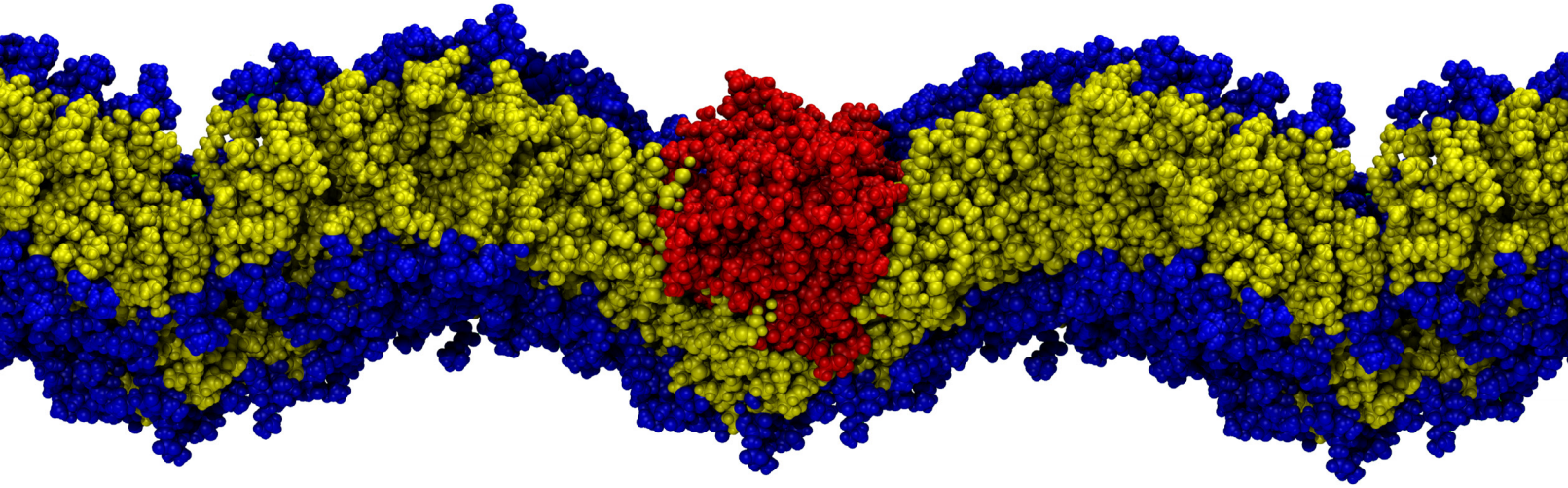
GUIDELINES FOR

IMPLEMENTING A SAFE-BY-DESIGN APPROACH FOR MEDICINAL POLYMERIC NANOCARRIERS



NANOBIOMAT

COVER IMAGE: Tommaso Casalini „Nanocarrier passing through the cellular membrane“

Impressum

PROJECT COORDINATION: Peter Wick, Claudia Som

PROJECT MANAGEMENT: Mélanie Schmutz

AUTHORS: Mélanie Schmutz, Claudia Som

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CONSORTIUM:
Peter Wick, Claudia Som, Mélanie Schmutz, Bernd Nowack, Marina Hauser, Edgar Fernandez (Empa, Switzerland)
Gerrit Borchard, Van Nguyen (University of Geneva, Switzerland)
Olga Borges, Sandra Jesus, Patricia Marques (University of Coimbra, Portugal)
Giuseppe Perale, Tommaso Casalini, Carolina Yumi (SUPSI, Switzerland)
Manfred Zinn, Véronique Amstutz, Nils Hanik (HES-SO Valais-Wallis, Switzerland)
Adriana Isvora, Ostafe Vasile (University of West Timisoara, Romania)
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CONTENT

THE GoNanoBioMat FRAMEWORK

The GoNanoBioMat framework provides elaborate current knowledge to small and medium-sized enterprises (SMEs), their suppliers, service providers and research institutes at the interface of nanomaterials and nanomedicine. The aim is to support SMEs in their decision making when developing and producing polymeric nanobiomaterials[1] (NBMs) for drug delivery by implementing a Safe-by-Design (SbD) approach. The GoNanoBioMat framework contains:

- a knowledge base presenting the current state of the science, including trends, gaps and uncertainties;
- guidelines for implementing an SbD approach for medicinal polymeric nanocarriers;
- and case studies involving an in-depth investigation of three selected materials: chitosan, polylactic acid (PLA) and polyhydroxyalkanoates (PHAs).

GUIDELINES' GOALS

The guidelines' goals are to (1) support informed decision-making in the field of polymeric NBMs for use in drug delivery, (2) to improve and facilitate communication (develop a common language) between the different stakeholders contributing to the value chain and between industry and regulatory authorities, (3) to prevent misguided investments, and (4) to enable SMEs to deliver safe products in an internationally competitive market. These guidelines are not only addressed to SMEs developing nanocarriers, but also to SMEs having some link to the topic. These guidelines are intended to accompany SMEs through the implementation of an SbD approach in the early research and development phases of medicinal polymeric nanocarriers (being considered as nanomedicines).

The guidelines are based on the knowledge base built up from peer-reviewed scientific publications. All the scientific references for these publications can be found in the knowledge base, but are not mentioned in the guidelines to facilitate its reading. However, links to other guidance documents which may be useful when developing a nanomedicine are provided in these guidelines.

SCOPE AND LIMITATIONS

These guidelines focus on polymeric NBMs for use in drug delivery systems (nanocarriers), but the principles laid out in them could be extrapolated, to a certain extent, to others, e.g. inorganic NBMs, such as metal and metal oxide nanobiomaterials.

The guidelines:

- provide information on nanocarriers in general and not on nanocarrier systems for specific drugs;
- only take into account the early phases of development (early research and development and the pre-clinical phase);
- emphasise the safety aspects by implementing an SbD approach during the development of nanocarriers.

These guidelines also discuss certain aspects of the concept of Quality-by-Design (QbD) because this is mandatory for pharmaceutical market approval and because QbD and SbD are interconnected.

[1] Biomaterials are materials that interact with specific biological systems and can either be derived from nature or be synthetically produced. Nanobiomaterials are therefore biomaterials in the nanoscale (up to 1,000 nm). https://ec.europa.eu/research/industrial_technologies/pdf/biomaterials-roadmap-for-horizon-2020_en.pdf

SAFE-BY-DESIGN

THE ORIGINS OF SBD

Safe-by-Design (SbD) is a general approach or concept used to identify the risks and uncertainties involved in human health and environmental safety during the early stages of product development; it supports efficient processes towards creating safe products, safe production methods and safe handling. The general approach to SbD in the field of nanomaterials started with the EU's NANoREG project (www.nanoreg.eu) and was propagated by its H2020 ProSafe initiative (www.h2020-prosafe.eu) and H2020's NanoReg2 project. In the GoNanoBioMat project, a transnational effort has been made to implement an SbD approach in the development of NBMs for drug delivery systems. This challenging goal required drawing together knowledge from several different fields (chemistry, biology, medicine and pharmaceutical sciences).

SBD IN THE CONTEXT OF POLYMERIC NANOBIOMATERIALS FOR DRUG DELIVERY

Within the GoNanoBioMat framework, the SbD approach focuses on addressing human health and environmental safety throughout the development phase of nanocarriers (excluding use and disposal phases, as these are beyond the project's scope). The SbD approach described here is an iterative, interdisciplinary process including the following aspects (Figure 1):

I. *Safe Nanobiomaterials:* designing low-hazard nanocarriers for specific applications by assessing human health and environmental risks early on in the development process
II. *Safe Production:* manufacturing and control of nanocarriers to ensure their safety and quality
III. *Safe Storage and Transport:* ensuring the safety and quality of nanocarriers

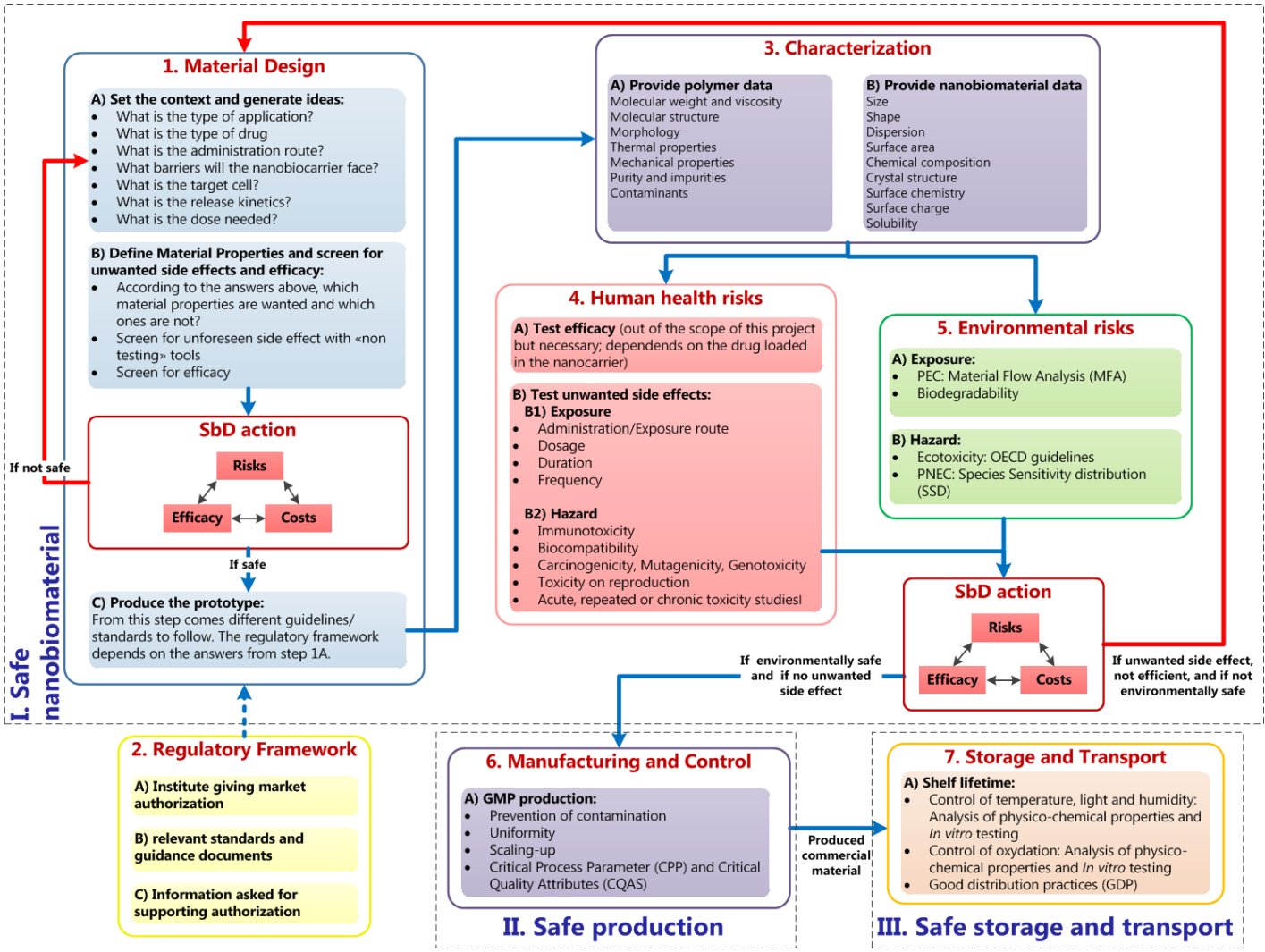
In addition, the regulatory frameworks applied in Switzerland and European Union are incorporated into the guidelines' different chapters (see the yellow box in Figure 1).

In Figure 1, the blue arrows represent the flow of polymeric nanobiomaterials for use in drug delivery from their design to their storage and transport. Feedback loops enable developers to go back to the design of the material (red arrows) after each SbD action. SbD actions are meant to maximise safety while optimising efficacy and costs. Bullet points inside boxes correspond to the possible methods, tools or endpoints that may be used or tested in each step.

The SbD approach begins by generating ideas for the design of NBMs as nanocarriers. This step can be seen as a brainstorming step and is meant to help set the context and open the door to exploring new opportunities by answering a few questions (see Set the context and generate ideas). The following steps define the desired material properties, collect information in the literature to screen for unwanted toxicity by using the answers from the previous step, and use "non-testing tools". Efficacy should also be screened for, as is mentioned in Figure 1, but this aspect is beyond this project's scope as efficacy is drug-carrier system specific (also depending on the drug that will be loaded onto the nanocarrier). A first SbD action is taken at this point. If no unwanted side effects or environmental toxicity have been found in the literature on a particular NBM or with "non-testing" tools, the selected prototype(s) can be produced. This step is followed by a first experimental evaluation of its safety profile (potential human health and environmental risks) by connecting the material's physicochemical properties to their effects. This means that a thorough characterisation of the NBM is needed to be able to correlate the NBM's properties to their effects and therefore enable an SbD approach. This can involve iterations until an optimum solution is found – one that is safer for both human health and the environment and which shows higher efficacy at lower costs (the *second SbD action*). Once the final candidate (experimental nanomedicine) is selected, the standards of Good Manufacturing Practice (GMP) (Manufacturing and Control box) have to be fulfilled in order to begin clinical trials. When safety and efficacy have been proven in clinical trials, information will be required about the nanomedicine's stability and shelf-life (Storage and Transport box) in accordance with Good Distribution Practice (GDP) standards. All the activities shown in Figure 1 – from the earliest stage of innovation – will have to follow the regulatory framework determined by the type of application planned (this was already answered in the first step of the Material Design).

All the boxes shown in Figure 1 correspond to a specific chapter in these guidelines. Each chapter provides the relevant current knowledge, an evaluation of that knowledge, useful methods and tools, and sometimes a case study as an example.

Figure 1
GoNanobioMat framework. Blue arrows correspond to the flow of polymeric nanobiomaterials as drug delivery systems from design to storage and transport, red arrows are feedback loops used whenever the nanobiomaterial product is unsafe, inefficient or has unwanted side effects, and bullet points represent the methods/tools or endpoints at each step.



MATERIAL DESIGN

WHAT ARE POLYMERIC NANOBIOMATERIALS FOR DRUG DELIVERY?

In medicine, a nanobiomaterial is a nanoscale material able to give an appropriate host response for a drug in a specific application. The definition of a nanomaterial differs according to the regulatory authorities around the world. For example, in medical applications, the European Medicines Agency (EMA) defines nanomaterials as being in the range of 1 nm to 100 nm, whereas the US Food and Drug Administration (FDA) has not established a regulatory definition. The latter, however, may consider a nanomaterial to be „a material or end product engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm)", or „a material or end product engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometre (1000 nm)"[2] . The GoNanoBioMat framework considers NBMs smaller than 1000 nm in the three dimensions.

Different materials can be used for drug delivery, and these can vary from lipid and polymer-based to inorganic NBMs. Polymer-based nanocarriers have interesting characteristics for drug delivery, as they can:

- Enable targeted drug delivery
- Increase the bioavailability of poorly water-soluble drugs
- Promote controlled drug delivery;
- Increase the stability of drugs in biological fluids
- Increase drug circulation time in the body
- Confer drugs protection from biological fluids
- Permeate through various biological barriers
- Enable surface modifications to increase interaction with biological targets

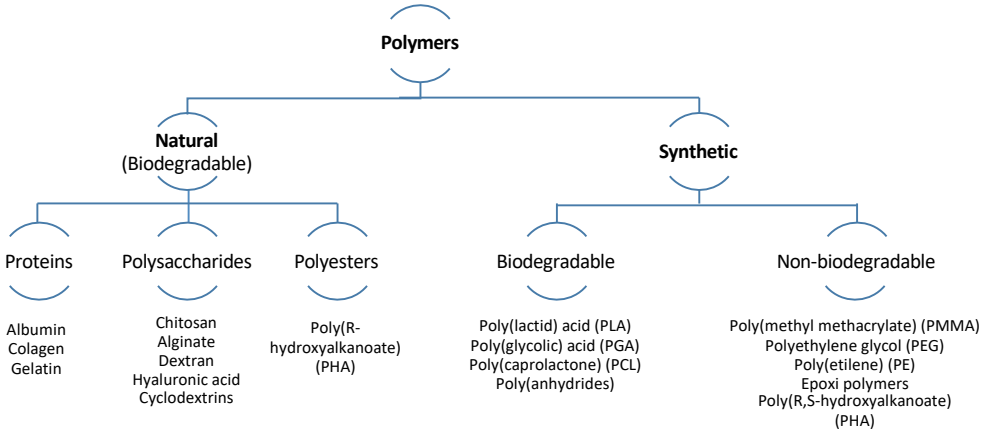


Figure 2
Examples of natural (the polymerisation step is made by a living organism) and synthetic (the polymerisation step is not made by a living organism) polymers used in drug delivery.

[2] FDA Draft Guidance Document: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/drug-products-including-biological-products-contain-nanomaterials-guidance-industry

Polymers are very versatile and can be either natural or synthetic, as shown in Figure 2. Natural polymers and their resultant NBMs generally suffer from problems of stability in biological media, and they also present poor batch-to-batch reproducibility. Because of their natural source and biodegradability, they are more prone to antigenicity and degradation. The chemical modification of certain natural polymers has generated some of the most widely used synthetic polymers, such as the poly (D,L -lactide).

The selection of the polymer used to produce a drug delivery system is dependent on several factors, such as the final product and its degradation product's antigenicity, biocompatibility and toxicity, the kinetics of its biodegradability[3], the drug release profile, solubility and stability of the encapsulated drug, and other physicochemical properties such as particle size and surface characteristics. The characteristics of most natural polymers are usually less reproducible than those of synthetic polymers. Importantly, a polymer's biodegradability influences the mechanisms by which it is eliminated from the body.

Polymeric NBMs can be assembled into different medicinal nanocarriers, such as polymeric nanoparticles, dendrimers, polymeric micelles and drug conjugates. Polymeric NPs, called thereafter polymeric nanocarriers, comprise both vesicular systems (nanocapsules) and matrix systems (nanospheres).

WHAT TO CONSIDER WHEN DESIGNING POLYMERIC NANOBIOMATERIALS FOR DRUG DELIVERY?

Designing NBMs for drug delivery means tailoring their physicochemical properties to the goal at hand. Physicochemical properties have an impact on the efficacy, safety and quality of the final product. NBMs as drug delivery systems should be fit for their intended use, that is, to consistently deliver their active substance at the site of action at the required dose and be stable throughout their shelf-life. In order to fulfil this objective, one should consider the following major factors (non-exhaustive) for a well-designed NBM:

- Type of disease and target population (patients)
- Type of drug (e.g. poor water solubility)
- Route of administration
- Type of barriers (e.g. blood-brain barrier, cell membranes, intestine)
- Target cell (e.g. tumour cells)
- Release kinetics
- Dose needed

All these factors influence the NBM design. For example, the design of NBMs will not be the same for treating diseases as it will be for vaccination. The disease to be treated will also determine the type of drug to be used, which in turn is the most important factor influencing nanocarrier design. Another important factor is the route of administration. Various routes of administration (see chapter on Human Health Risks) are used to deliver NBMs to the target, including the oral, parenteral (intravenous, subcutaneous, intradermal and intramuscular), respiratory and transdermal routes. On entering the body, drug nanocarriers need to pass through various biological barriers (e.g. epithelia, endothelia, cell membranes, and lysosomal and nuclear membranes) before reaching their site of action (target). Targeting can be achieved by passive diffusion (e.g. by exploiting specific physiological conditions, as seen in tumour tissues, which show enhanced permeation and retention effects for NBMs) and by active targeting. The latter includes the attachment of targeting moieties such as antibodies (or their fragments), aptamers or small molecules to the NBM's surface. These targeting moieties will specifically interact with proteins (over-)expressed on target-cell membranes and may thus trigger cellular uptake. On the one hand, the nanocarrier

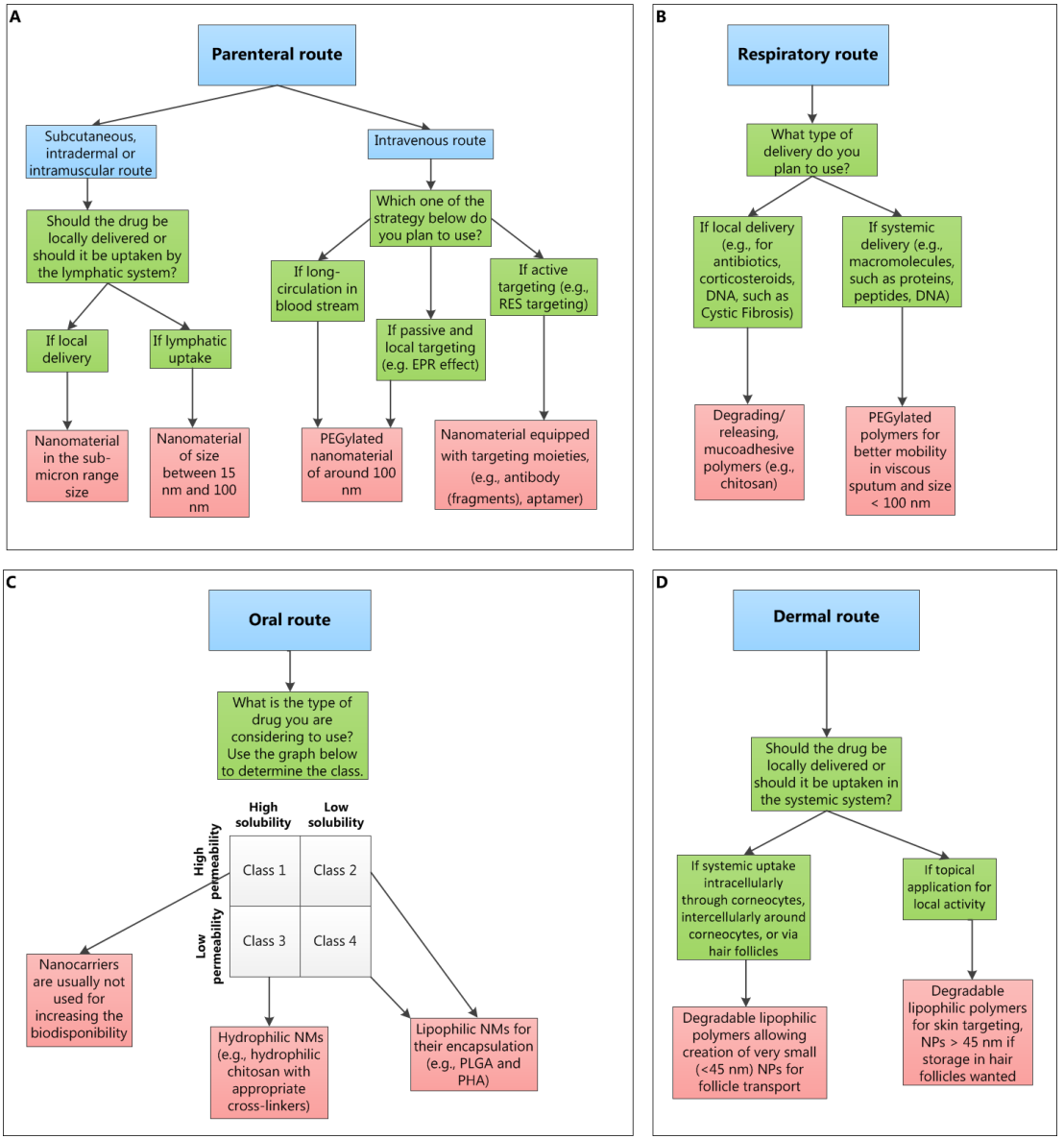
[3] Biodegradable means "susceptible of breakdown into simpler components by such biological processes as bacterial or other enzymatic action" https://medical-dictionary.thefreedictionary.com/biodegradable.

should be able to deliver the drug to the right site of action, and on the other hand, it should release the drug at a rate suitable to maintain an effective therapeutic concentration for a given period (release kinetics). Drug release kinetics may be modulatted by changing the type of biomaterial employed or by the formulation process of the NBMs. Finally, the dose of the active pharmaceutical ingredient is a decisive factor in any treatment's success. It can be influenced by the NBMs physicochemical properties, such as the size of the NBMs or the encapsulation efficiency, which in turn depend on the difference in lipo/hydrophilicity between the drug and the polymeric NBM.

Therapeutic or preventive vaccines utilise polymeric materials to form nano-sized materials as carriers for antigens and adjuvants. Indeed, the particulate form and shape of NBMs are recognised as being foreign to the body (resembling pathogens also in size) by the immune system. Like drug carriers, NBMs for vaccine delivery can be equipped with targeting moieties that interact directly with immune cell-specific receptors, which trigger their uptake or stimulate the targeted immune cell. Such carriers are being examined for the delivery of (adjuvant) subunit and DNA vaccines, with the latter only conferring the antigen's genetic information to the vaccinated individual. Physicochemical properties and other parameters (antigen/adjuvant loading and release, size, size distribution, surface charge, etc.) are being measured as described previously, and the choice of NBM is dependent on the application route, type and dose of the antigen to be delivered.

Figure 3 presents a general decision tree concerning the factors discussed above. It follows a methodological approach, is indicative and does not claim to be complete. However, it does offer a potential pathway for any application, and it supplies guidance on choosing polymeric NBMs for the preparation of nanocarriers for drug delivery.

Figure 3
Decision tree for choosing a nanobiomaterial taking into account the various factors discussed in this chapter. In blue, the route of administration; in green, the factors to consider; and in pink, examples of nanobiomaterials that can be used as delivery systems.



The immune system as a barrier to drug delivery

The key factor in the efficacy and safety of NBMs is their interaction with their physiological environment, or more precisely, with biomolecules, as these are the body's main constituents. In the case of drug delivery for treating disease, interaction with the immune system should be avoided (which is the contrary for vaccines). Moreover, most NBMs used as drug delivery vectors are administered parenterally. Thus, as soon as they are injected into the bloodstream, their surface becomes covered by plasma components, most of which are proteins which form the protein corona. The composition of this corona, and the kinetics which lead to its formation, determine the „biological identity" of the NBMs.

The protein corona is not a stable surface. It can be modulated according to the mobility and affinities of blood proteins (Vroman effect). The first proteins adsorbed (to form a "soft" corona) are later replaced by higher affinity proteins of lower mobility, which form a "harder" corona. The interaction of NBMs with plasma proteins depends strongly on the particles' physicochemical properties, especially their surface properties. This can affect their immunogenicity, their internalisation by immune cells, and their accumulation, degradation and toxicity. Proteins forming the corona may adopt another combination after interacting with the NBM modifying bindings with other proteins and influence signal transductions and gene transcriptions. Moreover, protein adsorption on the NBM surface can be recognised by the immune system, which then initiates an immune reaction (this process is also referred to as opsonisation).

To avoid recognition by the immune system and enable longer plasma circulation times for a drug's vector, it is important to design „stealth" NBMs that can at least temporarily avoid opsonisation. The most important factor is the architecture of the material's surface. The NBM surface may be functionalised with polyethylene glycol (PEG), polyethylene oxide (PEO) or surfactants such as poloxamers, poloxamines, polysorbates (Tween-80) and lauryl ethers (Brij-35). PEGylation is by far the most commonly used technique, with the „stealth" effect being attributed to the surface's higher hydrophilicity, which reduces or delays protein adsorption. The same effect is probably achieved via the steric hindrance induced by the PEG chains protruding from the NBM surface. In addition, recent studies have shown that in order to achieve specific targeting, the NBM surface can be „designed" to adsorb selected proteins, which then interact with specific receptors on the target site, or by preforming the corona with chosen proteins prior to injection[4].

MATERIAL PROPERTIES AND IMPACT ON SAFETY

Regulatory bodies require that the safety and efficacy of new drug-nanocarrier systems must be determined. This includes not only the evaluation of the drug-nanocarrier combination, but also the evaluation of the nanocarrier alone (the NBM). There are two ways of screening NBM's toxicity: firstly, based on current knowledge (literature review), and secondly, by using "non-testing tools".

Current knowledge of physicochemical properties and their effects on safety

At the nanoscale, small variations in physicochemical properties may have very significant effects on a NBM's biological interactions and its therapeutic efficacy and safety. Table 1 shows an overview of current knowledge about the influence of physicochemical properties (size, shape, surface charge and surface chemistry) on various factors which have an impact on safety.

[4] For further information, other barriers are described in the knowledge base on "Polymeric nanobiomaterials for drug delivery" www.empa.ch/gonanobiomat

Table 1
Physicochemical properties which have an impact on endpoints having an influence on the NBM' safety. The √s are based on scientific literature[5].

	Size	Shape	Surface chemistry and surface charge
Targeting efficacy	√	√	√
Stability	√		√
Biodistribution	√	√	√
Elimination and degradation	√		
Toxicity	√	√	√
Drug loading	√		√
Drug release	√		√
Surface area	√		
Protein corona	√		√
Cellular uptake	√	√	√
Biocompatibility		√	√
Blood circulation time	√	√	√
Aggregation	√		√
Drug interaction			√
Opsonisation			√

[5] See knowledge base on "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat

Non-testing tools

Assessing the safety of NBMs using non-traditional methods is being ever more greatly encouraged in order to reduce the need for animal testing. Various tools are regarded as "non-testing tools". For example, to evaluate NBM toxicity or better understand how nanocarriers interact with biological interfaces, the following tools may be used:

- (Q)SAR: (Quantitative) Structure-Activity-Relationship
- Grouping and Read-Across
- Molecular modelling

A (Q)SAR is a type of regression analysis traditionally used for drug discovery. It aims to find a correlation between a NBM's properties (extrinsic and/or intrinsic) and the desired activity (e.g. fewer side effects, greater efficacy or reduced toxicity) and it expresses this relationship in a quantitative manner. This means that given certain NBM characteristics as inputs, the model will give a numerical prediction which can be used to assess, for example, whether a material is safe for medical purposes.

The goals of Grouping and Read-Across are filling in data gaps, firstly by having groupings based on a certain NBM property or effect, and secondly by using this to interpolate where data may be missing. The premise is that similar materials behave in similar ways and have similar properties. Thus, by using the interpolation mentioned above, a material's endpoint can be predicted even if that material's data is not experimentally available.

The Organisation for Economic Cooperation and Development (OECD) published guidance on the validation of QSAR models in 2007[6]. Appendix R6.1 gives guidance on information requirements, and chemical safety assessment frameworks from ECHA may also be used[7].

Molecular modelling techniques[8] are powerful tools for investigating the interactions between polymer surfaces that mimic microparticles, nanoparticles and small or macromolecules (e.g. proteins and nucleic acids). Entire nanoparticles can be simulated, but their maximum size is restricted to 10 nm to 20 nm for computational reasons. Molecular modelling is an ideal complementary tool to laboratory experiments as it allows information to be gathered that is challenging or even impossible to achieve experimentally. For example, it can be used for better understanding: i) interactions between the nanocarrier and plasma proteins during the formation of the protein corona, and ii) interactions between the nanocarrier and the cellular membrane. However, it cannot replace laboratory experiments completely, nor can it currently be used purely as a prediction tool. This is due, on the one hand, to the intrinsic complexity of the system under investigation, and on the other hand, to the lack of any systematic validation with experimental data.

POLYMERIC NANOBIOMATERIAL PRODUCTION METHODS

There are two ways to prepare polymeric NBMs: from pre-formed polymers or by the polymerisation of monomers. The most common methods are:

- Emulsification/solvent evaporation or diffusion
- Spontaneous emulsification/solvent diffusion
- Emulsification/reverse salting-out
- Nanoprecipitation (or solvent displacement)
- Dialysis
- Freeze-drying
- Spray-drying
- Supercritical fluid techniques
- Emulsion/polymerisation
- Ionotropic gelation and polyelectrolyte complexion techniques

[6] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282007%2928&doclanguage=en

[7] https://echa.europa.eu/documents/10162/23036412/appendix_r6_nanomaterials_en.pdf

[8] More information in the knowledge base on "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat

Table 2
Advantages and disadvantages of the most commonly used NBM production methods.

Process	Advantages	Disadvantages
Single / double emulsion	• Particle size can be tuned acting on several variables (solvent, surfactant, shear rate, MW, NPs concentration, stabilizer concentration, and viscosity of the dispersed phase)	• High shear rate • High volumes of water to be removed
Nanoprecipitation[9]	• NBMshave a well-defined size and a narrow size distribution • Less toxic solvents	• Extensive optimization of polymer/solvent/non-solvent system
Salting out	• No heating process required • No hazardous / chlorinated solvents are employed	• Requires an extensive optimization of process conditions (salt type and concentration, type of polymer and solvent, and their ratios)
Spray drying	• The residual organic phase is immediately evaporated • Easy to set up • Possibility to scale up	• Difficult to control drug distribution into the NBM • Adhesion of nanoparticles to the inner walls of spray dryer • Broad size distribution
Ionotropic gelation and polyelectrolyte complexion technique[10]	• No expensive and toxic organic solvents needed	• Extensive optimization of polymer/counter ion concentration

Most of these preparation methods require two steps: first, the formation of the emulsion, and second, solvent elimination in order to obtain NBMs. The emulsion can be formed in the presence of two non-miscible solvents, where the smaller volume phase is dispersed into the larger one. Amphiphilic surfactants or emulsifying agents are generally added to stabilise the droplets inside the continuous phase (usually Tween® and Span).

The choice of the appropriate preparation method depends on the desired physico-chemical properties of polymeric NBMs being created, the drug to be encapsulated and the type of nanocarrier desired (nanospheres, nanocapsules, etc.). For example, the choice of solvent may influence the size of polymeric NBMs, but not the intrinsic properties of the polymer in terms of its composition or molecular weight. Moreover, this is crucial for the resultant drug-loading and drug-release profiles. The advantages and disadvantages of each method are described in Table 2[11].

[9] Method used for PLA nanoparticles in the case study. "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat
[10] Method used for chitosan nanoparticles in the case study. See knowledge base on "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat
[10] More information in the knowledge base on "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat

Table 3
Examples of methods to obtain solid NBMs.

Methods	Main principles	Advantages	Disadvantages
Freeze-drying (almost 50 % of biopharmaceuticals listed by FDA and EMA)	Elimination of water by sublimation	• In the presence of lyoprotectants and cryoprotectants, this method allows for resuspension of NBMs and preserves physiocochemical properties • Suitable for heat-sensitive molecules such as proteins or vaccines • Can be prepared in continuous mode or batches, depending on production needs	• Requires considerable energy for freezing • Needs a high vacuum • Long and expensive process • Vial-to-vial variations in polymorphs • Presence of residual moisture
Spray-dry	Elimination of water by product aerosolisation	• Rapid and cheap • Can be prepared in continuous mode or batches, depending on production needs	• Shear stress • Not suitable for heat-sensitive molecules

Almost all therapeutic NBMs are obtained in suspension using water-based solutions as dispersion medium. In order to obtain solid dosage forms, which are more stable than liquid dosage forms and help to ensure a long-term stability of nanomedicines, the methods in the Table 3 can be used.

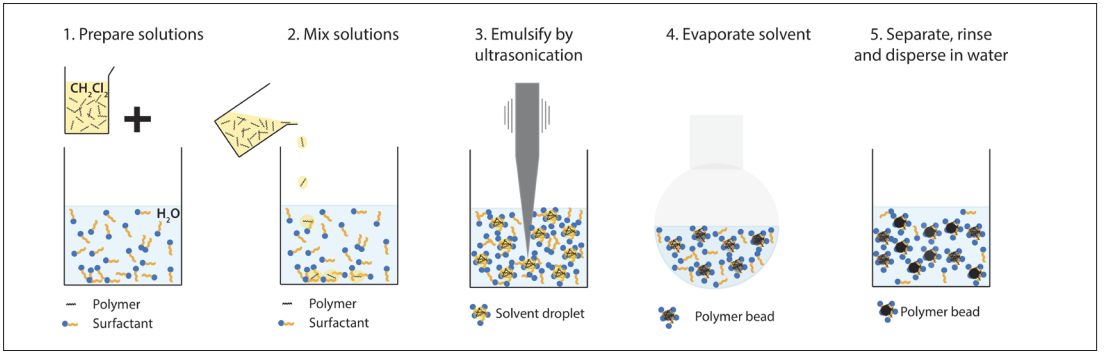


Figure 4
Emulsion-evaporation method for the preparation of tuneable PHA NBM.

CASE STUDY: PHA NANOBIOMATERIALS PREPARATION METHOD

Typical methods for producing polyhydroxyalkanoate (PHA)[12] micro- and nanobiomaterials are emulsion-evaporation, dialysis, nanoprecipitation, salting-out, supercritical fluid spray and electrospray. Emulsion-evaporation is the most commonly employed method due to the simplicity and flexibility of its synthesis parameters, which enable it to create a wide variety of NBMs. Figure 4 describes the main steps involved in a typical emulsion-evaporation process for producing surfactant-stabilised PHA micro- or nanobiomaterials.

It should be noted that for drug delivery applications, the production of PHA materials must reproduce constant quality. This can be achieved using a chemostat fermentation process i.e. a continuous culture with constant conditions. Moreover, a careful purification step is necessary to remove any endotoxins which could be transferred from the cell wall of the gram-negative producing bacteria.

The emulsion-evaporation method enables the preparation of polymeric PHA NBM with a wide range of physicochemical properties (size, density, surface charge and surface structure, stability in various media, etc.) that can be tuned by varying the following synthesis parameters:

- Chemical nature of the PHA polymer (side-chain length, chemical modification, etc.) and its molecular weight
- Nature of the surfactant (polarity, partition coefficient in both phases, size, chemical interaction with the polymer, etc.)
- Initial concentration of surfactant in the aqueous phase and of PHA in the solvent phase, as well as the surfactant-to-polymer mass ratio and solution-to-solvent volumetric ratio
- Means of generating the emulsion (e.g. stirring, high-speed stirring, ultrasonication), especially in terms of the total energy input
- Evaporation procedure, particularly the rate of evaporation (i.e. temperature, pressure, stirring)
- Remaining concentration of surfactant in the solution after rinsing and during storage
- When a drug is added, the chemical nature of the drug molecule – as well as its size, its partition coefficient and its interaction with the polymeric phase – may influence the physicochemical properties of the final NBM

[12] The complete PHA case study is found in the knowledge base "Polymeric nanobiomaterials for drug delivery": www.empa.ch/gonanobiomat

REGULATORY FRAMEWORK

WHAT ARE THE REGULATORY FRAMEWORKS IN SWITZERLAND AND IN THE EU?

One of the first steps towards developing a marketable nanomedicine is understanding the relevant regulatory frameworks and their requirements. These requirements are often underestimated and may put a product's success (e.g. a nanocarrier), or even that of its company, at risk. Compliance requirements, such as the time and money spent on product development, place a substantial burden on SMEs, despite this being in direct contradiction to the need for affordable drugs.

The main goals of medicine regulations are to ensure the safety, efficacy and quality of new nanomedicines or any other medicines. Any potential risks associated with a medicine should be eliminated or mitigated in order to protect medical personnel, patients and the environment. Different regulatory bodies are responsible for regulating nanomedicines depending on the region where the products are to be marketed and on their applications (Table 4).

Nanomedicine has been defined as the medical application of nanotechnology, and it can be divided into three different applications: (1) nanocarriers for drug delivery and pharmaceutical products themselves (nanopharmaceutical); (2) medical devices; and (3) *in vitro* and *in vivo* diagnostics. The focus is on nanopharmaceuticals. However, some information on medical devices is also given. The third type of application is outside of the scope of the guidelines and, therefore, will not be discussed further. The chemical branch is also represented in Table 4, as raw materials often start in their bulk form before being transformed into nanobiomaterials and ultimately used in a nanomedicine preparation.

WHAT IS SPECIAL ABOUT NANOMEDICINES?

Currently, there are no specific regulations regarding nanocarriers for drug delivery (nanopharmaceuticals). These products are monitored by applying the same regulations as for conventional medicines. However, the authorities do have the possibility to ask additional nano-specific questions. In the upcoming regulations for medical devices, the use of nanomaterials may require a specific and possibly higher classification depending on the risk of internal exposure (EU MDR 745/2017, Annex VIII, chapter III, rule 19). A notified body will have to decide whether clinical trials are needed. It is therefore highly recommended that SMEs contact their relevant regulatory authorities (if these authorities provide such services) or the newly established ContactPointNano. The ContactPointNano provides companies with contact to experts, organises trainings and acts as a platform for the exchange of information. (http://contactpointnano.ch/)

According to recently published draft guidance by the FDA[13], the following factors should be considered for safety, efficacy and quality in the development of a nanomedicine (medical device or nanopharmaceutical):

- The adequacy of the characterisation of the material structure and its function
- The complexity of the material structure
- The understanding of the mechanism by which the material's physicochemical properties have an impact on its biological effects (e.g. effects of particle size on pharmacokinetic parameters)
- The understanding of in vivo release mechanisms based on the material's physicochemical properties
- The predictability of *in vivo* release based upon established *in vitro* release methods

[13] FDA Draft Guidance Document: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/drug-products-including-biological-products-contain-nanomaterials-guidance-industry

Table 4:
Comparison between the authorities, laws and registration processes in Switzerland and European Union for any chemicals, pharmaceuticals and medical devices.

		Chemicals	Medicines	Medical Devices
SWITZERLAND	Authority	Federal Office of Public Health (FOPH); Federal Office for the Environment (FOEN); State Secretariat for Economic Affairs (SECO)	Swissmedic	Swissmedic
	Law	Chemicals Ordinance (ChemO)	Ordinance on Medicinal Products (OMed)	Medical Devices Ordinance (MedDO)[14]
	Notification and Reporting	Notification to notification authority at > 1 tonne/year	Authorisation through Swissmedic	Declaration of conformity by Conformity Assessment Bodies
EUROPE	Authority	Chemicals Agency	The marketing authorization (MA) is done by European Commission for centralised procedures[15]; The EMA (European Medicines Agency) is responsible for the scientific evaluation that supports the MA[16] National competent authorities are responsible for marketing authorization and scientific evaluation of non-centralised procedures	Regulation through National Competence Authorities in each member state[17]
	Law	REACH EC (1907/2006)	Directive 2001/83/EC and Regulation (EC) 726/2004	Regulation MDR 2017/745 and IVDR 2017/746 (transitional Directive 90/385/EEC, 93/42/EEC and 98/79/EC)[18]
	Registration process	Registration with ECHA at > 1 tonne/year	Authorisation through EMA	EC declaration of conformity by certified Notified Bodies

[14] The MedDO is being revised according to EU regulations and will be applied in 2020. (More info is available at: https://www.bag.admin.ch/bag/en/home/medizin-und-forschung/heilmittel/aktuelle-rechtsetzungsprojekte/revision-med-prod-verord-mepv.html)

[15] See the groups of medicines that are eligible for the centralised procedure https://www.ema.europa.eu/en/about-us/what-we-do/authorisation-medicines

[16] More information on MA under this link https://www.ema.europa.eu/en/human-regulatory/marketing-authorisation

[17] List of Medicine Regulation Authorities in each member state: https://www.ema.europa.eu/en/partners-networks/eu-partners/eu-member-states/national-competent-authorities-human

[18] Officially, MDR 745/2017 will be applied by 26 May 2020 (see MDR 745/2017 Art. 123) and IVDR (EU) 2017/746 by 26 May 2022. Before this date, the national laws and regulations of the member states are applicable. However, if devices comply with the new MDR, they can be registered according to MDR 745/2017, Art. 120, Section 5, before this date.

- Physical and chemical stability
- The maturity of the nanotechnology involved (including manufacturing and analytical methods)
- The potential impact of manufacturing changes, including in-process controls and the robustness of the control strategy on the drug product's critical quality attributes
- The material's physical state upon administration
- The route of administration
- The material's dissolution, bioavailability, distribution, biodegradation and accumulation, as well as the predictability of these elements based on physicochemical parameters and animal studies

In the case of nanocarriers, registration with the relevant authority requires a full set of pre-clinical and clinical studies because they are all considered to be new drug entities, even if the drug or the nanocarrier material used have previously been approved. The rationale behind this is that nanocarriers can be used to change a drug's bioavailability, for example, thus changing its pharmacokinetic and pharmacodynamic profiles, which may ultimately have an impact on its safety.

Due to their complex structure, drug-loaded nanocarriers are considered to be non-biological complex drugs (NBCDs). The combination of the nanobiomaterial and the drug is decisive for the efficacy and safety of this drug class, and in its entirety it represents the active pharmaceutical ingredient. As with their biological counterparts (e.g. therapeutic proteins), NBCDs cannot be fully characterised, and therefore the manufacture and registration of "follow-on" drug nanomedicines as generics appear to be impossible. Although such nanomedicine follow-on products have received marketing authorisations in the past, by following the generic pathway, discussions among stakeholders are ongoing about putting in place a regulatory strategy for "nanosimilars" – in analogy to the biosimilars for complex biological drugs. This will represent an additional hurdle in the development and marketing of future follow-on nanomedicines as they will require studies going beyond the demonstration of bioequivalence between the originator drug and the intended "nanosimilar".

The European Commission's conformity assessments for medical devices incorporating or consisting of nanobiomaterials or a nanoscale coating are identical to those for conventional medical devices. This means that conformity is dependent solely on the class of medical device, which for devices incorporating or consisting of nanobiomaterials are classes III, IIa or IIb, depending on their potential for internal exposure. However, the certified body responsible for the European Commission declaration of conformity must be accredited for the certification of devices incorporating or consisting of nanobiomaterials. For medical devices, this audit process may be problematic because of the limited availability of accredited notified bodies for medical devices of all classes. Currently, 57 notified bodies exist in the EU and affiliated countries. A few years ago, they were more than 80 and this number is expected to become significantly lower in the next few years, as this is the current trend with regards to EU Regulations 2017/745 and 2017/746. Switzerland has just two conformity assessment bodies (equivalent to Europe Commission's notified bodies). However, neither Switzerland nor the EU has yet issued accreditations to audit medical devices containing nanoscale parts[19], which means that the developer has to get accreditation from already existing notified bodies or conformity assessment bodies.

[19] The list of accredited notified bodies under 93/42/EEC and 2017/745 can be found on the "NANDO" platform, under this link: https://ec.europa.eu/growth/tools-databases/nando/index.cfm?fuseaction=directive.pdf&refe_cd=93%2F42%2FEEC&requesttimeout=900

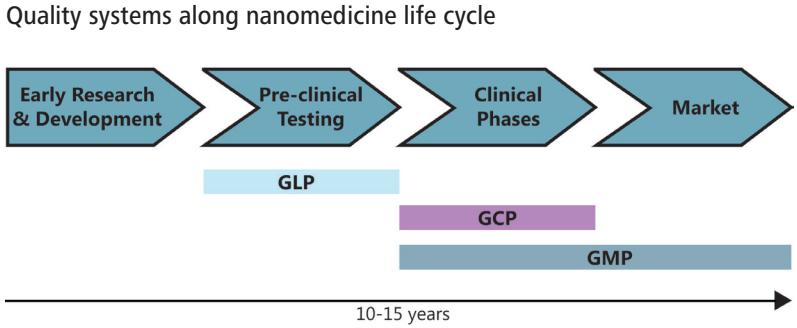


Figure 5
Quality systems for nanopharmaceuticals.

WHICH QUALITY SYSTEM SHOULD BE FOLLOWED?

As with any pharmaceutical, developing a successful nanopharmaceutical requires strict adherence to quality-system regulations. Different quality systems apply (Figure 5) depending on the phase of development. During the preclinical testing phase, all tests must be done using the principles of Good Laboratory Practice (GLP), which were developed in accordance with the OECD[20]. They concern the organisational processes and conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported, and they ensure the quality and vali-dity of data produced during this phase.

Before entering the phase of clinical trials, a nanopharmaceutical must be manufactured according to the principles of GMP[21]. Indeed, this aspect should already have been considered in earlier phases, meaning that collaboration with the manufacturer should begin as soon as possible in order to ensure the quality required for the clinical phases. GMP is also the standard for meeting the requirements of a marketing authorisation (MA). Another important aspect to remember is that changes in the value chain (e.g. production processes, suppliers) may cause new test requirements.

Before entering clinical phases, a nanopharmaceutical must be agreed by a competent Ethics Committee. Clinical phases must follow the standards of Good Clinical Practice (GCP)[22]. GCP encompasses the design, recording and reporting of trials involving human subjects. This ultimately ensures that the rights, safety and well-being of the trial's participants are protected and that the data produced during clinical trials are credible.

Quality management systems of medical device manufacturers and the technical document file of the product shall comply with ISO 13485:2016[23].

ARE THERE ANY NANO-SPECIFIC GUIDELINES?

Various organisations have drafted guidelines to help companies through the different steps in the development of a medicinal product:

ECHA: "Guidance describing the information requirements under REACH with regard to substance properties, exposure, use and risk management measures, in the context of the chemical safety assessment.

[20] OECD good laboratory webpage: http://www.oecd.org/env/ehs/testing/goodlaboratorypracticeglp.htm
[21] More information under this link: https://www.swissmedic.ch/swissmedic/en/home/news/mitteilungen/good-manufacturing-practices-gmp-vorgehen-abweichungen-zwischen-eu-und-pics-gmp.html
[22] ICH efficacy guidelines E6: https://www.ich.org/products/guidelines/efficacy/article/efficacy-guidelines.html
[23] https://www.iso.org/standard/59752.html

It is part of a series of guidance documents that aim to help all stakeholders with their preparation for fulfilling their obligations under the REACH Regulation. This document gives specific guidance regarding the testing of nanomaterials[24].

OECD guidelines: The OECD guidelines enable the assessment of the potential effects of chemicals on human health and the environment. The OECD has also produced a Guidance Manual for the Testing of Manufactured Nanomaterials[25] as well as Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials[26].

ICH guidelines: The International Conference on Harmonisation's goal is to harmonise the testing carried out during research and development of new medicines. The ICH has created diverse guidelines encompassing quality, efficacy and safety as well as multidisciplinary guidelines[27]. For example, it has created the Common Technical Document (CTD), which assembles all quality, safety and efficacy information in a common format.

EMA: The European Medicines Agency has created guidelines on nanomedicines in order to help medicine developers prepare MA applications for human medicines. For example, a reflection paper was produced about the Development of block-copolymer-micelle medicinal products[28].

SCENIHR: The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks has established Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices[29].

FDA: The US Food and Drug Administration established a draft guidance document in 2017 about Drug Products, Including Biological Products, that Contain Nanomaterials – Guidance for Industry[30].

CASE STUDY: POLYMERS AND REGULATION

Polymers are regulated differently depending on the type of application and the countries in which they will be marketed. The three decision trees below (Figures 6, 7 and 8) represent three case studies:

- Companies producing monomers and/or polymers in Switzerland
- Companies producing monomers and/or polymers in the European Union (EU)
- Companies developing either polymeric nano-platforms or polymeric nanocarriers for drug delivery in Switzerland and EU

In Switzerland and European Union, polymers which are only used for therapeutic products (e.g. polymeric nanocarriers for drug delivery) are exempt from notification (Switzerland) and registration (EU) because these polymers (which, here, are intermediary products) are regulated by other regulations.

[24] https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment

[25] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en

[26] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)40&docLanguage=En

[27] http://www.ich.org/products/guidelines.html

[28] http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000564.jsp&mid=WC0b01ac05806403e0

[29] SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), Final Opinion on the Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medi Devices, January 2015. https://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_045.pdf

[30] https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM588857.pdf

Figure 6
Decision tree for companies producing monomers or polymers in Switzerland.

Decision tree for companies producing monomers or polymers in Switzerland

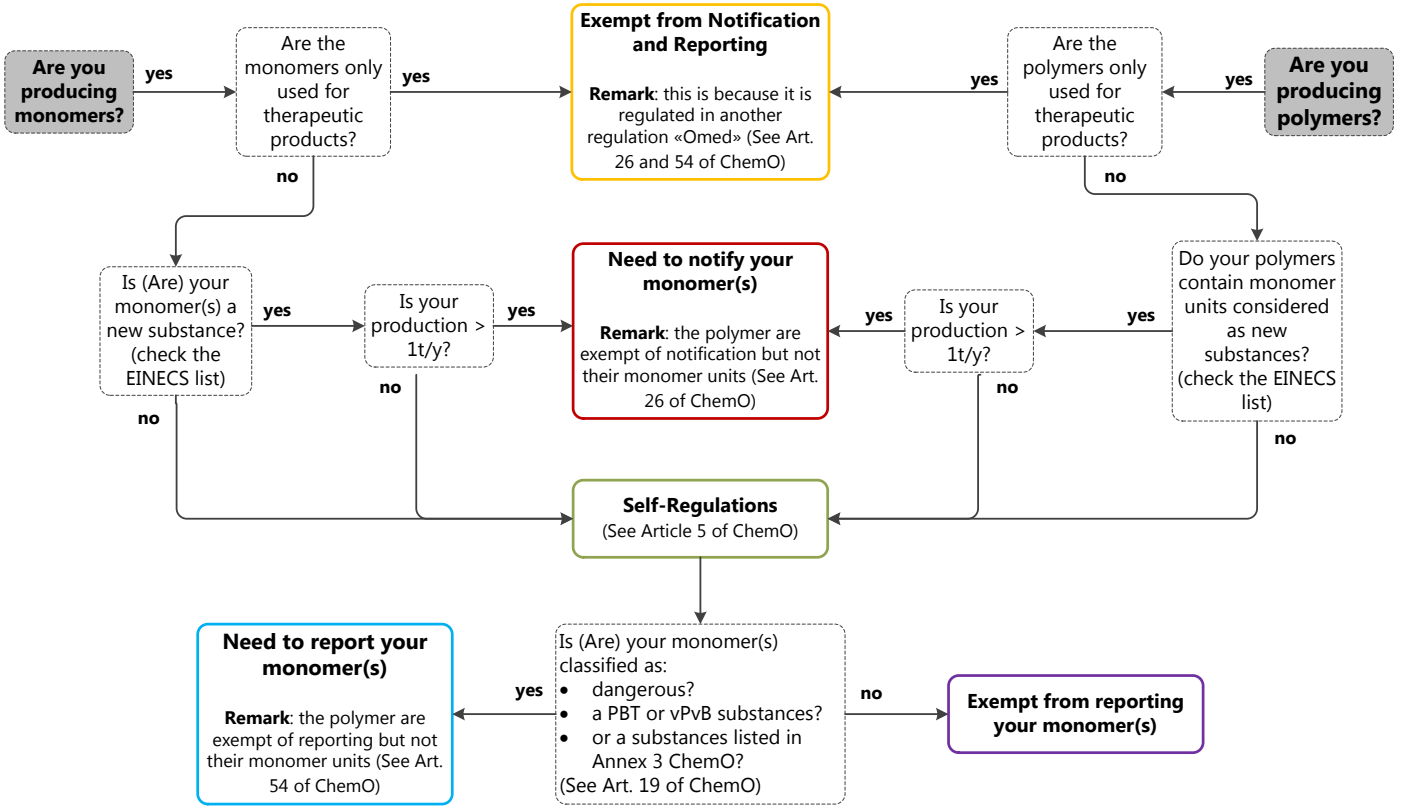


Figure 7
Decision tree for companies producing monomers or polymers in the EU.

Decision tree for companies producing monomers or polymers in the EU

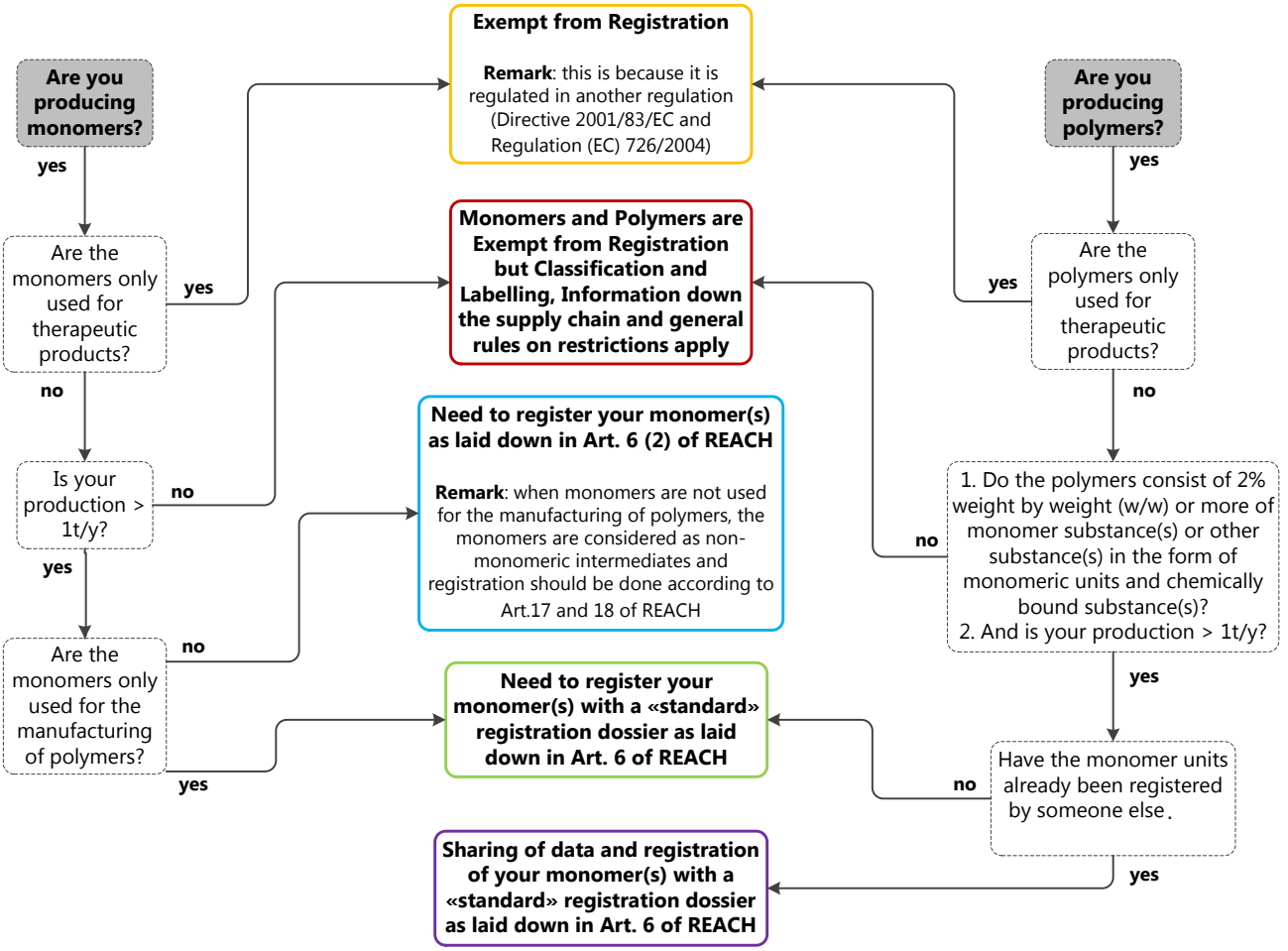
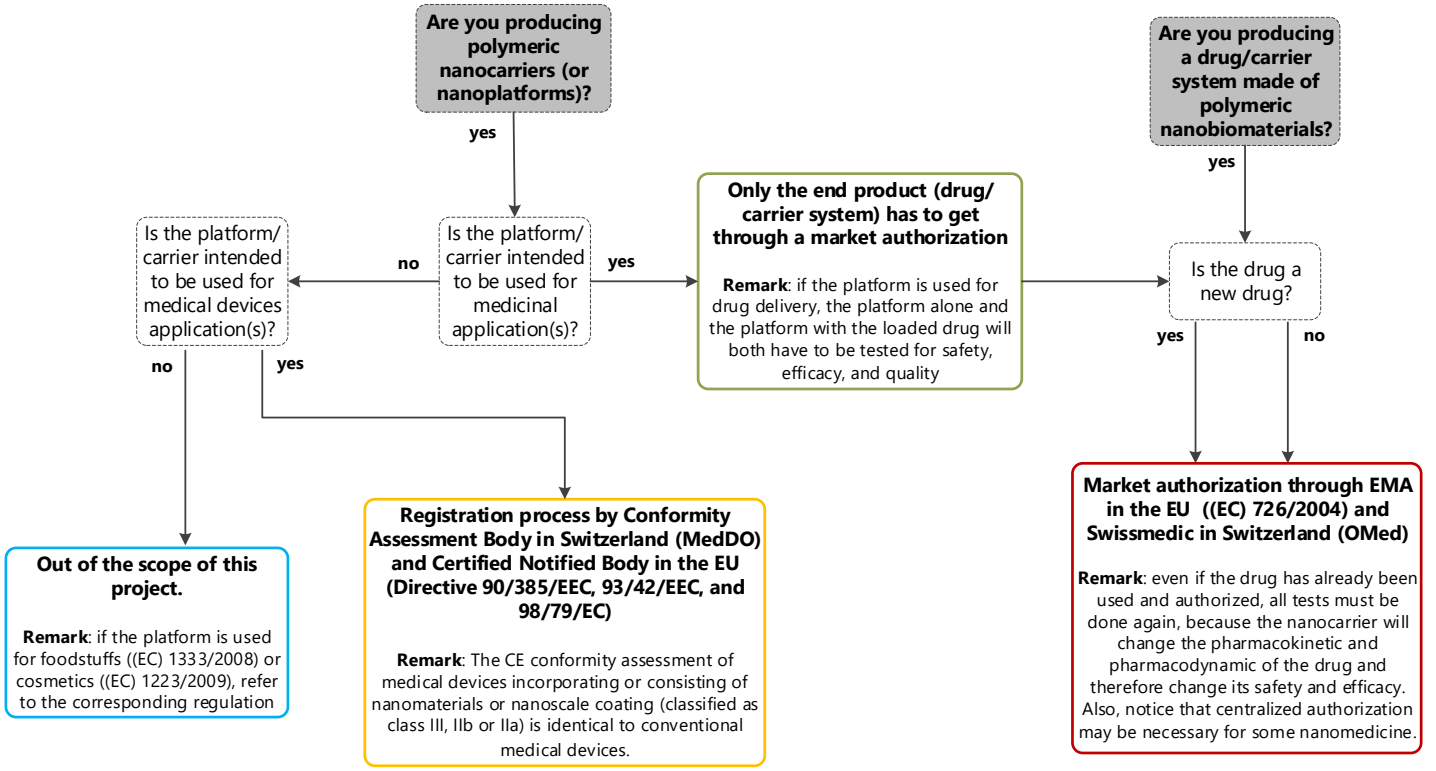


Figure 8
Decision tree for companies producing either polymeric nanoplatforms or drug/nanocarrier systems made of polymeric nanobiomaterials in Switzerland and in the EU.

Desision tree for companies producing either polymeric nanoplatforms or drug/nanocarrier system made of polymeric nanobiomaterials in Switzerland and in the EU



CHARACTERISATION OF POLYMERIC NANOBIOMATERIALS

WHEN AND HOW TO CHARACTERISE NANOBIOMATERIALS?

The characterisation of polymeric NBMs must include a meticulous evaluation of the physicochemical properties of both the raw polymer and the polymeric NBM as they affect the efficacy, safety and quality of the final product. As already mentioned in the regulatory framework chapter, according to the FDA's draft guidance, it is important to understand the mechanisms by which the physicochemical properties of the material influence its biological effects (e.g. effect of particle size on pharmacokinetic parameters). Characterising physicochemical properties is also critical to quality and finding the acceptable ranges for each attribute (Critical Quality Attribute, or CQA, refer to chapter on Chemistry, Manufacturing and Control).

Characterisations should be made at different time points in the development of the NBM, as listed in Table 5. It is generally recommended to use at least two orthogonal methods for each endpoint in order to identify and avoid any potential artefacts created by either method.

It should also be noted that the determination of properties – such as size and charge – which are highly dependent on dispersion medium, must also be performed in the final formulation diluted in cell culture medium. Furthermore, any predicted chemical or physical modifications which might occur during the polymeric NBM's lifetime should also be considered, as they could influence its biological effects.

Table 5
Important physicochemical properties to be evaluated and examples of the analytical techniques to be used for: A) unprocessed polymers (pristine); B) the nanobiomaterial's final formulation (polymeric nanocarriers suspended in e.g. water, buffers or other solvents); C) nanobiomaterial suspended in the medium used during *in vitro* assays. The √s indicate when these measures should be performed and constitute an expert consensus opinion of the GoNanoBioMat consortium.

	Polymer	Nanobiomaterial		Analytical Techniques (examples)
	A Pristine	B Nanobiomaterial final formulation	C Nanobiomaterial suspended in the medium[31]	
Molecular Weight (MW) and MW distribution	√			Size Exclusion Chromatography (SEC)
Molecular Structure	√			• Ultraviolet–Visible • Infrared • Raman • Nuclear Magnetic Resonance • Electron Spin Resonance • X-ray Diffraction and Mass Spectroscopy
Purity, Impurities, Contaminants, Endotoxins	√	√	√	Depends on impurity or contaminant: • Trace metals – Inductively Coupled Plasma/Optical Emission • Spectrometry (ICP/OES) • Endotoxins – the Limulus amebocyte lysate (LAL) method
Solubility	√			• Shake-flask method with an appropriate solvent, followed by quantification of the soluble polymer
Thermal Properties	√	√		• Glass transition, crystallisation and melting temperatures measured using Differential Scanning Calorimetry (DSC) • TGA thermos gravimetric analysis
Mechanical Properties	√			• Hardness (particle deformation) measured using Atomic Force Microscopy (AFM) • Viscoelastic behaviour measured using Dynamic Mechanical Analysis (DMA)
Chemical Composition	√	√		• Energy Dispersive X-ray analysis (EDX)
NBM Size, Size Distribution/ Polydispersion		√	√	• Dynamic Light Scattering (DLS) • Asymmetric Flow Field Flow Fractionation (AF4) • Transmission Electron Microscopy (TEM) • Scanning Electron Microscopy (SEM)

[31] Some of the physicochemical properties of nanobiomaterials are influenced by the external phase of the suspension (dispersion). To better understand the results of in vitro tests performed with nanobiomaterials, the NBM characterisation must be done in the medium used to carry out these tests, e.g. cell culture medium.

Table 5 (continued)

	Polymer	Nanobiomaterial		Analytical Techniques (examples)
	A Pristine	B Nanobiomaterial final formulation	C Nanobiomaterial suspended in the medium[31]	
Shape/Morphology		√	√	• TEM • SEM • Multi-Angle Laser Light Scattering (MALLS)
Surface Charge		√	√	• Electrophoretic Light Scattering (ELS)
Surface Area		√	√	• Gas adsorption using the Brunauer-Emmett-Teller (BET) technique
Surface Chemistry		√	√	• X-Ray Photoelectron Spectroscopy (XPS)
Aggregation/ Agglomeration		√	√	• Turbidity • DLS • TEM • SEM
In vitro Degradation	√	√	√	• Can be measured by changes in properties: tensile strength, colour, shape or molecular weight • Detection of degradation products (e.g., monomers) with HPLC or Gas Chromatography (GC)
Drug Loading Capacity		√		• Depends on the drug; HPLC is frequently used to quantify the drug
In vitro Drug Release		√	√	• Dissolution apparatus described in Pharmacopeias associated with a suitable drug detection method

[31]Some of the physicochemical properties of nanobiomaterials are influenced by the external phase of the suspension (dispersion). To better understand the results of in vitro tests performed with nanobiomaterials, the NBM characterisation must be done in the medium used to carry out these tests, e.g. cell culture medium.

HUMAN HEALTH RISKS OF POLYMERIC NANOBIOMATERIALS

HUMAN HEALTH RISKS – AN OVERVIEW

The physicochemical properties of polymeric NBMs, such as their smaller size and higher surface area ratio compared to their bulk material, make them attractive novel drug-carriers. However, these same NBM properties hinder any extrapolation of knowledge on the toxicity risks of their bulk material. It is thus essential to assess the potential human health risks of NBMs.

When it comes to nanomedicine, two types of exposure can be distinguished: firstly, intended exposure via administration to patients, and secondly, unintended exposure, mainly via the occupational exposure of staff. Exposure assessments are defined by the type of administration or the exposure route, the dose, and the duration of the treatment or exposure. Whereas patient exposure scenarios are well defined, occupational exposure can also occur through multiple, unexpected routes, resulting in potentially cumulative levels of exposure and accumulation in organs whose impact on human health might be very different from the one initially predicted. Moreover, workers' exposure to empty nanocarriers, preliminary forms of the nanocarrier or even by-products, can also have undesired side-effects distinct from those of the final drug formulation.

The polymer's properties should be assessed, including, for instance, its molecular weight, any chemical modifications, purity and contaminants, as well as (among other factors) the resulting NBM size, zeta potential, shape and surface chemistry. All these properties have the potential to have a critical impact on the safety profile of polymeric NBMs. Therefore, a thorough NBM hazard characterisation should comprise the quantitative and qualitative descriptions of all the possible toxicological effects generated via *in vitro* and *in vivo* toxicity studies, and dose-response assessments should be included whenever possible.

The following sub-chapter will go into more depth about the current status of research into exposure and hazards, poining out both the associated challenges and trends. The end of the chapter introduces a decision-making tool that will enable readers to evaluate the human health risks resulting from intentional (patient) or occupational exposure to polymeric NBMs.

EXPOSURE

With polymeric NBMs, just as with conventional medicines, patients or staff may be exposed via different routes, including the respiratory, oral, ocular, dermal and parenteral (injectable and implantable) routes. Each route has its own biodistribution pattern and consequently has different effects on human health. Table 6 summarises the most common administration/exposure routes, together with the most important NBM properties related to each one.

Table 6[32]
Common routes of administration/exposure: important considerations relating nanobiomaterial properties to various routes of exposure.

Route of Exposure	Considerations on the Exposure Route	Nanobiomaterial Properties and their Relationships to the Exposure Route	
Respiratory	• The most common route of exposure in the workplace • NBMs inhaled for drug delivery must overcome bronchial mucociliary clearance • Inhaled NBMs may translocate to various regions of the brain, without crossing the blood–brain barrier • Inhaled NBMs can cross the alveoli–blood barrier, reaching the systemic-circulation portion of the cardiovascular system, without gastric passage or a first-pass metabolism	Size	• NBMs of about 20 nm have the highest proportional deposition rate in the alveolar region • NBMs smaller than 55 nm will penetrate the alveoli more efficiently than NBMs of 200 nm or greater
		Charge	• Positively charged NBMs will exhibit greater interaction with the mucus' negative charge, thus avoiding fast mucociliary clearance
		Others	• Inhalation flow-rate influences which region of the respiratory tract NBMs will reach • The mucoadhesive properties of NBMs may increase their residence time in nasal mucosa, increasing drug absorption
Oral	• The first choice, non-invasive route • Inhaled NBMs cleared by the mucociliary system may be ingested • Ingested NBMs can reach and interact with different organs of the gastrointestinal (GI) tract such as the oesophagus, stomach, small and large intestine and colon • Ingested NBMs can translocate into the systemic circulation portion of the cardiovascular system, but to do so, they must resist a wide range of pH environments and enzymatic degradation until they reach the small intestine • The absorption of ingested NBMs can be hindered by the poor permeability of the intestinal epithelium • Before reaching systemic circulation, ingested NBMs and cargo drugs will undergo a first-pass metabolism in the liver	Size	• Particles with a diameter of less than 50 nm are known to cross epithelial barriers via paracellular passage, whereas larger particles are endocytosed by intestinal enterocytes (< 500 nm) or taken up by M cells in Peyer's patches (< 5 µm)
		Charge	• Positively charged NBMs may exhibit greater interaction with intestinal mucus, therefore improving NBM retention, but also decreasing NBM absorption • Neutrally charged NBMs diffuse more efficiently through the mucus layers
		Others	• Surface-coating NBMs with enteric polymers improves their resistance in the GI tract • Hydrophilicity and poor chemical or enzymatic stability in the GI tract diminish intestinal absorption

[32] The references can be found in the knowledge base report on "Human health risks of polymeric nanobiomaterials": www.empa.ch/gonanobiomat

Table 6[33] continued

Route of Exposure	Considerations on the Exposure Route	Nanobiomaterial Properties and their Relationships to the Exposure Route	
Injection	• Most commonly used routes for injectables include intravenous, intramuscular, subcutaneous and intradermal administration • Injectables are the first choice for active pharmaceutical ingredients with narrow therapeutic indices, poor bioavailability or administration to unconscious patients • Intravenously injected NBMs are distributed throughout the circulatory system, reaching different organs • Intradermal injection leads to uptake by the lymphatic system • Intramuscularly injected particles are taken up via the neuronal and lymphatic systems • Intravenously injected NBMs are rapidly cleared by the kidneys and liver, or via the reticuloendothelial system (RES)	Size	• Smaller NBMs are mostly absorbed into capillaries, whereas larger NBMs are drained by the lymphatic system
		Charge	• NBMs with positively charged surfaces exhibit greater interactions with blood components and are therefore more rapidly cleared by the mononuclear phagocyte system • NBMs with neutral and negatively charged surfaces have longer circulation half-lives
		Others	• NBMs surface-hydrophobicity increases interaction with blood components and therefore increases nanomaterial clearance via the mononuclear phagocyte system • NBMs surfaces coated with hydrophilic polymers or surfactants exhibit decreased clearance by opsonisation
Dermal	• Mostly used for the topical delivery of molecules intended to act locally (sunscreens, antifungals, antiinflammatory or keratolytic agents, etc.) • Accumulation in hair follicles can increase the penetration of NBMs and cargo drugs • Damaged skin is more permeable to larger NBMs • Small, lipophilic molecules can penetrate easily into the skin and eventually reach the bloodstream or the lymphatic system	Size	• NBMs < 20 nm may penetrate or permeate intact skin • NBMs < 45 nm may penetrate damaged skin • NBMs > 45 nm may translocate or be stored in skin appendages (i.e. hair follicles)
		Charge	• Cationic NBMs have an affinity for negatively charged skin pores (which can limit their subsequent diffusion)
		Others	• Physicochemical methods, such as the application of low-frequency ultrasound or surfactants (i.e. sodium lauryl sulphate), are used to disturb the skin barrier and promote NBM absorption

[33] The references can be found in the knowledge base report on "Human health risks of polymeric nanobiomaterials": www.empa.ch/gonanobiomat

Pharmacokinetics and pharmacodynamics of polymeric nanobiomaterials

The routes of polymeric NBM exposure or administration influence its pharmacokinetics (absorption, distribution, metabolism and excretion), as depicted in Figure 9. In addition, when used as delivery systems, their distinctive physicochemical properties, such as size, surface charge and chemistry, have a major influence on the pharmacokinetics of the drugs they deliver (Table 7). Indeed, NBMs may increase the absorption of low-bioavailability drugs by promoting their dissolution or by augmenting their half-life in systemic circulation and therefore enhancing the therapeutic effect. However, these changes can simultaneously potentiate the drug's toxicity profile in comparison to the original drug formulation. The NBM or its degradation products may reach and accumulate in different tissues than the bulk material, which makes predicting impacts on human health from bulk material distribution patterns difficult or even impossible.

In addition to the physicochemical properties mentioned in Table 7, the routes of administration or exposure also influence the drug's pharmacokinetics (as depicted in Figure 9). This, in turn, will affect the final therapeutic or toxicological outcome (pharmacodynamics). The pharmacokinetics of both the drug and the drug-loaded nanocarriers are crucial to understanding and predicting the formulation's efficacy and toxicity. Indeed, the EMA recommends evaluating and comparing the pharmacokinetics of the drug formulation (drug + carrier) and the pharmacokinetics of the drug alone.

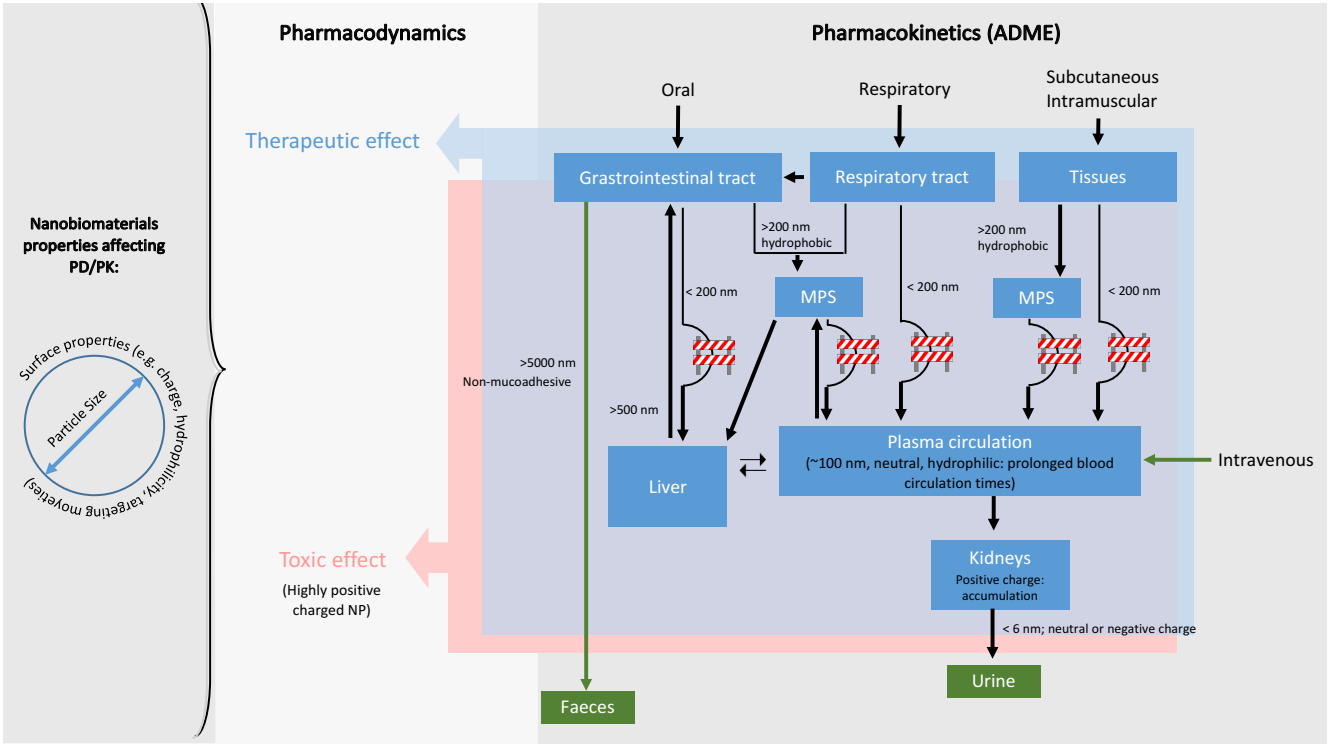


Figure 9
Representative illustration of the general pharmacodynamics and pharmacokinetics of nanobiomaterials and their cargo drugs. Depending on the route of administration and the nanobiomaterial's properties, Absorption pathways, Distribution, Metabolism and Elimination (ADME-pharmacokinetics) are depicted in the right-hand panel. The sizes and surface properties of the nanobiomaterials mentioned in the image are intended to illustrate their impact on its biodistribution. These series of events will culminate in therapeutic effects and/or undesired side-effects (pharmacodynamics) – left-hand panel. (MPS: mononuclear phagocyte system).

Table 7[34]
Influence of various nanobiomaterial properties on pharmacokinetics (PK) and pharmacodynamics (PD).

Properties	Influence on PK and PD
Composition	• Mesoporous silica NBMs are more likely than polymeric NBMs to reach the lungs
Size	• NBMs of ~100 nm have prolonged circulation times • NBMs < 6 nm are quickly eliminated through renal filtration • NBMs of 10 nm to 12 nm exhibit high permeation and low accumulation in tissues/organs • NBMs > 200 nm are recognised by the mononuclearphagocyte system (MPS) • NBMs > 200 nm are retained by splenic filtration
Shape	• Deviation from the spherical shape enhances circulation time • Rod-shaped particles are more easily taken up by cells (e.g. phagocytes)
Surface charge	• Positively charged NBMs have been known to form aggregates in the presence of negatively charged serum proteins: - aggregates may cause transient embolisms in the lung capillaries - protein corona formation may lead to particle clearance by the MPS • Neutral and negatively charged surfaces are associated with longer circulation half-lives • In the majority of cell types, non-specific uptake of positively charged NBMs is generally higher than that of neutral or negatively charged NBMs
Surface chemistry / modifications	• The modification of a NBM surface using a neutral non-ionic polymer decreases its opsonisation, increases blood circulation time, but also reduces interactions between the NBMs and the target cells • The modification of a NBM surface, using targeting moieties that bind specifically to cellular receptors, can modify the NBM's PK/PD profile by increasing specific cellular interactions

[34] The references can be found in the knowledge base report on "Human health risks of polymeric nanobiomaterials": www.empa.ch/gonanobiomat

Nanobiomaterial degradation and elimination

Polymeric NBMs may be eliminated from the body through biodegradation and/or degradation, or they can be directly expelled by the liver, kidneys or colon. Several factors affect the elimination of polymeric NBMs from the body, such as the polymer's composition and molecular weight, or the NBM's size and surface properties.

The term "biodegradable" refers to the material's property of being decomposed or mineralised into end products via biological activity as part of its degradation process. The most common polymer degradation mechanisms, which can play important roles in NBM elimination, are hydro-lysis, oxidation and enzymatic reactions. Polymers with hydrolysable backbones, such as polyesters (PLA, PCL, PHA, PLGA) or polyanhydrides, are susceptible to hydrolytic biodegradation under certain conditions. Other biopolymers, such as polyethylene or PEG, are more suitable to degradation by an oxidation reaction because their structures can easily generate free radicals. Enzymatic degradation involves hydrolysis catalysed by enzymes known as hydrolases and lipases, such as proteases, glycosidases and phosphatases. It is widely accepted that natural polymers such as chitosan or starch undergo enzymatic degradation. Several techniques can be used to reduce the enzymatic degradation of natural polymeric NBMs, e.g. acetylation of starch, chemical cross-linking of PHAs or grafting PEG onto chitosan.

The use of biodegradable polymers has advantages over that of non-biodegradable ones since the products of degradation are generally non-toxic and can be completely eliminated from the body via natural metabolic pathways. On the other hand, it cannot be excluded that some products of polymer degradation may generate cell alterations, such as inflammatory responses, and this should be taken into account when evaluating the biocompatibility of any degradable polymer. The degradation rate plays a key role in biocompatibility: a fast-degrading material can lead to such a rapid accumulation of degradation products that they overwhelm the removal mechanisms of the surrounding tissue. For instance, PLA-based polymers undergo hydrolytic degradation which may lead to an accumulation of lactic acid that decreases the tissue's pH and results in acidic toxic effects. However, some material properties, such as molecular weight, can be tuned by the developer to achieve the desired degradation rate for each specific application.

In contrast, if no degradation of the NBM occurs, it has to be excreted from the body. Only NBMs smaller than 6 nm can be eliminated through glomerular filtration in the kidneys and secretion in urine. Nevertheless, highly positively-charged NBMs or bigger nanoparticles may accumulate in tissues, causing toxicity. In fact, nanoparticles larger than 6 nm are more likely to be taken up by the mononuclear phagocyte system (MPS). In this case, if they are not degradable, nanoparticles will remain within those cells and be sequestered in the spleen and liver for more than 6 months. Alternatively, NBMs are believed to be excreted from hepatocytes when they empty their lysosomal contents into the biliary canaliculus. Depending on their composition, NBMs may be excreted into bile, transit through bile ducts, and pass ultimately into the small intestine to be excreted.

How can exposure to a polymeric nanobiomaterial be evaluated?

An exposure assessment should include a detailed estimation of the dosage (dose and frequency) and duration of exposure and, significantly, the predicted administration and/or exposure route. These parameters are the key difference between the planned administration of a patient's exposure and unplanned occupational exposure. The route, dosage and duration of

the patient's exposure are well defined since these parameters are decided upon based on what is necessary for the desired therapeutic effect and specifically for the delivered drug. However, there is a lack of appropriate methods for detecting and quantifying the unintentionally absorbed cumulative doses of these materials in workers' organisms during occupational exposures. This significantly complicates the design of predictive toxicological assays. In the context of the FP7 NanoReg project, a number of exposure tools for nanomaterials, such as the CB NanoTool and the Nanosafer, have been examined, and a new, two-box, nano-specific exposure model has been implemented. The main problems identified with these tools are their lack of quantitative estimates of exposure and the need to rely on detailed input data (rate of particulate release from the source, as well as the particle size distribution), which are not always available.

What are the challenges of testing realistic exposure *in vitro*?

The *in vitro* simulation of realistic human exposure is challenging not only for general medicines but also for nanomedicines. It is also essential for the accomplishment of adequate toxicity studies. In addition to the difficulty in quantifying occupational exposure, there is also the problem of accurately transposing actual human doses to *in vitro* settings. Furthermore, it is difficult to construct complex *in vitro* systems, based on human cells or primary cell lines, which can mimic the physiological complexity of the human body and its interactions with the NBMs. Indeed, since most of these *in vitro* studies use much higher concentrations of polymeric NBMs than those which could be used in *in vivo* experiments, they may not reflect realistic exposure scenarios. Moreover, *in vitro* tests commonly use a mass-based exposure metric, and this is believed to be a factor limiting accuracy as particle numbers, surface areas and the agglomerates formed in suspension greatly influence the effective concentration delivered to cells.

HAZARD

The hazards or toxicological effects of NBMs mainly result from their smaller particle size and greater particle surface area in comparison to their bulk material (e.g. as powders, flakes, surfaces in devices, etc.). These two properties increase the material's reactivity, ultimately resulting in augmented toxicity. Additionally, several other properties can contribute to the effects induced by nano-sized carriers, such as chemical composition, surface chemistry, surface charge or shape. The most relevant mechanisms through which NBMs interact and affect biological systems are: (1) cellular uptake; (2) oxidative stress, redox activities, the generation of reactive oxygen species; (3) cellular membrane damage; (4) inflammation, inflammasome activation, inflammatory cytokine and chemokine release; and (5) DNA damage. These mechanisms, whether alone or in combination or in synergisms, contribute to several toxicological endpoints which could have a significant impact on human health. Table 8 shows a qualitative overview of the hazard potential of various polymeric NBMs.

As can be seen, some of the results are ambiguous. This is probably due to the great variety of methodologies used: animal or cellular models, dose or concentration, assay duration and, notably, the differences between the physicochemical properties of the polymeric NBMs used in the studies and the lack of appropriate characterisation and controls in some of them. For instance, regarding oxidative stress, 6 studies were found for chitosan NBMs (bare). Of these, 3 studies reported no induction of ROS production with any of the concentrations tested (green), 1 study reported ROS production only with a higher concentration (orange) and the last 2 studies reported induction of ROS production for all the concentrations tested (red). It should be noted that this information deserves further analysis, especially considering the exhaustive table in the knowledge base report on "Human health risks of polymeric nanobiomaterials"; the concentrations and cellular models used in

each study are described in detail there. These issues derive from the absence of standardised methodologies and guidelines for evaluating NBMs. This makes the comparison of safety/toxicity assessments in different reports more complicated and, ultimately, makes it difficult to extrapolate safety profiles for human health.

Most studies are performed with drug-loaded formulations, without a simultaneous evaluation of the unloaded polymeric NBMs. In these situations, it is difficult to know whether effects are due to the drug, the NBM or both. Furthermore, testing for contaminants – particularly endotoxins such as LPS that may not be eliminated using common sterilising techniques – is almost always missing from reports. *In vitro* testing of LPS-contaminated polymeric NBMs could thus generate misleading results and false assumptions about bioactivity or toxicity, ultimately affecting a robust evaluation of the possible effects on human health. The gaps in the literature, identified above, have made it difficult to establish trends in the toxicity of most of the polymeric NBMs studied. Nonetheless, for chitosan, which was chosen as a reference case due to its common use in the field, the authors found significant relevant information and were able to formulate some general conclusions.

Chitosan-based NBM:

- do not induce oral toxicity (within the dose regimen tested);
- induce reactive oxygen species in a concentration-dependent manner
- should be tested using different but complementary genotoxicity assays, since results may be contradictory;
- induce embryonic toxicity in a dose-dependent manner.

It is important to highlight that the current lack of toxicity data – more precisely, the lack of consistent toxicity data – is preventing early safer material design based on literature. Therefore, experiments are still necessary to clarify inconsistencies and to fill the gaps. As this Safe-by-Design approach is made of iterations, the experimental data – that should have been previously been well-characterized – can then be used for refining material design and fed into (Q)SAR models for example.

Hazard potential	Chitosan				PLA				PHA				PLGA			
	Bare		Blend		Bare		Blend		Bare		Blend		Bare		Blend	
	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk
Acute toxicity																
- Via inhalation			1												1	
- Via ingestion		1	2													
- Via ocular contact	1															
- Via injection (i.v.)			1		1	1	1	1					1		3 / 1	
- Via injection (others)				1											1	
- Via implantation				1												
Repeated-dose toxicity																
- Via ingestion		1	6												2	
- Via injection (i.v.)	1		1												2	
- Via injection (others)															1	
Inflammation		1	2	1 / 2	1	1	1	1					3		1 / 2	
Oxidative stress	3 1 2	1 1 1	1 1 1		1	1	1	1					1		4	
Carcinogenicity		2	1 / 1	1											2 / 1	
Mutagenicity																
Genotoxicity																
Reproduction	2 / 2	1	1 / 1												2	
Hemolysis	2 1 1	3	1	2						1					3 2 1	

Table 8[35]
Systematisation of the toxicity results described in the literature for chitosan, PLA, PHA, PLGA, PCL, PEG, alginate, PVA and Pluronic®. The number in each cell represents the number of studies supporting each conclusion according to the following colour scheme: red indicates studies where all the concentrations tested induced an effect; orange indicates studies where at least one concentration tested induced an effect; green indicates studies that revealed no toxicity for any of the concentrations tested; (blank) no data available. (Bare: polymer materials whose nanobiomaterials were produced using crosslinkers or surfactants only, and which were not loaded with drugs, genes or proteins; Blend: blend of polymers, functionalised/chemically modified polymers or particles loaded with drugs, genes or proteins; NBM: nanobiomaterial).

[35] For further details on the tested concentrations and materials, please refer to the knowledge base report on "Human health risks of polymeric nanobiomaterials": www.empa.ch/gonanobiomat

Hazard potential	PCL				PEG				Alginate				PVA or Pluronic			
	Bare		Blend		Bare		Blend		Bare		Blend		Bare		Blend	
	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk	NBM	Bulk
Acute toxicity																
- Via inhalation															1	
- Via ingestion											2					
- Via ocular contact																
- Via injection (i.v.)	1		2				2 / 1									
- Via injection (others)							1									
- Via implantation																
Repeated-dose toxicity																
- Via ingestion							1				3					
- Via injection (i.v.)							2 / 1									
- Via injection (others)							1									
Inflammation	1														1	
Oxidative stress	1															
Carcinogenicity							1				1					
Mutagenicity																
Genotoxicity																
Reproduction							1									
Hemolysis							5 / 1									

Table 8 continued

How can a polymeric nanobiomaterial's hazard potential be assessed?

A hazard characterisation should consider suitable *in vitro* and, if necessary, *in vivo* assays, whose results can be interpreted in the context of human health risks. *In vitro* and *in vivo* assays may include the following endpoints (as described in Table 9):

- Immunotoxicity studies, such as cellular damage assessment (oxidative stress and inflammation)
- Genotoxicity
- Toxicity on reproduction
- Biocompatibility (haemocompatibility)
- Acute, repeated or chronic toxicity studies

It would be preferable for researchers to use standardised methodologies, validated for NBMs and with suitable controls in order to minimise the discrepancies in the results confirmed so far by different research groups. To date, no guidelines or reflection papers on polymeric NBMs have been released by competent authorities. However, it could be worth taking the reflection papers on coated nanomedicine products and block copolymer-micelle medicinal products[36], released by the EMA as examples, as they anticipate the important parameters which should be included in applications for marketing authorisation in those specific cases. Furthermore, for marketing authorisation, more endpoints are needed than those proposed above for human health risk assessment. In that regard, the ICH Safety Guidelines[37] should also be considered, especially for nanopharmaceuticals and in the context of assembling an Electronic Common Technical Document (eCTD). Likewise, regarding medical devices, it is also important to consider ISO 10993, which refers to several standards for the biological evaluation of these devices.

Finally, the OECD Working Party on Manufactured Nanomaterials (WPMN) aims to promote international cooperation on the safety of nanomaterials with regards to human health and the environment. This involves safety testing and risk assessments. Over the years, the WPMN has published numerous reports and some test guidelines, all of which are published in the OECD Series on the Safety of Manufactured Nanomaterials[38]. Similarly, the International Organization for Standardization has created Technical Committee ISO/TC 229 with the aim of bringing standardisation to the varied fields of nanotechnology. The committee's specific tasks include developing standards for terminology and nomenclature, metrology and instrumentation, test methodologies, modelling and simulations, and science-based health, safety and environmental practices[39].

[36] https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/multidisciplinary/multidisciplinary-nanomedicines

[37] https://www.ich.org/products/guidelines/safety/article/safety-guidelines.html

[38] http://www.oecd.org/env/ehs/nanosafety/publications-series-safety-manufactured-nanomaterials.htm.

[39] https://www.iso.org/committee/381983.html.

Table 9
Endpoints and protocols for *in vitro* and *in vivo* assays for hazard characterisation.

Endpoints	Protocols	Notes
In vitro assays		
Immunotoxicity	**Oxidative stress:** ISO/TS 19006: 2016 – CM-H2DCF-DA assay for evaluating nanoparticle-induced, intracellular reactive oxygen species (ROS) production in the RAW 264.7 macrophage cell line. **Inflammation:** No standard protocol for nanomaterials. Usually, evaluate the release of inflammatory cytokines in different cell lines using ELISA. **Metabolic activity:** ISO 19007: 2018 – In vitro MTS assay for measuring the cytotoxic effect of nanoparticles.	LPS contamination may induce cytokine release. Numbers of released cytokines may be underestimated due to the NBM's ability to adsorb biomolecules at its surface. Appropriate experimental controls should be performed.
Genotoxicity	No standard protocol for nanomaterials.	The OECD has published some considerations: "2018 Report No. 85 – Evaluation of in vitro methods for human hazard assessment applied in the OECD Testing Programme for the Safety of Manufactured Nanomaterials" and "2014 Report No. 43 – Genotoxicity of Manufactured Nanomaterials: Report of the OECD expert meeting".
Toxicity on reproduction	No standard protocol for nanomaterials.	The zebrafish embryo culture is one of the most commonly used animal models.
Haemocompatibility (biocompatibility)	ASTM E2524 – 08(2013) on Standard Test Method for Analysis of Hemolytic Properties of Nanoparticles	A material is defined as haemolytic if haemolysis values are above 5 % and as moderately haemolytic if they are between 2 % and 5 %.
In vivo assays		
Acute, repeated or chronic toxicity	OECD Test Guideline 412: 28 days (subacute) Inhalation Toxicity Study and OECD Test Guideline 413: 90 days (Subchronic) Inhalation Toxicity Study	These guidelines were revised in 2018 to accommodate the testing of nanomaterials.

What are the challenges of toxicity testing studies and the evaluation of their results?
Some of the challenges in testing the toxicity of NBMs have already been identified:

- Realistic exposure scenarios are difficult to simulate via *in vitro* studies (doses cannot be transposed to the concentrations administered to cells)
- The intrinsic and distinctive properties inherent to the nanoscale can interfere with the reagents and detection methods of the *in vitro* assays recommended for bulk materials because polymeric NBMs go through modifications (e.g. protein corona formation, aggregation/agglomeration, dissolution, generation of new nano-sized particles) when in contact with biological matrices
- Polymeric NBMs may interfere with endotoxin quantification assays. Moreover, *in vitro* testing of endotoxin-contaminated polymeric NBMs might generate misleading results and false assumptions about bioactivity or toxicity, ultimately affecting the evaluation of any potential human health effects
- Numerous toxicity studies lack the positive and negative controls designed for nanoscale material
- Results depend significantly on the chosen cell line (commonly, immortalised cancer cells), incubation time, cell culture media and cell culture supplementation. For instance, cell culture media supplementation with serum is highly likely to induce a protein corona at the surface of positively charged nanoparticles, thus changing their size and zeta potential, and therefore modifying both NBM cell interaction and uptake, and ultimately their biological effect

Case study: immunotoxicity of chitosan nanobiomaterial

As the available literature showed contradictory results, a case study was performed[40] in order to provide a better insight into chitosan's immunotoxicity. The case study's goals were to: 1) better understand how the physicochemical properties of chitosan (bulk material) and chitosan NBMs affect the immune system, 2) evaluate whether chitosan NBMs interfere with traditional assays, and 3) find appropriate positive and negative controls to avoid misinterpretation of the assays.

To test chitosan's immunotoxicity, two chitosans with 80 % and 93 % degrees of deacetylation (DD) were used to create two different chitosan NBMs of 127 nm ± 5 nm and 292 nm ± 52 nm, respectively. The results can be seen in Table 10. The tests were made using immune cells isolated from the blood of healthy donors, with immortalised macrophages (RAW 264.7 cell line) and the total human blood of healthy donors. Overall, both chitosan NBM species were more toxic than their bulk material. Chitosan NBM with an 80 % DD was more cytotoxic than chitosan NBM with a 93 % DD, and it showed longer coagulation times.

All the assays in Table 10 tested whether chitosan NBMs were interfering with their readouts as this can lead to misinterpreted results. Interference was found in the platelet aggregation study (not shown) performed through flow cytometry where NBMs were erroneously counted as platelets. Therefore, platelet aggregation study was repeated through microscopy analysis (hemocytometer platelet count) of the samples. With this assay it was possible to distinguish between platelets and NBMs preventing erroneous results.

[40] www.empa.ch/gonanobiomat

Table 10
Summary of the overall conclusions obtained from chitosan immunotoxicity studies. Blue squares represent assays where no effect was found, and brown squares represent assays where the respective test sample induced an effect at the stated concentration. Except for cytotoxicity assays, all assays were performed with a range of bulk polymer and Nanobiomaterial (NBM) concentrations that did not induce cytotoxicity. All formulations were free of endotoxin contamination.

Chit 80 % DD MW 60 kDa			Chit 93 % DD MW 122 kDa	
Polymer 612 µm ± 4 µm	Nanobiomaterial 127 nm ± 5 nm + 29 ± 1 mV		Polymer 608 µm ± 2 µm	Nanobiomaterial 292 nm ± 52 nm + 20 ± 6 mV
	IC50: 720 µg/mL	**PBMC Cytotoxicity** 2.44 µg/mL to 5 000 µg/mL		IC50: 2 104 µg/mL
		Il-6 and TNF-α 100 µg/mL		
		Hemolysis 100 µg/mL to 2 000 µg/mL		
	1 000 µg/mL	**Coagulation** 100 µg/mL to 1 000 µg/mL		
	IC50: 4 949 µg/mL	**RAW 264.7 Cytotoxicity** 312.5 µg/mL to 5 000 µg/mL		IC50: 4 858 µg/mL
		NO production 39 µg/mL to 156 µg/mL		
39 µg/mL to 156 µg/mL	39 µg/mL to 156 µg/mL	**NO inhibition** 39 µg/mL to 156 µg/mL	39 µg/mL to 156 µg/mL	39 µg/mL to 156 µg/mL
156 µg/mL	156 µg/mL	**ROS production** 39 µg/mL to 156 µg/mL		
		ROS inhibition 39 µg/mL to 156 µg/mL		

No effect
Effect
Concentration range tested

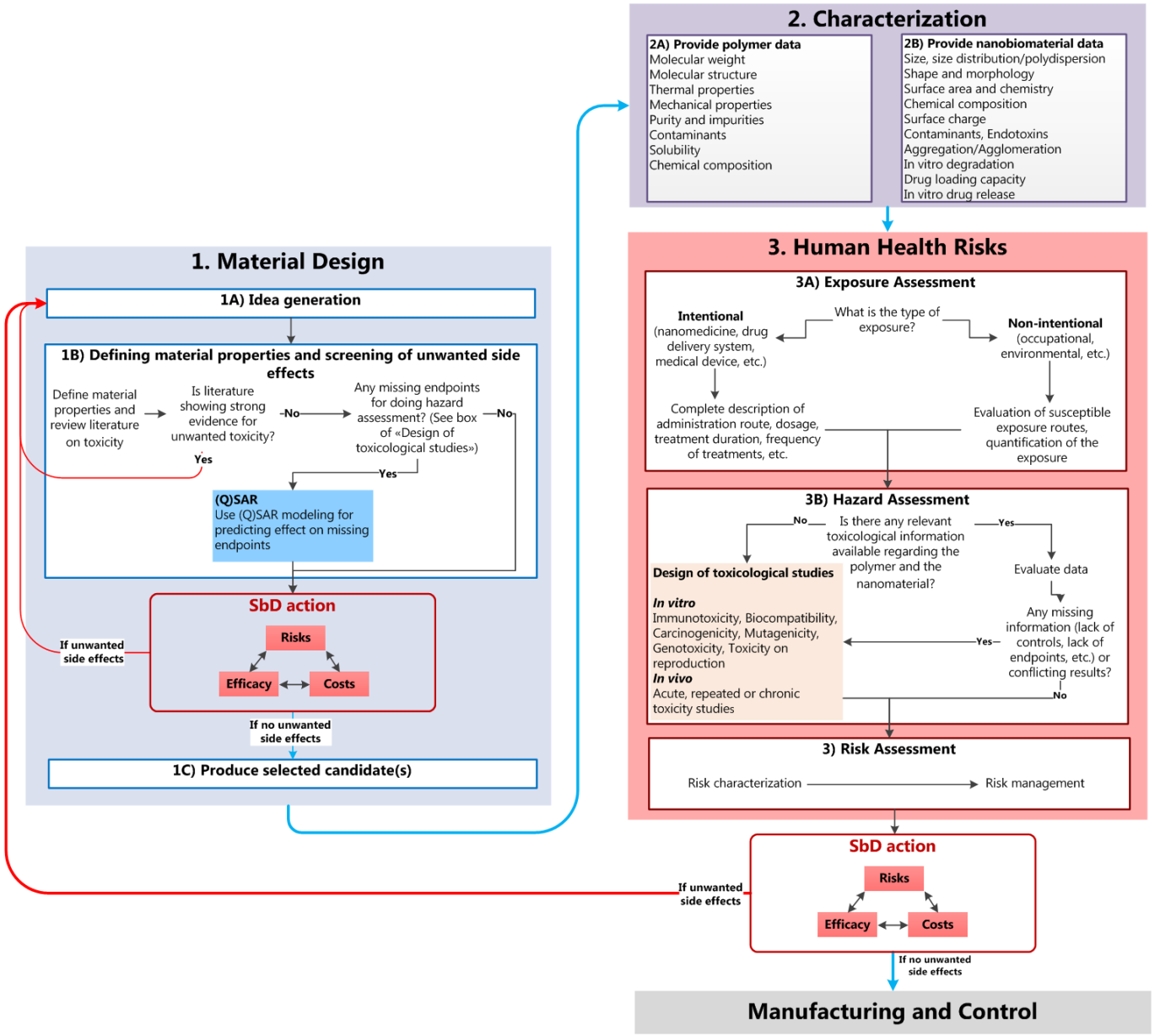
As exemplified, overcoming interferences requires the use of different assays to evaluate the same endpoint. Furthermore, all the results must be validated by carrying out appropriate experimental controls. These controls can consist of screening potential false-positive responses using one of the following strategies:

- Using NBMs only, without the biological matrix, to detect any interference with the assay readout, such as absorbance, luminescence or fluorescence;
- Always checking for cell viability when performing a cellular assay. Cell viability experiments to calculate IC50 are generally performed before any other cellular assay, however, they may use different culturing conditions (cell number, NBM concentrations, culture time, cell-plate type), and therefore NBM concentrations might not be extrapolated;
- Always testing the NBM solvent separately in every assay, as this allows the evaluation of whether results are due to the NBMs themselves or to the solvent in which they are dispersed.

HUMAN HEALTH RISKS

The decision tree illustrated on the next page (Figure 10) can be applied to an evaluation of the health risks posed by a polymeric NBM and the degree of information needed to complete a risk assessment analysis. Two SbD actions can be found in the decision tree: one before producing the selected candidates and one after the experimental evaluation of those candidates. The first SbD action is based on existing relevant literature and (Quantitative) Structure-Activity Relationship ((Q)SAR) tools. After this first SbD action, the selected candidates – those maximising safety while optimising efficacy and costs – are produced and characterised. The potential human health risks of each candidate NBM are sub-sequently evaluated and compared using experimental data and is followed by a second SbD action. An evaluation of environmental risks should also be taken into account, even though Figure 10 does not show this (see Figure 1). After each SbD action, a feedback loop returns to the material design phase should candidates show any unwanted side effects. This iterative process enables developers to find the optimum safety, efficacy and cost.

Figure 10
Decision tree for evaluating the human health risks of polymeric nanobiomaterials.



ENVIRONMENTAL RISKS OF POLYMERIC NANOBIOMATERIALS

AN OVERVIEW OF ENVIRONMENTAL RISKS

It is known that conventional pharmaceuticals can leak into the environment and have an impact on it. Some of their desired effects on humans, however, may be undesirable for other species. This is why the environmental risks of NBMs must be evaluated.

Risk is a function of hazard and exposure. This means that if there is either no hazard or no exposure, there will be no risk. An environmental hazard can be identified by the material's predicted no-effect concentration (PNEC), and environmental exposure can be identified by its predicted environmental concentration (PEC). PNEC is a concentration below which no adverse effects are expected in the environment, and PEC is the concentration in the environment. The risk can then be calculated by dividing the PEC by the PNEC. When the ratio is above 1, there is a non-negligible risk:

$$[1] \quad \frac{\text{PEC}}{\text{PNEC}} > 1 = \text{Risk}$$

CURRENT KNOWLEDGE OF ENVIRONMENTAL EXPOSURE

In order to evaluate potential environmental exposure to polymeric NBMs used in drug delivery, the first step is to evaluate their behaviour inside the human body (e.g. if NBMs are completely biodegraded before being excreted, there will be no release to the environment). In that regard, drug developers should evaluate **A**bsorption after administration, **D**istribution throughout the body, including accumulation in tissues and organs, **M**etabolism into different metabolites and **E**xcretion of NBMs and their transformation products. This is the **ADME** principle at the heart of pharmacokinetic (PK) studies[41]. When developing a nanomedicine, the European Medicines Agency (EMA) recommends studying the PK of the nanocarrier alone and the PK of the nanocarrier-drug system.

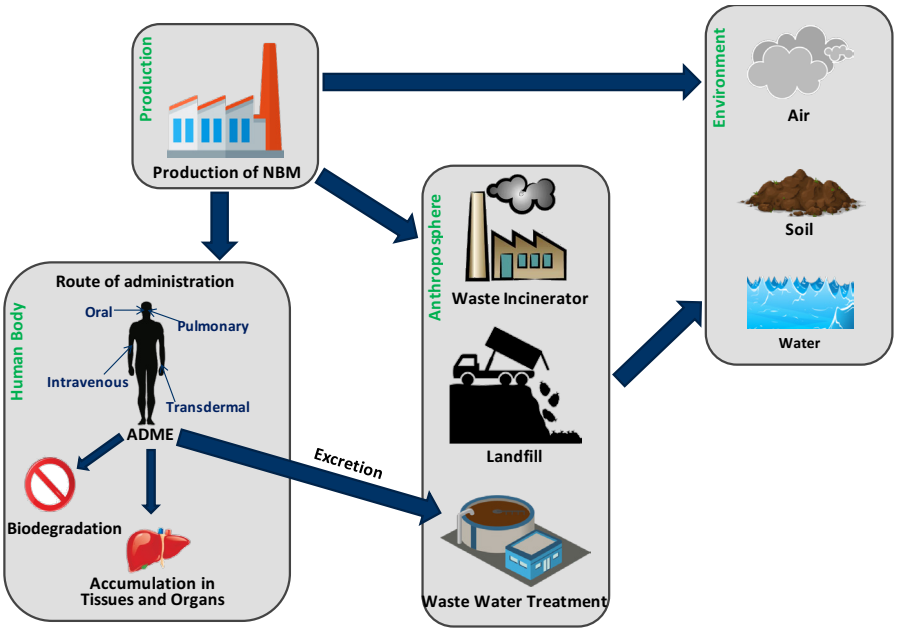
Once these materials are released to the environment, they are exposed to outdoor conditions and can undergo further transformations. These transformations generally involve the breakdown of the polymer (degradation) and generate oligomers and monomers. Polymeric NBMs may degrade through either abiotic or biotic mechanisms. This aspect should therefore also be considered in the environmental risk assessments carried out when designing polymeric NBMs for drug delivery. However, to the best of the authors' knowledge, no studies regarding the biodegradation of polymeric NBMs in the environment have yet been published.

After identifying the forms in which the NBM can be found in the environment, the next step is to calculate the PEC[42]. As it is impossible to measure the NBMs concentrations directly in the environment, Material Flow Analysis (MFA) and Environmental Fate Modelling (EFM) can be used to model and calculate PECs. Modelling the fate of NBMs most commonly uses multi-compartment models, described by different boxes (or technical and environmental compartments). The fate and environmental exposure of NBMs are very much dependent on human pharmacokinetics and the amount of material produced. Figure 11 shows a simplified flow-scheme for NBMs throughout their life-cycles. Like pharmaceuticals, NBMs are excreted in urine and faeces and so enter the sewage system. From there, pharmaceuticals usually reach wastewater treatment plants where some of them are removed and the rest are discharged into surface waters.

[41] Refer to chapter on human health risks

[42] REACH's "Guidance on information requirements and Chemical Safety Assessment, Chapter R.16: Environmental exposure" can be used for estimating environmental exposure. https://echa.europa.eu/documents/10162/13632/information_requirements_r16_en.pdf

Figure 11
A multi-compartment model for nanobiomaterials (NBM). ADME is Administration, Distribution, Metabolism and Excretion.



Those surface waters further distribute pharmaceuticals and NBMs throughout the biosphere, potentially reaching all the different compartments, including soil, groundwater, the ocean and the atmosphere.

To the best of the authors' knowledge, no studies to date have modelled flows of polymeric NBMs into the human body or the environment.

CURRENT KNOWLEDGE OF ENVIRONMENTAL HAZARDS

The goal of hazard assessment is to derive the material's PNEC in several environmental compartments (freshwater, soil, sediment). Different methods are available to derive a PNEC, as described in the REACH regulations[43].

Figure 12 compares the PNECs for three NBMs (red dots) and several common pollutants (brown dots for nanomaterials (NMs), green for pharmaceuticals and blue for other pollutants) in the freshwater compartment.

Chitosan is the NBM of highest concern regarding the freshwater compartment, whereas polyacrylonitrile (PAN) and hydroxyapatite (HAP) do not exhibit significant toxicity. However, it should be noted that, in freshwater, even the most toxic of the NBMs selected – chitosan – is less toxic than fullerenes, nano-ZnO and nano-Ag NMs, doxycycline- and amoxicillin-based antibiotics, estrogen, the heavy metals Cu, Pb, Cd and Hg, and organic pollutants such as triclosan, dibutyl phthalate (DBP) and dichlorvos[44].

In a SbD approach, SbD actions include comparing NBM toxicities, as shown in Figure 12, to determine which material has the lowest toxicity and is the most appropriate for a specific application.

WHAT CONCLUSIONS CAN BE DRAWN ABOUT ENVIRONMENTAL RISKS?

The following uncertainties have to be overcome in order to assess environmental risks:

- Lack of data on pharmacokinetics, exposure and potential environmental hazards
- Contradictory experimental data about effects on environmental organisms
- Uncertainty regarding the physico-chemical properties of NBMs that may be responsible for specific toxicity or hazards
- Often, the only data available is for a generic NBM and not a specific material (i.e. disregarding size, surface charge/coatings etc.)
- Uncertainty about the NBM's dose metric

[43] REACH's "Guidance on information requirements and Chemical Safety Assessment, Chapter R.10: characterization of dose [concentration]-response for environment" can be used for deriving the PNEC. There is also an Appendix to Chapter R10 with recommendations for nanomaterials. https://echa.europa.eu/documents/10162/13632/information_requirements_r10_en.pdf/bb902be7-a503-4ab7-9036-d866b8ddce69 and https://echa.europa.eu/documents/10162/13643/appendix_r10_05-2012_en.pdf/d5bc0038-0b76-4045-b101-b4cdfd47c7c6

[44] More information in the knowledge base "Environmental risks of polymeric nanobiomaterials": www.empa.ch/gonanobiomat

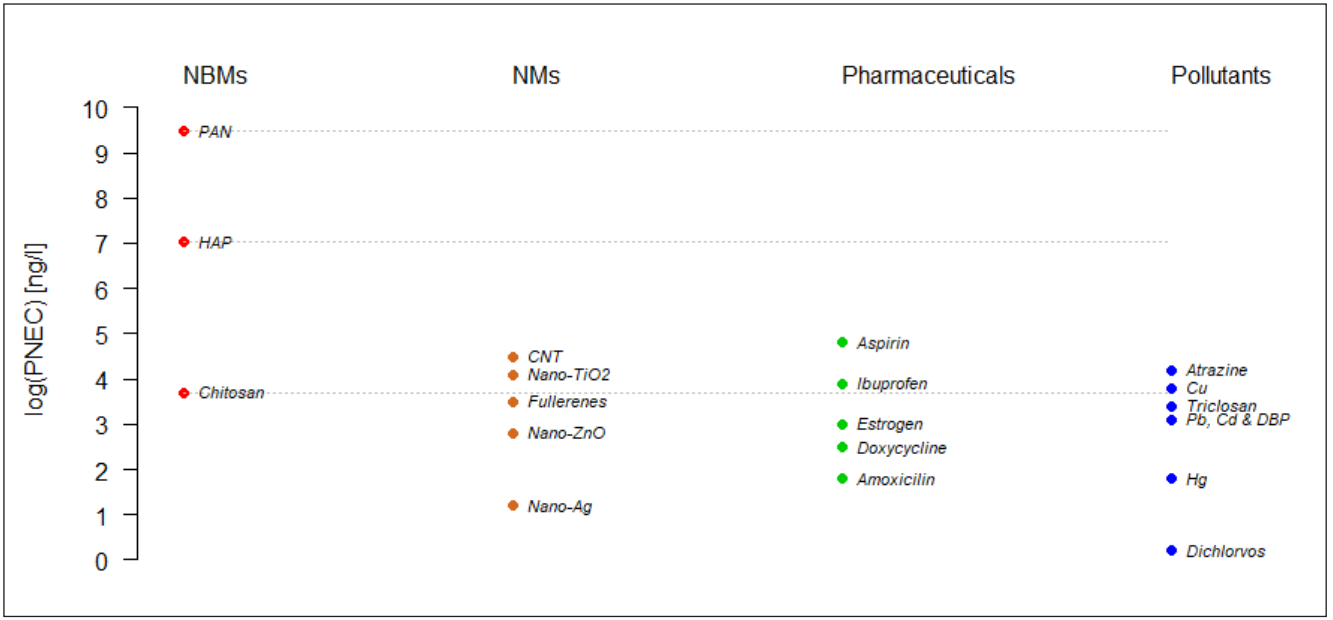


Figure 12
Predicted No-Effect Concentration (PNEC) of various nanobiomaterials, nanomaterials, pharmaceuticals and pollutants.

CHEMISTRY, MANUFACTURING AND CONTROL (CMC)

Chemistry, Manufacturing and Control (CMC) regulations must be fulfilled for any drug approval process. Several guidelines and regulatory documents describe these well for "conventional" drugs (see below). However, the particularities of nanomedicines, and especially polymeric NBMs, require the development of specific guidance to ensure the safety and efficacy of such drugs. As the chemistry has been discussed above, this chapter focuses on the manufacture of nanomedicine and the control of such processes.

As with other pharmaceutical products, a Quality by Design (QbD) approach can be applied to the manufacture of nanomedicine in order to obtain products of consistent quality and safety. A QbD process requires a technical understanding of the product and how to control the manufacturing process, both of which are based on sound science and high-quality risk management. Table 11 suggests a systematic approach to a QbD strategy.

In order to obtain a NBM with the required properties, a Target Product Profile should be established to list all of the final product's desired qualities (safety, efficacy, PK/PD profile, etc.). These qualities may be affected by the NBM's physicochemical properties – its Critical Quality Attributes (CQAs) – and their limits or specifications should be determined at an early stage of development, through close collaboration between basic research work and pre-clinical development. The link between CQAs and the Target Product Profile can be seen in Figure 13. However, the figure does not present an exhaustive list of parameters, and these should be considered on a case-by-case basis.

CQAs, in turn, can be affected by Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs). CMAs are defined as the „physical, chemical, biological or microbiological properties of an input material that should be within an appropriate limit, range, or distribution to ensure the desired quality of the output material/product".CPPs are defined as the "process parameters that influence CQAs and therefore should be monitored or controlled to ensure the process produces the desired quality" (ICH Q8 (R2))[45]. The database of information built up through these processes will subsequently be used in later development phases to meet regulatory requirements for the CMC part of the dossier submitted to the regulatory authority.

[45] https://www.ich.org/products/guidelines/quality/article/quality-guidelines.html

Table 11
A systematic approach to a QbD-driven drug development process.

Systematic QbD approach	
Predefined objectives	• Define the Quality Target Product Profile (QTPP) • Identify Critical Quality Attributes (CQAs)
Product and process understanding	• Identify Critical Material Attributes (CMA) and Critical Process Parameters (CPP) • Establish functional relationships linking CMA/CPP and CQA
Process control	• Develop an appropriate Control Strategy, including justifications
Sound science	• Science-driven development (scientific literature, prior knowledge, DOEs, etc.)
Quality risk management	• Risk-based development (according to ICH Q9)

Table 12
Implications of CQAs, CMAs and CPPs on polymeric NBMs.

	Implications for polymeric NBMs
CQAs	The link between physicochemical properties and efficacy and toxicity in clinical use does not currently exist. However, the chapter on characterisation described additional parameters, not shown in Figure 13, which the authors believe should also be considered as CQAs.
CMAs	Materials (polymers, active principle and excipients) should be selected carefully, be of a prescribed quality and be sourced from established suppliers.
CPPs	In the context of preparing polymeric NBMs, several CPPs – such as temperature, volumes, stirring speed, pH values, etc. – should be explicitly named and defined for each polymeric material in combination with the drug to be encapsulated.

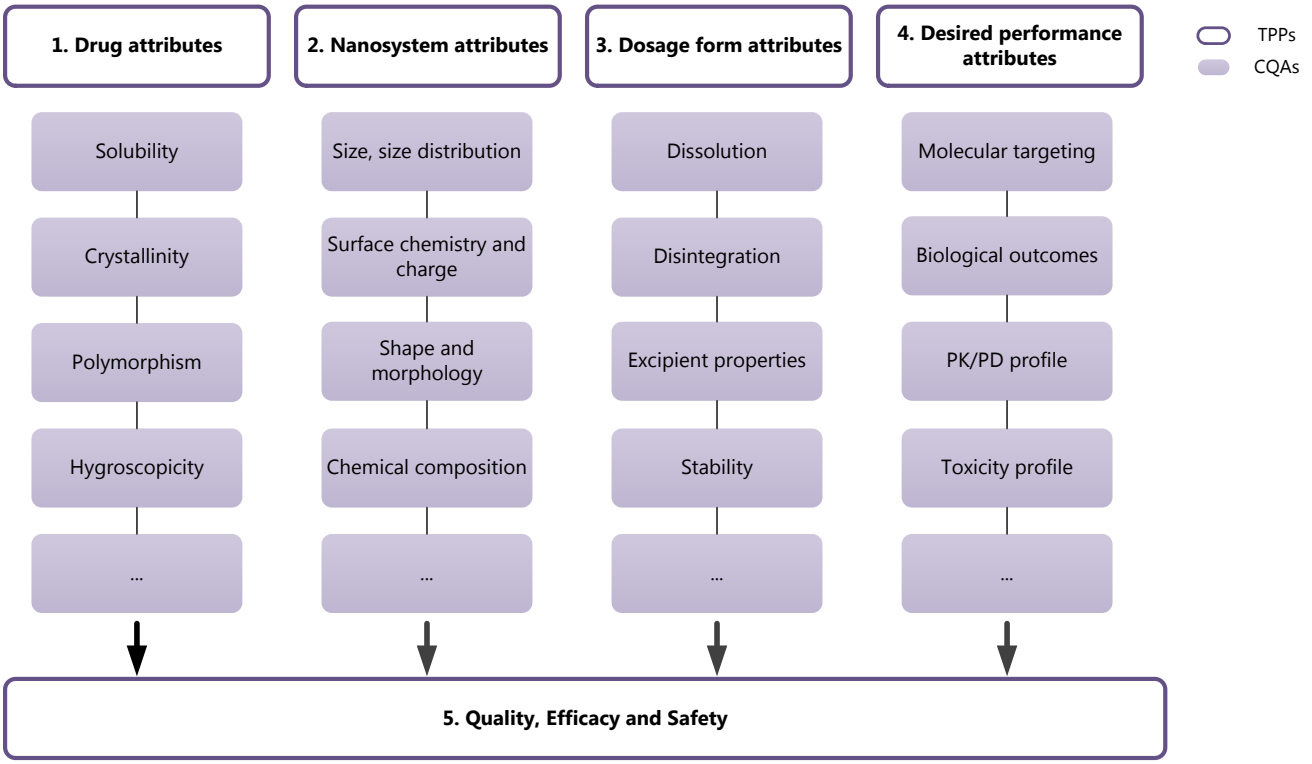


Figure 13
Critical Quality Attributes (CQAs) to be considered to support the drug's Target Product Profile (TPP).

STORAGE AND TRANSPORT

The stability of nanomedicines is one of the main factors influencing decisions on their possible commercialisation since their original physicochemical properties and quality must be ensured throughout their whole life-cycle, including storage and transport. NBM instability may induce lower efficacy and undesired effects.

Stability studies (long-term, intermediate and accelerated) should be planned soon enough in advance, just after producing the prototype; there is no need to wait for the scale-up process. The stability studies should be conducted on the drug substance packaged in a container closure system that is the same as or simulates the packaging proposed for storage and distribution. This should answer the following questions:

- What are the right storage conditions (temperature, humidity, photostability)?
- What is the right expiration date?

The International Conference on Harmonization (ICH) has established industry guidelines for testing the stability of pharmaceutical products (e.g. ICH Q1A Q1F)[46]. The WHO has published a guidance document on the storage and transport of pharmaceutical products (WHO Technical Report Series, No. 961, 2011, Annex 9)[47]. This document focuses on the key requirements for the safe storage and distribution of time- and temperature-sensitive pharmaceutical products (TTSPPs).

Another relevant document for European countries is the "Good Distribution Practice of medicinal products for human use", published by the Official Journal of the European Union (2013/C 343/01)[48]. This document is part of quality assurance and ensure that the quality of medicinal procedure is maintained through every stage in the supply chain, from the site of manufacture to the pharmacy. It informs pharmaceutical companies and wholesale distributors of medicines of their obligations to have suitable equipment and procedures in place to monitor the environmental conditions (including temperature, light, humidity and cleanliness) in storage areas for medicinal products and in transport vehicles.

The same document also provides instructions for wholesale distributors involved in the destruction of medicinal products (e.g. expired shelf-life or defective products). Destruction must be performed by specialised companies which can ensure that medicines will not contaminate the environment. In this regard, nanomedicines may require additional attentiveness, and there is still some work to be done in this field, both by official entities and the companies involved.

[46] https://www.ich.org/products/guidelines/quality/article/quality-guidelines.html
[47] http://apps.who.int/medicinedocs/en/m/abstract/Js21896en/
[48] https://ec.europa.eu/health/human-use/good_manufacturing_distribution_practices_fr

GLOSSARY[49]

Active Pharmaceutical Ingredient (API)	Any substance, or mixture of substances, used in the manufacture of a medicinal product, that becomes an active ingredient of it. Such substances are intended to induce a pharmacological activity or other direct effects on the diagnosis, cure, mitigation, treatment or prevention of disease or to affect the structure or function of the body.
Biomaterials	In healthcare, biomaterials are materials that are entities, surfaces or constructs that interact with specific biological systems. They can be either derived from nature or synthesised in the laboratory.
Bulk polymer	A polymer not in the form of nanoparticles.
Container closure system	The sum of the packaging components that together contain and protect the active substance or the dosage form. This includes immediate packaging components and secondary packaging components if the latter are intended to provide additional protection to the active substance or the drug product.
Critical Material Attributes (CMAs)	The physical, chemical, biological or microbiological properties or characteristics of a material that should be within an appropriate limit, range or distribution so as to ensure the desired material quality.
Critical Process Parameters (CPPs)	The process parameters whose variability has an impact on a Critical Quality Attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.
Critical Quality Attributes (CQAs)	The physical, chemical, biological or microbiological properties or characteristics of a drug or drug formulation that should be within an appropriate limit, range or distribution so as to ensure the desired product quality.
Exposure assessment	The determination or estimation (qualitative or quantitative) of the magnitude, frequency, duration and route of exposure to a nanobiomaterial.
Hazard assessment	A process designed to determine the possible adverse health and/or environmental effects of nanomaterials by using hazard identification and hazard characterisation. A hazard assessment should consider the analysis of suitable *in vitro* and, if necessary, *in vivo* toxicity assays.
Material Flow Analysis	The systematic assessment of the flows and stocks of materials within a system defined in space and time. Material Flow Analysis connects a material's sources and pathways to its intermediate and final sinks.
Medicinal product	A substance, or combination of substances, intended to treat, prevent or diagnose a disease, or to restore, correct or modify particular physiological functions by exerting a pharmacological, immunological or metabolic action.
Monomer	A substance capable of forming covalent bonds with a sequence of additional like or unlike molecules under the conditions of the relevant polymer-forming reaction used in the particular process.
Nanocarriers	A nanocarrier is a nanobiomaterial used as a transport module for another substance, such as a drug or vaccine.
Nanobiomaterial (NBM)	Nanobiomaterials are biomaterials at the nanoscale. Within the GoNanoBioMat framework, we consider spherical nanobiomaterials to be smaller than 1,000 nm in diameter.
Nanomedicine	Nanomedicine is the application of nanotechnology to achieve innovation in healthcare. Nanomedicine uses the properties expressed by materials at a nanometric scale as these often differ from the same bulk material in terms of their physical, chemical or biological attributes.

[49] The definitions found in this glossary are based on official and commonly used definitions, but they have been adapted to our understanding of each term and the needs of the guidelines' contents.

Oligomer	An oligomer is a chemical compound formed by repeating units, called monomers, connected by covalent bonds. Oligomers can contain a number of similar monomers. In the field of nanomedicine, an oligomer is a chemical compound resulting from the degradation/hydrolysis of polymers.
Pharmacodynamics (PD)	The relationship between an unbound drug's concentration over time and its resulting effects and mechanisms of action in an organism's body.
Pharmacokinetics (PK)	The study of the absorption, distribution, metabolism and excretion (ADME) of a foreign substance (e.g. a drug or pollutant) in an organism's body over time.
Polymeric nanomaterial	A polymeric material in the nano-sized range (please also see "nanobiomaterial").
Predicted Environmental Concentration (PEC)	A substance's predicted concentration in the environment.
Predicted No-Effect Concentration (PNEC)	The predicted concentration of a substance below which no adverse effects are expected to occur in the environment.
Quality-by-Design (QbD)	A systematic approach to product development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management.
Quality Target Product Profile (QTPP)	The prospective summary of the ideal quality characteristics of a drug product—those which should be achieved to ensure the desired quality, safety and efficacy of that drug product.
Risk assessment	A process intended to calculate or estimate human and/or environmental risks. Risk assessment consists of hazard identification, hazard characterisation, exposure assessment and risk characterisation.
Risk characterisation	The qualitative and, wherever possible, quantitative determination (including uncertainties) of the probability of occurrence of the known and potential adverse effects of nanomaterials on humans and/or the environment under defined exposure conditions.
Safe-by-Design (SbD)	An approach for identifying the risks and uncertainties relating to human and environmental safety at an early phase of the innovation process so as to minimise uncertainty, hazard(s) and/or exposure. In the GoNanoBioMat framework, the SbD approach includes: I. Safer Nanobiomaterials: designing low-hazard nanocarriers for specific applications by assessing human health and environmental risks early on in the development process; II. Safer Production: manufacturing and control nanocarriers to ensure their safety and quality; III. Safer Storage and Transport: ensuring the safety and quality of nanocarriers. The SbD approach is iterative in order to maximise safety while optimising efficacy and costs.

LEGAL AND PUBLISHING DETAILS

DISCLAIMER

These guidelines were designed based on the best currently available knowledge on nanobiomaterials. Updates and corrections are not planned. However, knowledge in the field of nanobiomaterials and nanomedicine is developing fast: Empa and the GoNanoBioMat Consortium can, therefore, provide no guarantee as to the correctness of the information contained herein. Empa and the GoNanoBioMat Consortium accept no liability for any consequences resulting from the application of the information provided in the GoNanoBioMat Guidelines. We reject all responsibility for any references and links to third-party websites and their contents.

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LAYOUT AT EMPA

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Empa
Überlandstrasse 129
CH-8600 Dübendorf
Switzerland
+41 58 765 11 11
www.empa.ch

Empa
Lerchenfeldstrasse 5
CH-9014 St. Gallen
Switzerland
+41 58 765 74 74
www.empa.ch

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