



*Agence française de sécurité sanitaire  
des produits de santé*

**RECOMMENDATIONS FOR TOXICOLOGICAL EVALUATION  
OF NANOPARTICLE MEDICINAL PRODUCTS**

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### **INTRODUCTION**

Nanoparticles are defined as particles at dimensions between approximately 1 and 100 nanometres. They have considerable general industrial applications and although only a few nanoparticle medicinal products are available at the present time, a spectacular development of these products can be expected over the years to come. In the short and medium term, the main use of nanoparticle medicinal products (NMP) is for vectorization of active principles, corresponding to several products already marketed.

Three types of vectors are distinguished at the present time:

- First generation vectors: nanospheres and nanocapsules (the best known and most accessible),
- Second generation vectors: nanoparticles coated with hydrophilic polymers such as polyethylene glycol (PEG), pegylated nanoparticles
- Third generation vectors, still under development, combining a biodegradable core and a polymer envelope (PEG) with a membrane recognition ligand.

Very generally, nanoparticle medicinal products are colloidal systems that can be classified into three classes:

- 1) biodegradable nanoparticles,
- 2) soluble nanoparticles,
- 3) insoluble or slowly soluble nanoparticles.

Biodegradability or solubility is an important property, determining elimination of nanoparticles introduced into the body. Other parameters than the conventional parameters (mass, mass per unit volume) must also be taken into account in order to relate their effects to exposure: for example, the relative size of the particles, their active surface area (total external and possibly internal surface area of exchange), number of particles per unit volume, etc. These parameters largely determine uptake, distribution, and elimination of NMPs. It is also absolutely essential to take into account the formation of nanoparticle agglomerates, that subsequently form aggregates the characteristics and potential risks of which being very different from those of the initial nanoparticles.

In addition to the specific toxicity of the vectorized active principle, the structure in which it is contained could also considerably modify this toxicity. Consequently, it would often be preferable to consider the NMP as a distinct entity that needs to be evaluated as a largely new "total" drug substance. The nanoparticle form can also induce specific risks (formation of agglomerates), transport impurities by adsorption, generate toxic substances by degradation or dissolution of the constituents of the NMP, cross physiological barriers (blood-brain, foeto-placental, cell and nuclear membranes, etc.). This illustrates the magnitude of the task of toxicological evaluation of NMPs, especially since part of this field is only very poorly documented, on the one hand, and no reference material is available to evaluate nanoparticles, on the other hand. Under these conditions, to ensure better efficacy (especially for screening) and for ethical reasons (unjustified and extensive use of laboratory

animals), the use of validated *in vitro* methods must be strongly encouraged. These methods must ensure relevant evaluation of genotoxicity, cytotoxicity, free radical formation, biopersistence, phagocytic capacity, etc.

It should be stressed that although vectorization of active principles is probably the major potential use of nanoparticles in medicine, other very valuable applications must be considered: tissue engineering and diagnostic tests, for example. Nanoparticle formulations are also widely used in cosmetology (sunscreens) and are a subject of controversy in some countries. Finally, it is also somewhat surprising to find so many scientific publications concerning the occupational and environmental risks of nanotechnologies and nanoparticles, compared to the limited number of documents concerning NMPs.

## **OBJECTIVE**

The recommendations presented in this position paper reflect the opinion of the Afssaps Working Group on New Approaches to Non-clinical Evaluation of the Safety of Health Products. They are therefore opened to reflection and discussion, especially as some proposals are essentially pragmatic, sometimes even empirical. In view of the potentially extensive nature of the field of NMPs, the Working Group has decided to limit its investigations to three sectors already developed or under development:

- Use of NMPs in medical imaging (MRI and ultrasound),
- Vectorization of drug substances (anticancer drugs, antibiotics, antifungal agents, etc.), by introduction of NMPs into the body,
- Use of NMPs by topical routes (skin, lung, eye, etc.) in order to obtain systemic exposure or a local effect.

Recommendations for the toxicological evaluation of NMPs will be formulated in the usual order of other guidelines. The Task Force emphasizes that, in view of the wide range of structures, physicochemical and biological properties, therapeutic uses, etc., case-by-case assessment of the most relevant study programme for a given NMP will always be essential.

## **RECOMMENDATIONS**

As indicated above, general scientific and/or regulatory data related to the toxicological evaluation of NMPs are currently lacking. However, we can refer to the European Commission (Health and Consumer Protection) document prepared by the SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks) entitled "Opinion on the Appropriateness of the Risk Assessment Methodology in Accordance with the Technical Guidance Documents for New and Existing Substances for Assessing the Risks of Nanomaterials" and approved by public consultation on 29 March 2007. Although not specifically dealing with NMPs, this document contains a number of considerations that can apply to NMPs. Also note that the "conventional" toxicological approach proposed by current guidelines for medicinal products in general (ICH, FDA, EMEA) has been accepted up until now for approved NMPs or NMPs currently under evaluation by health authorities. However, some criticisms have been raised concerning the currently available methods of experimental evaluation that are considered to not adequately assess the properties of nanoparticle products. The most frequent criticisms essentially concern pharmacokinetic and toxicokinetic studies, which are considered to not realistically take into account the specificities related to the nanoparticle structure. The relevance of *in vitro* tests has also been questioned, as the sedimentation rate and diffusion capacity of nanoparticles must modify the conditions of exposure (dose-duration) of the models used (for example, genotoxicity tests). Finally, the lack of data on long-term effects is often emphasized.

Consequently, like certain consumer groups in the USA, we may need to recommend the development of completely new regulations based on “adapted” safety assessment tests for nanomaterials, including NMPs. This maximalist proposal is totally idealistic and scientifically unjustified according to the very great majority of the scientific community. How many years for development and validation would be necessary to achieve such a result? This major revision also does not appear to be justified by the available scientific data.

Some manufacturers consulted and most of the task force also consider that toxicological evaluation of NMPs should not be appreciably different from “conventional” evaluation, but with certain specific adaptations (inappropriate nature of repeated-dose studies for NMP used as a single dose in man, such as in medical imaging). The plan adopted for elaboration of these recommendations is based on this approach, i.e. adapt the safety assessment strategy, when necessary, without modifying the basic principles.

### **1. Pharmacokinetic studies**

Evaluation of absorption, distribution, metabolism and excretion (ADME) of nanoparticles and of their degradation or solubilization products is essential and must be performed prior to safety studies. The pharmacokinetic properties of nanoparticles are very different from those of conventional molecules but are nevertheless studied in similar ways. Four factors essentially determine the pharmacokinetics of nanoparticles: route of administration, particle size, nature of coating polymers and animal species. In particular, due to the importance and originality of the biodistribution study, it must be based on relevant methods (radioactive labelling). Scintigraphic imaging techniques and PET are adapted to the biodistribution study of parenterally, pulmonary, or even enterally administered NMPs, and to determination of sites of sequestration and translocation phenomena. Particular attention must be paid to the potential impact of labelling on the properties and outcome of NMPs.

NMPs are frequently administered parenterally (IV, sometimes SC or IM), but also by specific topical routes (lung, skin, eye). NMPs are recognized by the reticulo-endothelial system and are phagocytosed by macrophages (liver, spleen, lymph nodes, bone marrow, lungs, etc.). As opsonization phenomena vary enormously according to the animal species, the choice of species possibly predictive for Man is particularly difficult: dogs appear to be less relevant. The use of 2 animal species (rodent and non-rodent) may therefore be inappropriate. The case of a specific nanoparticle system associated with the active principle raises the question of whether this system should be studied alone and in the presence of the active principle. This question has not yet been resolved, but the list of required studies cannot be unreasonably increased.

In the current state of knowledge, apart from NMPs used in medical imaging, little is known about their metabolism and excretion. The formation of degradation products of polymers for example (“metabolite”) currently remains hypothetical for a large number of compounds.

The essential pharmacokinetic studies of NMPs must therefore be based on a case-by-case scientific approach, possibly by referring to the studies conducted on NMP already developed, by resolving any defects of these studies.

Observed effects should preferably be expressed per unit surface area to rather than unit of mass as is usually the case, as the smaller the particle, the greater the proportion of atoms exposed to the environment.

## **2. TOXICOLOGICAL STUDIES**

### **2.1 *IN VITRO* TOXICITY**

For the reasons indicated above (rapidity - ethics), and also in view of the absence of *in silico* data, it is highly recommended to develop and validate *in vitro* methods that are able to provide information on cytotoxicity, phagocytic capacity and macrophage activation, activation of complement pathways, biopersistence, generation of toxic free radicals, topical cutaneous, pulmonary and ocular tolerance (when these routes are used) etc. right from the prerequisite stage. Specific pharmacological tests, especially concerning the action on nerve cells and myocardial fibres, should be considered.

### **2.2 SINGLE DOSE TOXICITY**

Evaluation of single dose toxicity provides a wealth of information on the adverse effects of NMPs also administered as a single dose in man (imaging). These studies should be designed not as acute toxicity studies in which the endpoint is death, but as complete toxicity studies including evaluation of biochemical, haematological and histological parameters, as in repeated dose studies (extended single dose study). These studies could also be useful to rapidly compare active principles in nanoparticles and conventional forms, intended to be administered repeatedly in Man (anti-cancer drugs) and for topical uses.

### **2.3 REPEATED DOSE TOXICITY**

Repeated dose toxicity cannot be evaluated according to conventional study plans due to the structural and physicochemical differences of the particles, the animal species used, the indications and conditions of administration in therapeutics, etc. It will therefore be recommended to propose case-by-case protocols adapted to the above characteristics, reproducing human exposure conditions as closely as possible; standard protocols inducing massive exposure of the animals and consequently uninterpretable adverse effects should be excluded. Potential target organs or systems, due to the capacity of NMPs to cross physiological barriers should be investigated as a priority. In particular, potential targets include:

- Liver and organs of the reticulo-endothelial system (uptake),
- Kidney (e.g.: possibility of urolithiasis, tubular lesions),
- Central nervous system (various mechanisms have been proposed, especially passage across the blood-brain barrier, to evaluate the risk of neuronal degeneration),
- Reproductive organs (potential impairment of fertility),
- Cardiovascular system (e.g.: formation of aggregates),
- Development of inflammatory reactions, which appear to constitute a major risk for the respiratory tract, related to the formation of agglomerates and aggregates, due to its long-term consequences: cancer (DNA damage) and fibrosis (role of cytokines). Pulmonary inflammation also plays a major role in translocation phenomena, leading to exposure of other target organs, especially the brain. There is a high risk of induction of pulmonary intravascular macrophages, because of their phagocytic activity on NMPs or their microaggregates following intravenous administration. This could cause major pulmonary haemodynamic disorders. Scintigraphic and ultrasound imaging techniques are very suitable for *in vivo* assessment of these phenomena.

Evaluation of systemic exposure during animal studies in order to define safety margins for human exposures obviously remains to be investigated.

## **2.4 PARTICULAR TOXICITIES**

Certain forms of toxicity may occur according to the characteristics of the NMP or according to the route of administration and it would be highly recommended to pay particular attention to these aspects.

### *2.4.1 IMMUNOTOXICITY*

The immune response to a foreign substance introduced into the body can be globally divided into two compartments: the adaptive response specific to the antigen introduced and the innate immune response not specific to the antigen. The structure and properties of NMPs suggest that these products are able to modify both of these types of immune response. The recognition of NMPs by “scavenger” receptors located on macrophages and polynuclear neutrophils can induce release of cytokines responsible for an inhalation pulmonary inflammatory response. Particulate matter, especially fine particles, is known to possess adjuvant properties that can induce exacerbation or modification of the type of immune response to a given antigen (Th1 response versus Th2 response). This type of response could possibly induce hypersensitivity or allergic reactions. Finally, uptake of NMPs or their recognition by human dendritic cells could also lead to immunosuppression, and NMPs may also be able to modify self antigens, inducing autoimmune reactions.

Evaluation of the immunotoxic potential of NMPs is therefore recommended, particularly for medicinal products administered by inhalation. This evaluation must be based on appropriate, validated methods. Determination of dermal sensitization by the LLNA (local lymph node assay) test in mice is the test most widely used at the present time for nanoparticle products (OECD guideline 429), for example for the various forms of titanium dioxide in cosmetology. The development of cell models, particularly to study the effects on macrophages and polynuclear cells and dendritic cells, should be actively encouraged.

### *2.4.2 RISKS RELATED TO THE FORMATION OF AGGLOMERATES*

This is a classical risk, identified right from the use of the first NMPs (liposomes). The formation of agglomerates can affect various territories in the body, especially in smaller vessels (peripheral microcirculation, cerebral vessels, etc.) by inducing embolic phenomena. This potential should be evaluated by appropriate techniques, especially histological techniques.

### *2.4.3 LOCAL EFFECTS*

Nanoparticle systems can induce severe irritation and inflammation phenomena via direct mechanisms or mechanisms mediated by signalling pathways. This should be investigated for all routes of administration, particularly topical routes: skin, eye, and especially lung, as indicated above. It is reasonable to propose that OECD guidelines 404 and 405 can be applied to evaluation of the irritating potential on the skin and the eye after single administration in rabbits. The preferred technique to assess local effects on the lung consists of intratracheal administration of the test substance in rats followed by bronchoalveolar lavage (evaluation of biological markers of inflammation in the lavage fluid), evaluation of cellular proliferation and histological examination. This type of study could be associated with evaluation of reversibility of the observed phenomena (after one to four weeks for example).

The haemolytic potential by IV administration of NMPs should be evaluated, if this route of administration is used in therapeutics.

## **2.5 REPRODUCTION TOXICITY**

No published data are currently available concerning the potential effects of nanoparticles on reproduction, fertility and their teratogenicity, and these aspects need to be evaluated. For example, passage of NMPs across the foeto-placental barrier makes evaluation of embryofetal toxicity and the teratogenic potential essential. The protocols described in current guidelines should therefore be used, but may need to be adapted to NMPs. Maternotoxic and teratogenic effects have been described for medical imaging products (probably due to iron overload).

## **2.6 GENOTOXICITY**

Although the ability of nanoparticles to cross cell membranes has been established, much less is known about their ability to reach the cell nucleus at the appropriate time of the cell cycle to interact directly on DNA, especially during cell division when the nuclear envelope is lost. Various mechanisms can be proposed to explain a genotoxic effect of nanoparticles, which would be fairly unpredictable because of their particular physicochemical characteristics:

- A direct effect by inducing DNA damage, chromosomal alterations, disturbances of cell division (aneugenic potential),
- An indirect effect via alteration of membranes and especially by the formation of free radicals, toxic oxygen metabolites or lipid peroxidation products, especially related to inflammatory processes.

In practice, the essential question concerns the relevance of the tests recommended by international guidelines to evaluate the genotoxic potential. Conventional batteries of *in vitro* tests are generally used (Ames, MLA, chromosomal aberrations, *in vitro* micronucleus). Use of these tests and the conclusions drawn must be particularly cautious, especially as the relevance of these tests is currently controversial. There is a current tendency in favour of *in vivo* tests, although they are generally criticized for focussing on bone marrow (micronucleus) and liver (UDS test), while the targets of NMPs could also include the gastrointestinal tract, lungs and skin. From this point of view, the comet test that can be applied to many tissues appears to be more appropriate. A micronucleus test on rat alveolar epithelial cells has been proposed to test genotoxicity via the respiratory route and a test on reconstituted human skin has been proposed for products applied to the skin.

Evaluation of the genotoxic potential of NMPs therefore remains absolutely necessary, bearing in mind that although conventional tests must still be performed, they must be modified to improve their relevance in relation to the particular characteristics of the products evaluated.

## **2.7 CARCINOGENIC POTENTIAL**

Evaluation of the experimental carcinogenic potential of NMPs is currently a controversial issue:

- On the one hand, it is clear that, in view of their structure, their potential to induce DNA damage and inflammatory reactions and their bioaccumulation, NMPs could induce tumours, especially lung tumours.
- On the other hand, the protocols recommended by guidelines are complex, time-consuming, and poorly adapted to exposure to nanoparticles (metrology, control of exposure, etc.). Furthermore, carcinogenesis studies do not appear to be necessary in view of the current applications of NMPs (single dose in medical imaging, vectorization of anti-cancer drugs).

Consequently, evaluation of the carcinogenic potential of a NMP should not be systematic, and should be justified by detailed consideration of the potential hazard and risk assessment, in order to avoid drawing hasty and inappropriate conclusions. An adaptation of current protocols is highly recommended: for example shorter studies, studies with a limited number of administrations, use of transgenic mice, etc. Particularly relevant evaluation of the genotoxic potential of NMPs would obviously be very useful as part of risk assessment.

## CONCLUSION

The recommendations formulated in this document are based on the following concept: evaluation of the safety of NMPs, taking into account scientific and practical considerations such as the need to be immediately operational, must not fundamentally differ from the conventional strategy of safety evaluation of medicinal products. However, the methods of this evaluation must be adapted when necessary and the results must be expressed in relation with the particular characteristics of the nanoparticle structure.

Nevertheless, a much longer term view cannot be excluded and it will also be recommended to apply the conclusions of the document “Nanotechnology: A report of the US FDA Nanotechnology Task Force” published on 25 July 2007, which proposed the following long-term objectives to the FDA:

- Evaluate the adequacy of current testing approaches to assess safety, effectiveness, and quality of products that use nanoscale materials;
- Promote and participate in the development of characterization methods and standards for nanoscale materials;
- Promote and participate in the development of models for the behaviour of nanoscale particles *in vitro* and *in vivo*.

This ambitious objective could be confided b Afssaps to a Task Force composed of academic scientists, members of regulatory authorities and obviously manufacturers specialized in this field.

**N.B.** : This document is not designed to formulate recommendations for the evaluation of environmental toxicity.