking advantage of them for therapeutic purposes. Nanocarrier usually have better distribution and efficacy profiles than bulk drugs as well as higher reactivity, due to larger surface-to-volume ratios, which can mean improved solubility and cell uptake. Size alone can also grant to PEGASEMP™ enhanced permeability and retention (EPR), which results in the nanoproduct penetration of leaky tumour vasculature and accumulation in the tumorous tissue.

PEGASEMP™ is also a product of the latest nanoengineering advances. The surface which is covered by an hydrophilic polymer (PEG) is key to overcome solubility issues, and among other effects it also shields the nanocarrier from proteolytic enzymes and avoids its opsonanization by immune cells, which means longer half-life circulation and improved pharmacokinetics [7]. PEGASEMP™ is also endowed of specific targeting moieties on its coating which facilitate the delivery to tumour cells overexpressing the receptor through active transport.

Proliferative cancer cells exhibit an increased metabolic rate, which resorts to glycolysis most of the times, producing an acidic environment[8]. PEGASEMP's pH sensitive controlled burst release takes advantage of that peculiarity for an increased therapeutic efficacy. Moreover, it decreases cancer cell invasion to healthy tissues and reduces the drug's overall toxicity, even after the cell lysis[9].

Overall PEGASEMP™ can turn a doxorubicin chemotherapy into safe and efficient one, the free-drug therapy would have high risk-benefit ratios with major cardiotoxic issues[10].

The drug delivery and targeting features here listed are combined into one single nanosystem, which classifies PEGASEMP™ as a multifunctional nanocarrier [11, 12] .PEGASEMP™ is also versatile enough to encapsulate other small weight drugs, small interfering RNA, plasmids or recombinant viruses. These pose major advantages over similar competing drugs such as Doxil®, the first FDA approved liposome[13].

2.2.Proof-of-principle studies[14]

The results, conducted in an orthotropic model of human breast cancer, demonstrated that PEGASEMP™ is capable of targeting the tumour at two distinct levels, both the cancer cells and endothelial cells from blood vessels that nurture the tumour. In addition, it shows that pH-dependent triggered release mechanism maximises the specific accumulation of the drug and increases therapeutic efficacy. In view of the promising results, TREAT U took the decision to proceed to further clinical development of PEGASEMP.

In Vitro*1 (PEGASEMP™ Vs Control*2)	In Vivo*4 biodistribution and efficacy studies (PEGASEMP™ Vs Control*3)
• Specific cellular association in cell lines overexpressing cell membrane nucleolin • Increased delivery of payload	• 4-fold increase in doxorubicin tumour accumulation (24h after IV injection) • Tumour-to-heart ratio of 80.8 Vs 3.3 for the control (24h after IV injection)

• 177- and 162- fold increase in cytotoxicity of doxorubicin against human breast cancer and tumour endothelial cell lines, respectively • 71% decrease in vessel formation vs 29% for the control • Higher levels of association with cancer cells harvested from human patients diagnosed with breast cancer	• 50% reduction of the viable peripheral area of the tumour Vs 25% for the control • More evident signs of extensive cell death at tumour periphery • 75% decrease in microvascular density within the tumour, Vs 20% for the control • More marked suppression of the tumour invasion to adjacent healthy tissue

Table 1 - Major findings of Preliminary pharmacologial studies

*[1]-results generated with PEGASEMP™ in breast cancer and tumour endothelial cell lines

*[2]-Non-targeted non-pH-sensitive lipossomes

*[3]-Non-targeted non-pH-sensitive doxorubicin formulation

*[4]-performed in an orthotopic model of human breast cancer with tumours implanted in the mammary fat pad of Balb/c nude mice

The results from these preliminary studies are needed to effectively design the more definitive preclinical studies, particularly the toxicology and drug metabolism evaluations needed to support an authorization for first-in-human studies[15].

2.3. Present strategies and future perspectives

TREAT U aims at the internalization of PEGASEMP™ in the European and American market, as well as the clinical approval for cancer with different histological origins. Moreover, PEGASEMP is a platform suitable for incorporation of other drugs and combination of drugs. TREAT U is preparing a Clinical Trial Application (CTA) to be submitted for the approval of PEGASEMP™ clinical trials for advanced breast cancer to be conducted in EU. The Investigational Medicinal Product Dossier (IMPD) is the core document that composes the CTA. The dossier will compile information related to the quality, manufacture and control of PEGASEMP™, data from non-clinical studies and from its clinical use (upon availability). This monograph will focus on the preclinical characterization and will expressly indicate the psychochemical characterization for the Chemical Manufacturing Control (CMC) that will be part of the IMPD's Quality Chapter.

3. Regulatory framework

3.1. Regulatory Gap

Nanomedicine and nanotechnology are not a consensual topic among scientists. The definitions and the nanoproduct's jurisdiction are one major divisive issue. The most quoted definitions (e.g. National Nanotechnology Initiative) imposes inaccurate size limitations, as scientists argue that it leaves many nanoproducts behind. These medical products , that are outside the range (1-100nm), still display nanotechnological properties though [16]. Regarding current jurisdiction, a "drug" employs "chemical action" while "medical-device" employs

"mechanical action". However, at a molecular level, such as the nanoscale, it becomes virtually impossible to distinguish mechanical from chemical or electrical effect[17].

Besides the adequacy of regulatory oversight, another dissenting point is the level of uncertainty about the risks that nanoproducts may carry. The mistrust about nanoproducts seems to be fuelled by two unexpected toxicity effects: the increased reactivity of nanomaterials relative to bulk material as a function of a greater relative surface area; and the enhanced potential for nanomaterials to cross biological barriers. Ironically, these same characteristics are explored by nanoengineering in medicine. Occupational and environmental risks associated with the manufacture and disposal of nanodrugs are yet to be thoroughly studied as well[18].

Overall, owing to nanotechnology particular nature and its infancy state, the understanding of nanotechnology is inadequate and consequently the normal course of the nanoproducts' development is strongly affected.

3.2. Regulatory agencies' approach to nanotechnology

The first generation of nanoproducts was able to get the approval by meeting general standards, it is not likely, however, that new products will have the same regulatory framework as the scientific community strongly advocates its amendment[19]. The above-mentioned safety and legal concerns plus upcoming challenges regarding follow-on nanomedicine products, as first-generation products come off patent, and the advent of even more complex hybrid structures[20] , left nanodevelopers somewhat clueless on how to proceed to get the approval. This can cause an unwanted delay on innovation and on alternative therapies for deadly diseases. More recently, main regulatory agencies convened work groups to address this type of situations.

In 2006, FDA created a Nanotechnology Task Force to evaluate the agency's regulatory approach to nanotechnology. The take of this work group on nanotechnology was that "existing regulatory requirements were sufficient to ensure the safety of nanodrugs (...) on the assumption that such products would undergo pre-market testing and pre-market approval as new drugs requiring a New Drug Approval (NDA)"[21]. Such requirements are thought to detect any toxicity in the required clinical studies even if caused by a novel mechanism unique to nanotechnology, according to FDA task force.

Similarly, the European Medicines Agency (EMA) currently regulates nanotherapeutic products within the conventional regulatory framework. This attitude towards regulatory

approval among other agency's views on nanotechnology are stated on a Reflection Paper (RP), issued on June 2006, on *Nanotechnology-Based Medicinal products for human use*. On the RP it is acknowledged the current discussions on nanoparticles and it is stressed EMA's experience with nanoproducts. The agency also puts forward a definition for nanotechnology in which "the nanometre scale ranges from the atomic level at around 0.2 nm (2Å) up to around 100nm". Although many complex nanomedicinal products do not fit into that description, in agreement with EMA's working definition[22] for nanomedicines any systems purposely designed for clinical applications with at least one component at nano-scale size can be qualified as such. Also, a nanomedicine needs to meet the definition of a medicinal product according to European legislation.

On a similar topic, with respect to nanotechnology jurisdiction, it accentuates, under present rule, that the mechanism of action is key to distinguish a medicinal product from a medical device. In addition, EMA admits that products of novel technology will require special consideration and that the development or update of specific guidance may be needed for certain cases[23]. Actually, EMA has begun to track its own regulatory path by issuing four RPs[24] on particular nanotechnology topics. The Committee for Medicinal Products for Human Use (CHMP) has also created a multidisciplinary expert groups such as The Expert Group and Drafting Group on Nanomedicines and the Innovation Task Force to support the agency with specialist input on new scientific knowledge and to review guidelines on nanomedicines.

Both regulatory agencies mentioned require similar documentation that will prove quality, safety, and efficacy of the nanomedicinal product. Since the advances in emerging technologies may be unpredictable and rapid, both the FDA[21] and EMA[25] use an adaptive and flexible case-by-case approach, using established principles of benefit/risk analysis, and encourage developers' interaction with the agencies. They also assure safety post-marketing with the implementation of specific pharmacovigilance systems and by activating a risk management response, if needed.

ICH has taken no official action to date to initiate the development of a guidelines but current guidance that applies to drug products generally also applies to nanotechnology-based drug products. Its initiatives like workshop, meetings and symposium could be an opportunity to build and implement a specific global set of regulation before being hampered by upcoming national regulations[19].

It is important to bear in mind as well, that there may not yet exist standardized characterization methods suitable for a rigorous preclinical characterization of a nanosized

particle such as PEGASEMP™. Until such standards are available, the nanotech developers have to design and validate their own novel characterization methods, demonstrating their appropriateness[26]. Although the regulatory entities have shown some flexibility on this issue there has to be a delay to get the regulatory approval since such entities have to assess and interpret unfamiliar techniques with little history of acceptance in the scientific world. It is noteworthy, though, that non-governmental standard organizations such as ISO and ASTM contributed to further clarification by publishing standards on the terminology, definition and environment studies for nanoparticles. These entities can be helpful, when developing methods for characterization of nanomaterials, where regulatory standards are not available.

To sum up, the nanotechnology based drugs' regulatory evaluation and framework is still not as straightforward as opposed to other drugs, such as small-molecule drugs. Nevertheless, all applicable extracts from existing regulation should be taken into account during for the preclinical characterization of PEGASEMP™.

3.3. Major guidance documents available and regulatory pathways

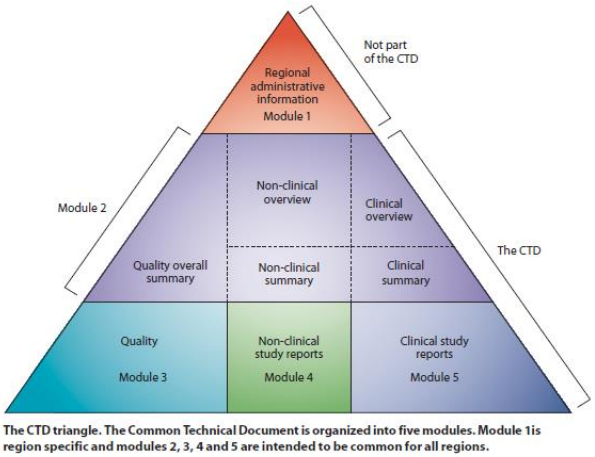
For the purpose of this monograph, the guidance documents herein discussed will cover the preclinical psychochemical characterization rather than preclinical characterization as a whole (which includes in vitro and in vivo parameters). Plus, this scope will only mention preclinical psychochemical characterization parameters that assure the quality of PEGASEMP™ (critical quality attributes) that may be submitted in the application for clinical trials. Its unique properties may warrant additional scientific considerations from a quality perspective during product regulatory review still. Furthermore, PEGASEMP™ does not fit into any particular classification as a result of its innovative factor. Therefore, the applying legislation will be dispersed and the underlying principles of the guidelines may need to be adapted.

3.3.1. Governing documents for the authorization of clinical trials

- ICH

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has developed a process that, over the past 21 years, has harmonized the criteria and documents necessary for submission of an application to conduct clinical trials in the US, Europe, and Japan[27]. A major benefit of the ICH process has been the harmonization of both the content and format of the submission

dossier with the purpose of facilitating that the work of sponsors and reviewers worldwide. Harmonization of format has been achieved to a large extent through development of the Common Technical Document (CTD).



The CTD triangle. The Common Technical Document is organized into five modules. Module 1is region specific and modules 2, 3, 4 and 5 are intended to be common for all regions.

Picture 2 - CTD triangle

The CTD gathers all types of data (clinical, non-clinical) which accumulates along the drug development towards the marketing authorization. The CTD format, organized in five modules, is accepted by most regulatory agencies specially FDA. Module 1 is region specific while Modules 2, 3, 4, and 5 are intended to be common for all regions. A submission in CTD format can be used to request the clinical trials authorization. The Quality section of the Common Technical Document (M4Q) provides a harmonised structure and format for presenting CMC information in a registration dossier, however, many CMC topics have not yet been the subject of ICH guidelines.

- <u>EMA</u>

PEGASEMP™ is currently applying for CTA, which requires the submission of an Investigational Medicinal Product Dossier (IMPD). The detailed guidance for the request for authorisation of a clinical trial on a medicinal product is currently regulated under ENTR/F2/BL D(2003)CT 1Revision 2. This guidance, elaborated by the European commission, gives us further insight of what data is required for the IMP, at point 4.1.6.. In this dossier an IMPD should "*include summaries of information related to the quality, manufacture and control of the IMP, data from non-clinical studies and from its clinical use*". The amount of data to be required will depend on many factors like the products nature, the state of development, the disease to be treated and so on. Since this guidance cannot cover all situations, it should be used as a starting point while applicable community guideline or commission decisions should be consulted. Moreover, in PEGASEMP™ case the guidance divides IMPD in two major parts: Non-clinical

pharmacology and toxicology data and Quality data. On the latter the sponsor "*should submit summaries of chemical, pharmaceutical and biological data*" on PEGASEMP, which meets the monograph's objectives. It selects CHMP/QWP/185401/2004 as one of the guidance sponsors should refer to, where applicable.

CHMP/QWP/185401/2004 was elaborated by CHMP to facilitate the implementation of 2001/20/EC Directive (to be replace by a new regulation in 2016), which defines a general pathway to be considered by the applicant. This document comprises two main parts: the drug substance and the drug product. These parts are further divided into subsections dedicated to detailed aspects such as structural information, batch analysis, analytical methods, and so on. Most of the information to be included in the IMPD should particularly focus on the risk aspects.

- FDA

An Investigational New Drug (IND) application is submitted by the sponsor responsible for developing the drug to the FDA and it can fall into two categories, either commercial or research. To move forward with the application, animal studies must assure that the proposed drug is reasonably safe for initial use in humans, and that it seems sufficiently promising as a treatment to justify such an investment. Unlike a New Drug Authorization (NDA), the FDA does not formally approve an Investigational New Drug (IND) submission. If the FDA reviewers believe that the proposed clinical trial submitted in the IND is acceptable from a safety and risk versus benefit viewpoint, the IND is in "effect" and specific clinical trials can be conducted [15]. The Guidance for Industry entitled, Content and Format of Investigational New Drug Applications for Phase 1 trials of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products clarifies many of the phase 1 IND requirements for an expedite entry of new drugs into clinical testing[28]. With regards to the Chemistry, Manufacturing and Controls section it states that the submitted data should "*assure the proper identification, quality, purity, and strength*" of the investigational drug. The required data gets more detailed and demanding along the clinical phase in order to rest assure the regulatory authorities that the sponsor has full understanding of the production process, can produce multiple lots in compliance with a strong set of specifications[29].

3.3.2. PEGASEMP™ as liposomal-base product

Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product (EMA/CHMP/806058/2009/Rev. 02)

This RP guides development of liposomal based drugs, which generally fall into the definition of nanomedicine based on particle size. Although it would be a stretch to identify PEGASEMP™ as a conventional liposome, in truth it shares several properties with the conventional counterpart since PEGASEMP™ is liposomal-based nanoparticle after all. At this RP's[30] introduction part some of the lipossome's properties listed do recall PEGASEMP.

This RP is intended to assist intravenous liposomal products, developed with reference to an innovator product, on gathering relevant quality, non-clinical and clinical data to support a marketing authorization. The relevance of this RP to the PEGASEMP™ case may be questioned, as PEGASEMP™ is an innovator molecule. However, the principles outline in the RP "*might also be considered to be applicable to other novel types of "liposome-like" and vesicular products which may be under development*". Plus, unlike some non-clinical and clinical requirements listed in this RP, the ones significant to the monograph do not ,necessarily, need to be referenced to an innovator product, as they address the quality of the liposome (at 3.). The RP argues that the "*critical quality attributes*" of a formulation may have an impact on the in vivo pharmacokinetic and pharmacodynamics properties. The RP exposes through example how quality attributes may affect it. At 3.1.1 the RP catalogues general parameters that should be addressed in the submission of all types of liposomal products. The general parameters range from manufacturing, finished product, intermediates, raw material, impurities, liposome specific functions, to stability and excipients .The following parameters are the ones that apply to the monograph's intend (PEGASEMP™ as a drug product):

- Liposome (PEGASEMP) morphology, mean size and size distribution, aggregates;
- Fraction of encapsulated active substance (amount of free/entrapped);
- Stability of the active substance, lipids and functional excipients in the finished product, including quantification of critical degradation products
- In vitro drug substance release rate from the liposome in physiologically/clinically relevant media;
- Maintenance of liposomal formulation integrity in plasma;
- characterisation of lipid bilayer phase transition behaviour (e.g. temperature and enthalpy of transitions);
- Determination of liposomal 'surface' charge;
- If relevant, characterisation of physical state of the active substance inside the liposome (e.g. precipitation in the case of doxorubicin);

- Distribution of drug substance within liposome (e.g. surface, bilayer, interior, etc.);
- Details of linkage chemistry (such as PEG-lipid or similar constructs with or without PEG);
- Disposition of PEG at surface;
- Stability of conjugation.

Guidance for Industry Liposome Drug Products

FDA has drafted a guidance for industry on "Liposomal Drug Products" as well. The draft provides recommendations on "*CMC, human pharmacokinetics and bioavailability; and labelling documentation for liposome drug products submitted in new drug applications (NDA)".* Nevertheless, it can be applicable to a certain extend when a sponsor is submitting an IND. The introductory note underlines how the liposome modulate the encapsulated drug's pharmacokinetics and/or biodistribution comparing to the uncapsulated drug's profile. The same is true for PEGASEMP™ in relation to the doxorubicin.

On the particular point about CMC psychochemical properties, the draft highlights that "*physicochemical properties of the liposome drug product are critical to ensuring drug product quality. Therefore, a detailed evaluation of these properties should be provided*." The properties raised by the draft almost match the ones put forward by the aforementioned CHMP's guideline.

- Morphology of the liposome
- Net charge
- Volume of entrapment in liposomal vesicles
- Particle size (mean and distribution profile)
- Phase transition temperature
- In vitro release of the drug substance from the liposome drug product
- Osmotic properties

Stability studies are weighty when assessing the quality of drug product. The topic F of this draft mentions that importance. The studies should not only be conducted on the drug product as a whole but also on its components individually. Therefore the stability studies should "*address both physical and chemical stability of the liposome drug product, including the liposome itself*", "*stability testing of the unloaded liposomes*" and "*stability of the encapsulated drug substance as well as stability of the lipids that compose the liposomal bilayer*". The concepts for the design of stability studies for liposome drug products can be found at CDER guidance for industry Submitting Documentation for the Stability of Human Drugs and Biologics and on the ICH guidance Q1A(R2) Stability Testing of new drug substances and products.

3.3.3. PEGASEMP™ as a coated nanoproduct

Reflection paper on surface coatings: general issues for consideration regarding parental administration of coated nanomedicine products

The noncovalent or covalent coating can be an integral component of a nanomedicines design, according to this RP. Such coatings have been typically used to minimize aggregation and improve stability, or in certain cases to minimise rapid clearance by the reticuloendothelial system. They have also been used to improve hematocompatibility and limit antigenicity. Both are phenomena that can arise due to the inherent physicochemical nature of the product or the surface adsorption of biomolecules from the physiological environment to which they are exposed. The RP also states that "*the physico-chemical nature of the coating, the uniformity of surface coverage, and the coating stability (…) will govern the pharmacokinetics, the bio-distribution of the product and its intracellular fate*". It also mentions that "*coating material may elicit new biological responses*". The RP adds that when designing surface modifications to facilitate cell-specific targeting (use of ligands) it should be "*carefully consider the chemistry used for their attachment*". The control of ligand orientation is also key to know the impact on the PK and biodistribution and therefore safety.

Considering the points raised above, the RP lists critical characteristics. For this monographs some should be taken into account during the PEGASEMP™ quality characterization.

- Complete characterization of the coating material, including its composition and control
- Orientation and conformational state of any ligand
- In vitro determination of the physic-chemical stability of the coating

3.3.4. Already marketed Medicinal Nanoproducts related to PEGASEMP

Draft Guidance on Doxorubicin Hydrochloride

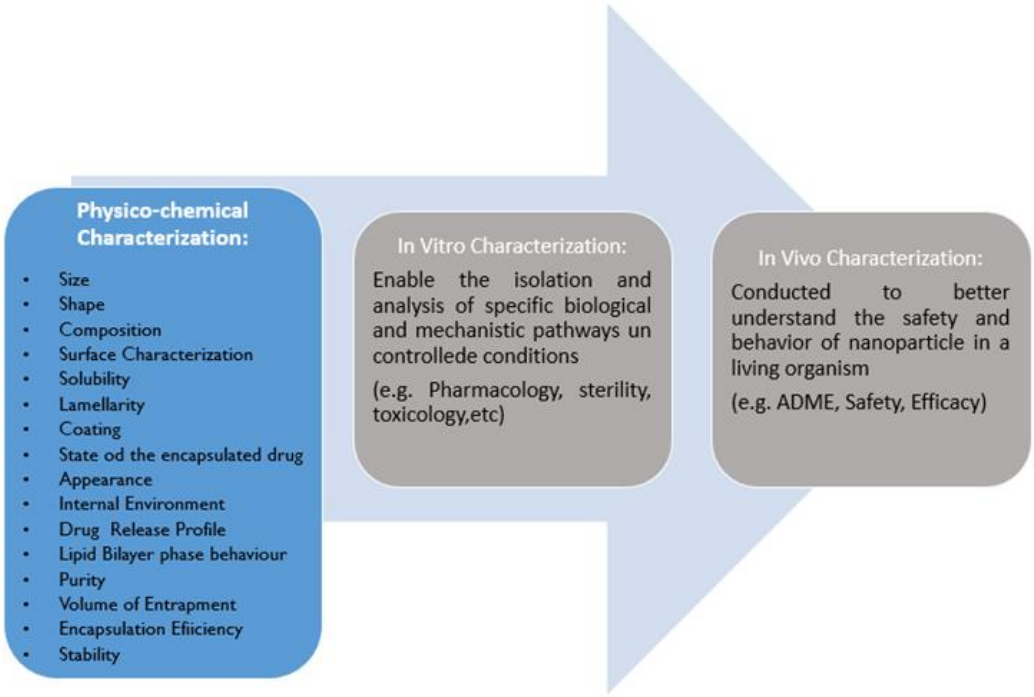
Doxil/Caelix, was the first FDA-approved nano-drug. Although PEGASEMP™ and Doxil/Caelix are two distinct nanodrugs, with different mechanism of action, manufacturing, ADME, bioavailability, etc. there are some common traits between them. They both are liposomal-based products, they both have a PEG coating, they both carry the same drug and have similar size scale. This draft guidance gives non-binding recommendations to demonstrate bioequivalence with Doxil/Caelix. It is not the intention of PEGASEMP's sponsor to undertake such studies, on the other hand the draft guidance mentions certain attributes used to claim

equivalence with Doxil/Caelix that may be perceived as critical quality attributes for Doxil/Caelix and therefore liposomal-based nanodrugs.

- Liposome composition: Liposome composition including lipid content, free and encapsulated drug (…).The drug-to-lipid ratio and the percentage of drug encapsulation…
- State of encapsulated drug
- Internal environment
- Liposome morphology and number of lamellae
- Lipid bilayer phase transitions
- Liposome size distribution
- Grafted PEG at the liposome surface: PEG layer thickness
- Electrical surface potential or charge
- In vitro leakage under multiple conditions

3.4. Suggested parameters for preclinical Characterization

In light of the above mentioned guidance documents plus the considerations from the application governing documents, it is possible to draw a suggestion for the quality parameters critical to product performance and therefore vital for the overall risk assessment[23]. Further predictive in vitro methodologies and in vivo using animal models needs to be done to adequately study efficacy and safety of the nanoproduct. In these cases, auxiliary guidance can be found in ICH documents like M3 (R2)(safety), S3A (toxicological assessments) and others on ICH's internet domain[31].



Scheme 1 - Suggested preclinical physicochemical characterization and other preclinical testing

4. Preclinical Characterization

4.1. Introduction

The preclinical characterization of a nanoparticle is the last stepping stone towards the clinical trials, where the developer can test the candidate drug's therapeutic/diagnostic potential in humans. No matter how promising the project seems to be that far, the preclinical characterization poses one of the greatest challenges a sponsor may face during the product development, especially if the product concerned is a nanoproduct.

The data amassed from the broad range of tests must be sufficient to thoroughly conclude that PEGASEMP™ will not expose humans to unreasonable risks and that it exhibits enough pharmacological activity to justify first-in-man clinical trials [26]. This will not be possible if the preclinical characterization does not follow the regulations at force or if it fails to elucidate on structure-activity relationships.

In order for the data acquired to be legally binding, the laboratory which is going to perform the preclinical characterization or part of it must abide to GMP and GLP protocols. GLP Regulations concern standard methods, facilities, and controls used in conducting preclinical and nonclinical laboratory studies and are used to assure the quality and integrity of generated data[15].

One possible drawback on the preclinical characterization of PEGASEMP™ is its multicomponent identity. Apart from being a therapeutic agent, PEGASEMP™ also serves as a scaffold for the attachment of chemical moieties on a specific target. The result is a much more complex and thus harder to assess molecule than the conventional small-molecule drugs[26].

The most rational approach used for the characterization of a nanoparticle is comprised of three components: the physicochemical characterization, in vitro assays, and in vivo studies. This type of strategy is being employed by the major characterization laboratories, including National Characterization Laboratory (NCL)[26]. The monograph will approach the physicochemical characterization component with respect to the nanoparticle PEGASEMP™.

4.2. Physicochemical characterization

It is not practical and it is time consuming to characterize the full spectrum of the nanoparticle system, instead the physicochemical characterization should assess a set of parameters that may influence the nanoparticle bioactivity through cellular uptake, blood

protein's binding, access to target sites, cytotoxicity, etc. It is also important to bear in mind that PEGASEMP, as a multifunctional particle, has its biological activity dramatically affected by its targeting agents in the coating and by the excipients used, which may play a more or less active part[26]. From a successful characterization it is possible to link certain nanoparticle's psychochemical properties to its in vivo behaviour. The characterization will also be useful to set up specifications that ensure the quality of each batch produced afterwards and will be helpful as well, when evaluating subsequent changes in the manufacturing.

As it was previously mentioned there are few examples of standard characterization criteria in the literature and the measurement criteria to be applied to any given nanomaterial is far from being consensual[32]. This is because nanomaterials characterization should not be object to a "one size fits-all" approach since there are multiple categories of nanomaterials (organic nanoparticles, inorganic nanoparticles, liposomes and other biological materials) with different compositions and therefore different physicochemical properties. Many of the techniques used for the characterization of small molecules (micelles, liposomes, emulsion) can be employed in the nanoparticle characterization, however additional characterization needs to be put in place to understand certain attributes like surface chemistry, surface area, polydispersity, zeta potential and so on[26].

4.2.1. Size and Shape

Size is the most basic information of a nanoproduct. Currently, it is the only parameter mentioned in the troublesome legal definitions of a nanomaterial. It is understandable why it is such a critical parameter, since it modules the absorption, biodistribution and route of elimination. Nevertheless, other attributes such as surface characteristics play its part.

The particle size is defined as the size of a hypothetical hard sphere that diffuses in the same fashion as that of the nanoparticle being measured. The most commonly used technique is the Dynamic Light Scattering (DLS)[33] where the sample is irradiated with a laser and the intensity fluctuations in the scattered light are analysed and related to the size of the suspended nanoparticles. The results are reported as mean particle size and homogeneity of size distribution. The latter is a relevant parameter because nanoparticles coming out of manufacturing are far from being homogenous. This parameter is expressed by the polydispersity index (PDI)[33] which describes the degree of "non-uniformity" of a distribution. Besides being determined by DLS, size can also be obtained from a fraction method. The asymmetric-flow field flow fraction (AFFF) would be the most suitable for a liposomal particle

since it does not use a stationary phase, which is useful while measuring less stable nanoparticle like liposomes[26]. Molecular weight, which is a required parameter, can be determined directly from the scattering behavior of the sample.

Other methods to be used for the size assessment could be microscopic ones[33] (scanning probe microscopy, transmission electron microscopy, scanning electron microscopy). Not only would they assess the size but they would also assess the shape of the nanoparticle and its distribution. The shape of a nanoparticle should not be neglected, it affects the bioavailability, surface energy and access to physiological structures moreover the spherical shape, in particular, is often assumed for nanoparticles by instruments. So, it is essential to know the shape in order to validate this assumptions or to validate adapted methods for non-spherical shaped nanoparticles. Some downsides of the microscopic approach would be its complicated sample preparation, which can modify the sample and/or create artifacts (e.g. nanoparticle agglomeration) and its limited throughput, which hampers the size distribution data.

The nanoparticle tracking analysis (NTA) is another method for size assessment based on single particle tracking by dark field or fluoresce microscopy and automatic imaging analysis. Its single tracking analysis provides a high resolution for multimodal samples and aggregations[33]. Prior to its use it is essential that the sample is adequately diluted so that the observation fields do not get excessively crowded. Methods using the sedimentation speed, such as disc centrifugation and analytical centrifugation, can also be effective on measuring nanoparticle size. They can determinate very small size differences between nanoparticles from a very broad range of particle size, however they take longer to run the samples than other methods. Plus, it requires that the nanoparticle be denser than the suspended fluid.

The physical principles and sample preparation are different from method to method, hence the slightly variance of results between each one. It should be noted that the sample status is not the same on every method. Little traits can be critical during the measurements. On the DLS, for instance, it must be assured that nanoparticles are well dispersed. High levels of agglomeration would render it almost impossible to have reliable results. The nanoparticle can be dispersed using sonication or vortex mixing, however increased duration and high levels of energy may promote agglomeration caused by the enhanced interactions of nanoparticle with high surface energy. On top of that, measurements should not be done by a light scattering instrument when the nanoparticles absorb in the same wavelength of the laser being used[33]. This same difficulty is often observed in many in vitro testing.

Furthermore, there is some degree of dependence on the pH and ionic strength of the nanoparticle suspension, regardless of the method being used. Both can affect the agglomeration state of the sample, additionally the distance of the pH from the isoelectric point can do the same to the hydrodynamic size.

4.2.2. Composition

The chemical composition of a nanoformulation is an obvious requirement to ascertain the product's properties, the purity state and the homogeneity of nanomaterial product preparation. Elemental analysis , CHN analysis in particular, Atomic absorption(AA), Atomic emission, High performance liquid chromatography and Mass Spectrometry have been used to define, with more or less precision, the composition and the ratio of different elements present in the sample[26].

4.2.3. Internal Environment

The measurements of total and free concentrations of components allow the inference of the internal concentrations inside the liposome. Internal component concentration involves measurement of the component external to the nanoparticle and the total component content in the formulation (i.e., internal plus external) using syringe filtration and subsequent chromatography analysis. The internal concentration is calculated by subtracting the external concentrations from the measured total. The totality of the encapsulated material can also be analysed when separation techniques are employed first. Minicolumn centrifugation and protamine aggregation methods are the separation procedures that are commonly used[34].

4.2.4. Surface Characteristics

The surface is the linchpin of the whole nanoparticle. Its psychochemical characteristics contribute to the nanoparticle's aggregation tendency, biological barriers crossing, solubility, biocompability, and targeting ability. For molecules as small as the nanoparticles the surface can take overwhelming importance as it can make up 50% of the mass of a 3nm particle, as an example. Through analytical measurements the nature and integrity of the surface must be established. The results can be used to assure product quality and anticipate surface-dependant effects[26].

4.2.4.1. Composition

The composition of the surface dictates much of the nanoparticle chemistry. It is challenging to directly measure the atomic composition of the surface because most surfaces

have trace contaminants that may not be detectable by general chemical analysis. Electron spectroscopy for chemical analysis (ESCA), x-ray photoelectron spectroscopy (XPS) and secondary ion mass spectroscopy (SIMS), in particular, have been extensively used for characterizing nanoparticles[35].

4.2.4.2. Structure

The PEGASEMP's external structure and texture can be determine by microscopic methods such as scanning electron microscopy (SEM) , atomic force microscopy (AFC), SEM-Raman and confocal Raman[36].

4.2.4.3. Surface Charge

The Surface Charge, which is expressed as zeta potential, strongly influences the interaction of a nanoparticle with the surrounding environment. Charged functionalities on the surface may increase nonspecific uptake and depending on the charge, a modification can occur on the nanoparticle's distribution and pharmacokinetics. For instance, negatively charged nanoparticles are believed to be cleared more slowly and other studies show that surface charge may alter the ability for a nanoparticle to penetrate the blood-brain barrier.

The zeta potential is generally measured by laser Doppler electrophoresis, which calculates the electrophoretic mobility of suspended nanoparticles in the medium, henceforth measuring the potential at the boundary of the outer layer. There are two liquid layers surrounding each nanoparticle; one strongly bound inner layer (Stern layer) and one weakly bound outer layer[33]. If the suspension consists of different sized nanoparticle groups, the zeta potential value of larger particles tends to overshadow the scattering signal of smaller particles. Potentiometric titrations can also be used to acquire surface charge information. This zeta potential value rests on the strength and the valency of the ions contained in the suspension. High ionic strength and high valency ions result on a more compressed electric double layer and then on a smaller zeta potential; and vice-versa. So, for a zeta potential value to be meaningful it should be indicated the solution pH. In addition, it is recommended that further information is accurately described regarding the nanosolution, including the ionic strength, composition of the medium, and pH[33].

4.2.4.4. Target moiety

PEGASEMP™ is an actively targeted nanosystems with small-molecule ligands, peptides, on the liposomal surface[8]. The targeting moieties are key to the mechanism of

cellular uptake. Its quantification by Fluorescent labelling, TEM-tomography and HPLC, gives a thorough grasp on target ability, biodistribution, pharmacokinetics, toxicity profile and so on[36].

4.2.4.5. Surface energy

Aggregation, dissolution and bioaccumulation are phenomenon that can be roughly predicted by the surface energy/wettability of the nanoparticle. Heat of immersion microcalorimetry studies or contact angle measurements with various liquids are methods that can be used to estimate that parameter.

4.3. Lamellarity

Lamellarity is the number of lipid bilayers surrounding the inner aqueous space of the lipid vesicles. Lamellarity of liposomes can be determined with direct microscopical observation[37].

4.4. Coating

Adequate methods should be shaped to determine the linkage chemistry, disposition and thickness of PEG. Likewise, orientation and conformation is expected to be known from the peptide ligands.

4.5. State of the encapsulated drug

The nanoproduct internal environment is responsible for doxorubicin precipitation. The precipitation occurs through drug self-association or through doxorubicin interaction with the salts present in the aqueous core[38]. Depending on the internal conditions, different precipitated structures can be formed, but it is unclear whether this affects the biological behavior of the resulting product (25). Through Cryotransmission electron microscopy (cTEM) [38, 39] the state of the doxorubicin can be assessed, which can draw conclusions on drug loading process, concentration, internal environment, and so on. It can also be measured by several techniques such as X-ray diffraction and fluorescence studies[40].

4.6. Appearance

It should be performed the way it is on any compendial appearance testing. A qualitative statement describing the physical state (e.g., solid, liquid) and colour of a drug substance is provided.

4.7. Internal Environment

The nanoproduct internal environment is responsible for doxorubicin precipitation. The precipitation occurs through drug self-association or through doxorubicin interaction with the salts present in the aqueous core[38]. Depending on the internal conditions, different precipitated structures can be formed, but it is unclear whether this affects the biological behavior of the resulting product (25). Through Cryotransmission electron microscopy [38, 39] the state of the doxorubicin can be assessed, which can draw conclusions on drug loading process, concentration, internal environment, and so on. It can also be measured by several techniques such as X-ray diffraction and fluorescence studies[40].

4.8. Drug Release profile

The stimuli-response of the release should be considered while developing the method. It should be tested under multiple conditions, including biorelevant medium. The drug release from nanoliposomal products can be followed by the usage of a well calibrated in vitro diffusion cell in order to predict pharmacokinetics and bioavailability of drug before expensive and time-consuming in vivo studies[34].

4.9. Lipid Bilayer phase behaviour

Lipid Bilayer phase transition give further insight into membrane fluidity, uniformity and fusion. An unexpected phase transition from closely packed arrangements (crystalline state) to a loose liquid state can result in drug leakage[41]. Thus the transition temperature is important in optimizing the storage conditions. Lipid bilayer phase behaviour can be evaluated by high-sensitivity differential scanning calorimetry (high-sensitivity DSC)[42] . Cholesterol plays a major role on managing the membrane fluidity and its mechanical strength[37].

4.10. Purity

Purity state must verify the presence of any artifact and side products of the preparation. The impurity concern can be caused by the presence of solvents, free material and chelates, unconjugated ligand, dimers, etc. Residual solvent, for instance, should be kept to a minimum since it is very undesirable in drug delivery formulations. As a result, gas chromatography (GC) should be done for the quantification of residual solvent. The purity testing must take into account that the PEGASEMP™ is comprised of coating, layers and encapsulated components[33].

4.11. Volume of entrapment

This parameter governs the morphology of liposomes. It is defined as the aqueous entrapped volume per unit quantity of lipids. The most successful way to determine this internal volume is to measure the quantity of water by replacing external medium (water) with a spectrophotometrically inert fluid (i.e. deuterium oxide) and then measuring water signal by Nuclear magnetic resonance (NMR)[34].

4.12. Encapsulation efficiency

Most of the reported experimental methods to determine liposomal encapsulation efficiency, require removal of the free (unencapsulated) bioactives from liposome encapsulated bioactives by column chromatography, size exclusion chromatography (SEC), ultracentrifugation, equilibrium dialysis (ED), ultrafilteration , before quantification of the entrapped material by analytical techniques such as UV/VIS Spectrometery, HPLC[37].

4.13. Stability

The quality attributes of PEGASEMP™ are defined, and its testing is developed. Stability studies are performed, in the form used for testing, to demonstrate that pre-clinical samples maintain their specifications for the time frame of the animal study. It is important to determine the stability of the sample under physiological and non-physiological condition to account for the effects of storage, ultrafiltration, pH variation, exposure to light, and so on[26]. Should drug properties change beyond the accepted criteria during a stability study, then the established safety and efficacy data may no longer be applicable. The formulation being tested must be stable to assure that all animals receive the nominal dose and purity from start to finish of the study[43].

Conclusions

This monographs highlights some critical aspects that should be included on the submission of PEGASEMP™ for clinical trials. However, it is debatable whether further or less testing should be done or not. On one hand, an extensive characterization of quality attributes can set a high bar for the product; on the other, an exhaustive list of measurables can require extensive resources and time for tests that may have no actual bearing on the quality, safety,

or efficacy of the end product. Quality by design and target product profiling can be two important strategies to overcome this kind of issues. They assure greater results at tackling major obstacles while keeping the regulators updated of the progress.

In addition, present regulatory framework does not seem to completely accommodate nanotechnology, yet it requires an extraordinary effort from multidisciplinary teams to create specific regulation for such a variety of materials that often have their unique properties. The same goes for standardized methods although a lot of organizations (e.g. NCL, ISO, ASTM) are working in that direction.

Science has the ability to defy the current thinking. Nanotechnology is an example of that. At present times adaptation from both sides, the regulator and sponsor, seems to be key to move forward.

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