Annex 1. Roadmap of the translational strategy and regulatory aspects for Soft nanomedicines in oncology

A complex and expensive process such as drug development requires a clear and coherent strategy from which a tactical action plan should be developed and implemented. Performing a thorough risk analysis is a critical component of any drug development program at early stages. This roadmap for risk analysis must be developed as early as possible and is a living document that is revisited regularly. It will help to identify the applicable Regulations and Guidances for each phase of the development from the initial inception of your research to the regulatory submission to the Competent Authority.

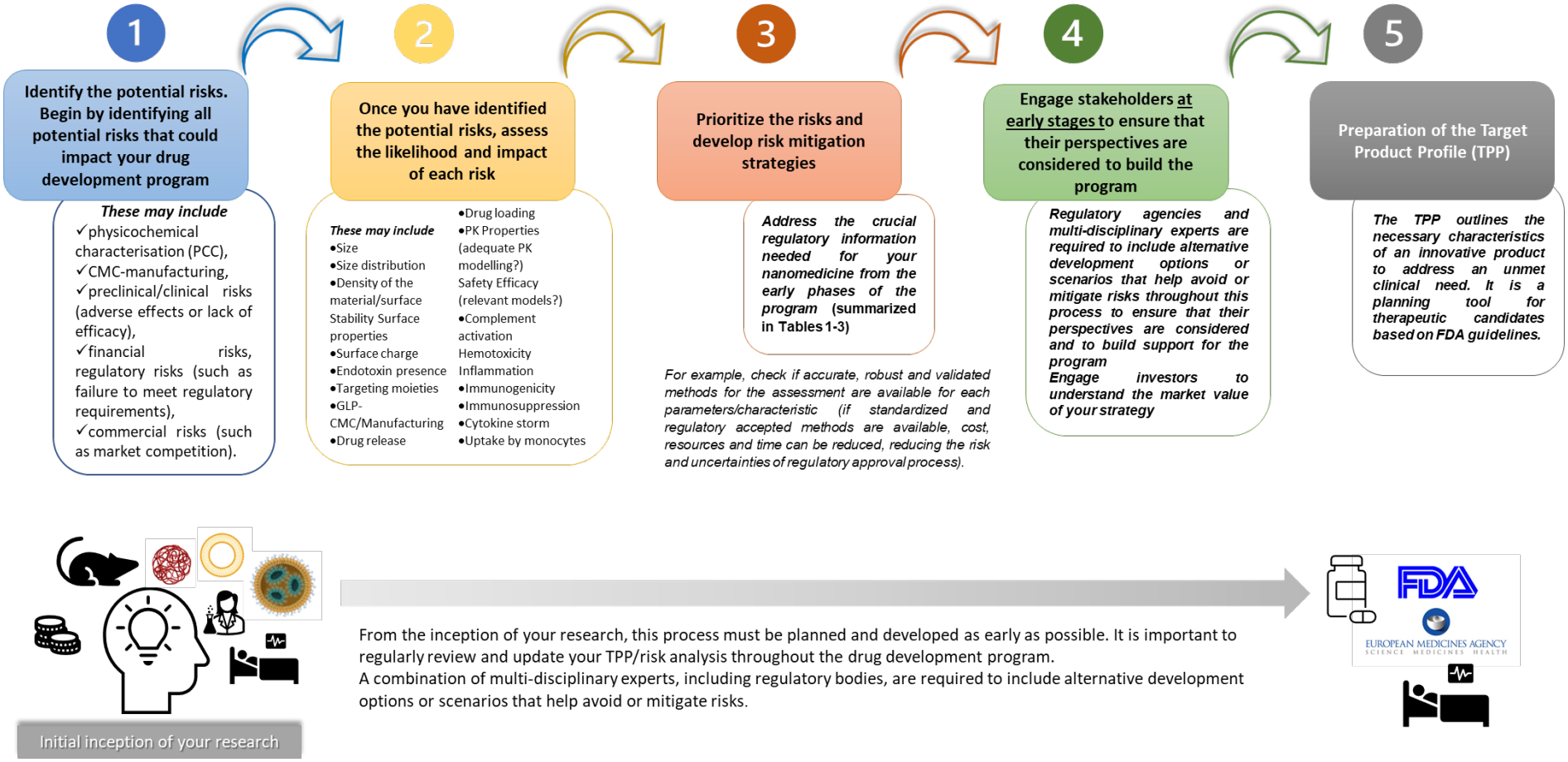

Table 1. Relevant Information of Physicochemical parameters required by Regulatory Agencies in the nanomedicines field.

PHYSICOCHEMICAL PARAMETERS	AVAILABLE STANDARDISED METHODS MATCHING REGULATORY NEEDS		CHALLENGES FOR DEVELOPERS	COMMENTS
Chemical composition	Yes	▪ ISO/TS 19590:2017 - "Nanotechnologies — Methodology for the detection and identification of nano-objects in complex matrices" ▪ ASTM E2490-15 - "Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA)" ▪ ASTM E2865-13 - "Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials" ▪ ISO/TS 21236-2:2020 - "Nanotechnologies — Electrical measurements for characterizing the properties of the surface of nanoparticles — Part 2: Particle size dependent electrical characterization of the surface" ▪ ASTM E2834-12 - "Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS)" ▪ ASTM E2921-19 - "Standard Test Method for Examination of Carbon Nanotube Sizing Agent Removal from Surfaces ▪ Others: ASTM WK54613; ISO/TS 13278:2011		
Chemical structure	Yes	▪ ISO 14187:2011 - Nanotechnologies - Materials specifications - Guidance on specifying nano-objects ▪ ASTM E2490-13 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS) ▪ ASTM E2834-12 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ASTM E2859-13 - Standard Guide for Size Measurement of Nanoparticles Using Atomic Force Microscopy ▪ ISO 19590:2017 - Nanotechnologies - Size distribution and concentration of inorganic nanoparticles in aqueous media via single particle inductively coupled plasma mass spectrometry		
Impurities	Yes	▪ ISO 11930:2012 - Nanotechnologies - Application of inductively coupled plasma mass spectrometry (ICP-MS) for the detection of metallic nanoparticles in vitro. ▪ ASTM E2456-06 - Standard Terminology for Nanotechnology.		

		▪ ISO/TR 13014:2012 - Nanotechnologies - Guidance on physicochemical characterization of engineered nanoscale materials for toxicologic assessment. ▪ ASTM E2490-06 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS). ▪ ISO/TR 13121:2011 - Nanotechnologies - Nanomaterial risk evaluation. ▪ ASTM E2834-12 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA). ▪ ISO 10801:2010 - Nanotechnologies - Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing. ▪ Others: ISO/TS 80004-11:2017; ASTM E2909-13(2019)		
Particle size and size distribution	Yes	▪ ISO 13317-1:2014 - Particle size analysis -- Image analysis methods ▪ ASTM E2834-12 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ISO 19430:2016 - Fine ceramics (advanced ceramics, advanced technical ceramics) -- Test method for particle size distribution of ceramic powders by centrifugal liquid sedimentation using dispersion stability ▪ ASTM E2490-09(2015) - Standard Guide for Measurement of Particle Size Distribution of Nanoparticles in Suspension by Photon Correlation Spectroscopy (PCS) ▪ ISO/TS 27687:2008 - Nanotechnologies -- Terminology and definitions for nano-objects -- Nanoparticle, nanofibre and nanoplate ▪ Several other test methods and guides ISO 13318-1:2001, -2:2007, -3:2004, ISO/TS 19590:2017, ASTM WK54872, ASTM WK54615, ASTM WK54872, ASTM WK54615, ISO 17867:2015, ISO 22412:2017, ASTM E2859-11 (2017)		
Shape and morphology	Yes	▪ ISO/TS 80004-3:2018: Nanotechnologies -- Vocabulary -- Part 3: Nano-object characterization This standard provides a vocabulary for the characterization of nanoscale objects, including terms related to shape and morphology. ▪ ASTM E2490-13: Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) This standard provides guidelines for using the nanoparticle tracking analysis (NTA) technique to measure the size and size distribution of nanoparticles in suspension. ▪ ASTM E2834-12: Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Dynamic Light Scattering (DLS) This standard provides guidelines for using the dynamic light scattering (DLS)		

		technique to measure the size and size distribution of nanoparticles in suspension. ▪ ASTM E2921-13: Standard Practice for Characterizing Uncertainty in Airborne Particle Size Measurements Using Nano-Particle Tracking Analysis This standard provides guidelines for assessing the uncertainty in size measurements of nanoparticles using the NTA technique. ▪ ASTM E2865-13: Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials This standard provides guidelines for using the electrophoretic mobility and zeta potential measurements to characterize the surface charge of nanoparticles. ▪ Others: STM E2859-11 (2017)		
Surface properties	Limited	▪ ISO 14187:2011 - Nanotechnologies -- Material specifications -- Guidance on measurement of surface area of nano-objects ▪ ASTM E2834-20 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ASTM E2865-20 - Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials	▪ Impact on the interaction with proteins, biomolecules, immune cells. ▪ Most of the existing methods are not standardised. ▪ Even though, standard guides are accessible for the analysis of the surface charge of NPs, currently no standardised methods are available for the analysis of the hydrophobicity, quantification and homogeneity of surface functionalities and surface area of NPs.	▪ Limited technological solutions for soft nanomaterials, and/or for surface properties in solution. ▪ No methods for the heterogeneity of surface coatings. ▪ Analytical methods should be available to: (a) analyse the chemical nature of the surface coating (b) quantify the amount of surface coating (quantitative), (c) determine the homogeneity of the coating coverage and the conformational structure of its molecules. ▪ Measuring the coating heterogeneity, is not possible with the existing technologies. ▪ Currently no standards exist for assessment of surface hydrophobicity. ▪ Standardised method ISO 9277:2010 describes how to measure the ▪ specific surface area of nanoparticle powders by gas adsorption — ▪ The BET method is only applicable to inorganic NPs but it is not reliable in the presence of a polymeric surface coating. ▪ No standardised methods exist to measure the surface area of organic particles and of particles dispersed in aqueous suspension.

Particle concentration	Yes	▪ ISO 15900:2009 - Nanotechnologies - Terminology and definitions for nano-objects - Nanoparticle, nanofibre and nanoplate. ▪ ASTM E2490-11 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS). ▪ ASTM E2865-12 - Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials.		
Porosity	Yes	▪ ISO 15901-1:2016 - Nanomaterials -- Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing -- Part 1: Sample preparation and dosimetry ▪ ASTM E2490-16 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ASTM E2834-11(2016) - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ASTM E2865-12(2017) - Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials ▪ ASTM E2866-12(2017) - Standard Test Method for Determination of Kinetic Reaction Order of Nanoparticle Catalysts in Solution ▪ ASTM E2867-12(2017) - Standard Test Method for Determination of Acidity in Nanoparticle Catalysts by Titration ▪ ASTM E2868-12(2017) - Standard Test Method for Determination of Metal and Metal Oxide Nanoparticle Purity by Total Elemental Analysis Using ICP-MS.		
Physical and chemical stability	Yes	▪ ISO/TS 80004-13:2017 - Nanotechnologies -- Vocabulary -- Part 13: Nanoparticle size distribution ▪ ISO/TR 10993-22:2017 - Biological evaluation of medical devices -- Part 22: Guidance on nanomaterials ▪ ASTM E2490-13 - Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Nanoparticle Tracking Analysis (NTA) ▪ ASTM E2834-12 - Standard Guide for Measurement of Particle Size Distribution of Nanoparticles in Suspension by Dynamic Light Scattering (DLS) ▪ ASTM E2865-12 - Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials		
General Guidances		▪ ISO/TR 13014:2012 Guidance on physicochemical characterization of engineered nanoscale materials for toxicological assessment ▪ CEN/TS 17010:2016. Nanotechnologies—Guidance on measurands for characterizing nano-objects and materials that contain them.		

Table 2. Relevant Information about the Drug Delivery system required by Regulatory Agencies in the nanomedicines field.

DRUG DELIVERY SYSTEM PARAMETERS	AVAILABLE STANDARDISED METHODS MATCHING REGULATORY NEEDS	CHALLENGES FOR DEVELOPERS	COMMENTS
Drug loading efficiency	No	▪ Need to adapt the methods to each nanocarrier/API combination. ▪ The analytical methods are substance-specific and cannot be generically standardised. ▪ Separation of API from nanocarrier prior to API quantification can induce artefacts. ▪ Needs of methods for monitoring of API leakage during storage and use (stability).	▪ The method for the separation of the free API from the nanocarrier should be able to (a) no affect the carrier integrity, (b) no induce leakage of drug and (c) no affect the concentration equilibrium of the drug between the encapsulated and the free state. EUNCL and NCI-NCL have developed and validated protocols for separation of free vs encapsulated drugs by ultrafiltration that are applicable to multiple nano-formulations encapsulating classical small drugs. ▪ Still major challenges for the development of analytical methods for novel classes of APIs, DNA or mRNAs, where techniques for identification and quantification and detection are still under development.
Presence and distribution of the API encapsulated in the NPs and free in solution	No		
In vitro drug substance release rate in physiologically/ clinically relevant media	No		
Kinetic properties in biological media	No	▪ Separation and quantification of encapsulated and unencapsulated drug fraction. ▪ For PK purposes, tissue distribution of all the fractions (a) API encapsulated in the NPs, (b) free API and (c) API bound to plasma proteins need to be evaluated, since the three species may have very different PK profiles in blood and tissues. ▪ Separation of particles from the blood proteins. ▪ Separation of NP–protein corona complexes.	▪ Molecular stability can be affected by the biological media, for example, by chemical or enzymatic hydrolysis of labile structures introducing critical methodological challenges. ▪ Despite the lack of standardised methods to assess all the fractions of the API/NPs a relevant method developed by NCI-NCL [1], employing a stable isotope analogue of the API, is now under evaluation for standardisation by the ASTM E56 committee. No solutions currently exist for the reliable quantification of large APIs (for example, mRNA, DNA) [2].
Drug release in blood/plasma	No		

[1] Skoczen S, McNeil SE, Stern ST. Stable isotope method to measure drug release from nanomedicines. J Control Release. 2015 Dec 28;220(Pt A):169-174.

[2] Halamoda-Kenzaoui B, Vandebriel RJ, Howarth A, Siccardi M, David CAW, Liptrott NJ, Santin M, Borgos SE, Bremer-Hoffmann S, Caputo F. Methodological needs in the quality and safety characterisation of nanotechnology-based health products: Priorities for method development and standardisation. J Control Release. 2021 Aug 10; 336:192-206.

Physical stability in biological media (particle size)	No	▪ Ionic strength, the proteins and the enzymes in blood and plasma can impact the physical stability of a nanomedicinal formulation.	▪ EUNCL and NCI-NCL laboratories have jointly developed multiple protocols for size measurements, that have been tested in complex biological media under specific conditions and suggest using them in a step-by-step approach. High resolution techniques are available such as PTA, AUC and AF4 can be used, where AF4 seems to be the most promising method [3]. ▪ No technical solutions are available to measure size changes of small soft organic particles in plasma (for example for dendrimers or small polymeric micelles with a size below 20–30 nm).
Protein corona formation	No	▪ Protein corona formation (amount and identification of bound proteins). ▪ Separation of NP–protein corona complexes.	▪ Two approaches are available to study the protein corona: (a) quantification of the total amount of proteins binding to the surface and (b) identification of individual corona proteins separated by gel electrophoresis and determined by MS. ▪ Quantification/composition analysis of the corona is limited to particles that can be separated from plasma by centrifugation. ▪ It is required in the field to define and to standardise the composition of the biological media to be used for corona studies, to assure comparability between different studies.

[3] Hu Y, Crist RM, Clogston JD. The utility of asymmetric flow field-flow fractionation for preclinical characterization of nanomedicines. Anal Bioanal Chem. 2020 Jan;412(2):425-438.

Table 3. Relevant Information about Biological Characterisation (PK and PD) required by Regulatory Agencies in the nanomedicines field.

BIOLOGICAL CHARACTERISATION PARAMETERS	AVAILABLE STANDARDISED METHODS MATCHING REGULATORY NEEDS		CHALLENGES FOR DEVELOPERS	COMMENTS
Sterility and endotoxin levels	Yes	▪ FDA, Drug Products, Including Biological Products, that Contain Nanomaterials, in: ▪ Guidance for Industry, 2017 ▪ FDA, Liposome Drug Products. Guidance for Industry ▪ MHLW, Guideline for the Development of Liposome Drug Products, 2016 ▪ ISO/TC 194; ISO /TR 10993	▪ Quantification of endotoxin ▪ and pyrogen ▪ Detection of encapsulated endotoxins	▪ For medicinal products exposure limits have been set. ▪ While the LAL is the reference method for endotoxin determination, interferences have been described. Other methods are available [4].
Pharmacokinetic parameters ▪ ADME ▪ Detection and quantification in biological matrices ▪ BioDistribution/accumulatin studies ▪ Plasma protein binding (protein corona) ▪ In vivo degradation/elimination ▪ Stability in blood and serum.	In general, it can be stated that methods are similar to those used for small-molecules On a case-by case basis analysis	▪ ICH S6(R1): Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals. ▪ ICH S9: Nonclinical Evaluation for Anticancer Pharmaceuticals. ▪ FDA. Pharmacokinetics in Patients with Impaired Renal Function: Study Design, Data Analysis, and Impact on Dosing and Labeling. ▪ FDA. Nanotechnology - Overcoming Scientific and Regulatory Challenges. ▪ MHLW. Guidelines for Nonclinical Safety Studies of Nanomedicines. ▪ MHLW. Guidelines for Clinical Trials of Nanomedicines.	▪ Characterization of PK profiles of NPs products that can significantly differ from small-molecule drugs requires the development of validation of suitable analytical methods. ▪ Need for method adaptation for each type of nanomaterials. ▪ Relevance for human biodistribution. ▪ Unknown Inter-species variation between animal models and human.	▪ Various imaging techniques can be used in the case of labelled NPs or occasionally bioanalytical methods such as ICP-MS which can be used to detect intact metal nanoparticles.

[4] Halamoda-Kenzaoui B, Vandebriel RJ, Howarth A, Siccardi M, David CAW, Liptrott NJ, Santin M, Borgos SE, Bremer-Hoffmann S, Caputo F. Methodological needs in the quality and safety characterisation of nanotechnology-based health products: Priorities for method development and standardisation. J Control Release. 2021 Aug 10;336:192-206.

Pharmacodynamic parameters ▪ Safety ▪ Toxicology	In general, it can be stated that methods are similar to those used for small-molecules On a case-by case basis analysis	ICH S6(R1) ICH S4 ICH S9 ICH M3(R2) ISO/TR 16197:2014 ISO/AWI TR 22019 Under development ISO/AWI TS 22455 ISO/TR 10993-22: 2017 FDA Guidance for Industry "Drug Products, Including Biological Products, That Contain Nanomaterials" FDA Guidance for Industry "Drug Development and Approval Process for Manufacturing and Controls Documentation" FDA Guidance for Industry "Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products MHLW "Guidelines for the Quality, Safety, and Efficacy Assurance of Nanopharmaceuticals" MHLW "Guidelines for the Nonclinical Safety Evaluation of Nanopharmaceuticals".		▪ Safety assessments are performed following ICH guidelines (medicinal products) and ISO guidance documents (medical devices) and currently, only few standardised methods are available to assess biological effects of nanotechnology-based products. ▪ The ISO guidance on the biological evaluation of medical devices contains a specific part dedicated to products containing nanomaterials (ISO/TR10993–22:2017), but it provides only general considerations and no specific method protocol. ▪ Compilation and description of toxicological screening methods for manufactured nanomaterials. ▪ Considerations in performing toxicokinetic studies of nanomaterials.
▪ Cytotoxicity	Yes	ASTM E2526-08(2013)		▪ Standard test method for evaluation of cytotoxicity of nanoparticulate materials in porcine kidney cells and human hepatocarcinoma cells. Cytotoxicity assessment using MTT and LDH.
		ISO/TS 18827:2017		▪ Electron spin resonance as a method for measuring ROS generated by metal oxide nanomaterials.
		ISO/TS 19006:2016		▪ 5-(and6)-Chloromethyl-2,7-dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW264.7 macrophage.
		ISO/FDIS 19007 Under development		▪ In vitro MTS assay for measuring the cytotoxic effect of nanoparticles.

		ISO/AWI TS 22455 Under development		▪ High throughput screening method for nanoparticles toxicity using 3D cells.
▪ Biocompatibility with blood and serum ▪ Haemocompatibility (including effects on red blood cells (haemolysis) and thrombogenicity potential)	Yes	▪ ISO 10993-4 ▪ ASTM F2064-20 ▪ ASTM F619-18 ▪ ICH S5A ▪ ICH S6(R1) ▪ Protocol developed by NCI-NCL and EUNCL laboratories (platelet aggregation).	▪ Quantification of uptake by phagocytes (unlabelled organic nanomaterials). ▪ Interference of NPs with commonly used readouts (fluorescence, absorbance, chemiluminescence).	
▪ CARPA and complement activation ▪ Inflammation and innate immune cell ▪ Effect on adaptive immune system ▪ Uptake by the MPS and cytotoxicity of NPs ▪ Interaction with enzymes ▪ Immunogenicity	Yes	▪ EMA/CHMP, Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, 2013. ▪ MHLW, Guideline for the Development of Liposome Drug Products, 2016. ▪ EMA/CHMP, Joint MHLW / EMA reflection paper on the development of block copolymer micelle medicinal products, 2013. ▪ MHLW, Reflection Paper on Nucleic Acids (siRNA)-Loaded Nanotechnology Based Drug Products, 2016. ▪ SCENIHR, Opinion on the guidance on the determination of potential health effects ▪ of nanomaterials used in medical devices, Final Opin. 2015. ▪ ASTM E2524-08 ▪ ICH S5(R3) guideline on detection of toxicity to reproduction for medicinal products & toxicity to male fertility. ▪ ISO/TR 10993-4.	▪ Well-documented interaction of nanomaterials with the immune system. ▪ The use of conventional methods to study the immune system can be hampered by interference of NPs with assay components or readouts. ▪ Activation of secretory cells. ▪ Hypersensitivity reactions/CARPA. ▪ Inflammation. ▪ Immunosuppression. ▪ Increased clearance from the body. ▪ Variability of results (whole blood, plasma, primary cells) ▪ Lack of advanced in vitro systems to test interactions between different immune cell types or to translate results among species.	▪ Activation of the complement system in vitro is the most used approach to evaluate the risk of CARPA in humans. ▪ Activation of the NLRP3 inflammasome is a widely studied effect of nanotechnology-based products. ▪ Several methods were developed by the European project Nanommune and are included in the Nanommune Quality Handbook [5]. ▪ The assay that currently is considered the “gold standard” for effects of compounds and drugs on the adaptive immune system is the T-cell dependent antibody response (TDAR). ▪ The complexity of the primary immune response cannot be mimicked by currently available in vitro methods or array of methods, more sophisticated in vitro models would be necessary.

[5] NANOMMUNE, Quality Handbook: Standard Procedures for Nanoparticle Testing, 2011.

		▪ SCENIHR, Risk Assessment of Products of Nanotechnologies, 2009. ▪ ICH-8, Immunotoxicity Studies for Human Pharmaceuticals S8, 2005.		