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EXPLORING THE *IN VIVO* TOXICITY OF NANOPARTICLES

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27 **Abstract**

28 Toxicological tests of a xenobiotic play a key role to determine the safety of the new
29 compound before it reaches the market. In this review article, we describe the main
30 types of toxicological studies that can be performed *in vivo* to detect a possible
31 undesired effect of a xenobiotic with especial emphasis on the data available for the
32 different types of nanoparticles. The different procedures described in this review allow
33 to obtain valuable information about the possible toxic effects of a xenobiotic in order
34 to minimize the possible risks for patients once the compound has been approved for
35 therapeutic use.

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50 **Keywords:** nanoparticles, *in vivo* toxicity, toxicology, experimental procedures

51 1. Introduction

52 Drug or chemical compounds toxicity in animals is one of the key steps in the approval
53 of a new drug and has the objective of identifying possible risks for human health
54 before the drug reaches the market. After thalidomide disaster, toxicity testing has been
55 routinely performed prior to a drug being released into the market. These studies with
56 regulatory purposes are performed following standardized protocols and meeting the
57 good laboratory practice and experimental animal's protection regulations ¹.
58 Toxicological tests focus on different aspects of the new drug candidate such as
59 molecular mechanisms and the biological targets of the possible toxicity of the
60 compound; species-specific toxic susceptibility, gender and population groups; kinetics
61 and metabolism in different organisms. Finally, all available information for risk
62 assessment of the use of xenobiotics in different therapeutic applications is integrated to
63 predict the undesired consequences in the interaction between xenobiotics and living
64 beings ².

65 In this review article, we describe the main types of toxicological studies *in vivo* to
66 detect a possible undesired effect of a xenobiotic with especial emphasis on the data
67 available for the different types of nanoparticles (NPs) (Figure 1).

69 2. Types of toxicological studies.

70 A good toxicological evaluation of a xenobiotic means that the toxicity of the
71 compound must be study using different tests and during different periods of time.
72 According to this last parameter, we can classify the toxicological studies in:

73 a) Acute toxicity studies.

74 The objective of this type of studies consists in completely characterize the acute
75 biological effects of the xenobiotic. They include not only the study on lethality rate
76 (the dose that causes the death in the animal species chosen) but also xenobiotic
77 effect on target organs and the clinical signs associated to those effects. This type of
78 studies also provides us information about the range of doses that can be used
79 during repeated administration toxicity studies in animals.

80

81 Acute toxicity studies require the use of animal groups that must be homogenous in age,
82 gender and weight. Most used specie is rat, although other rodents might be also used.
83 Regarding gender, females are preferred since literature surveys of conventional LD₅₀
84 tests show that, although there is little difference in sensitivity between sexes, in those
85 cases where differences are observed, females are generally slightly more sensitive ³.
86 However, if previous knowledge of toxicological properties of structurally related
87 chemicals indicates that males are likely to be more sensitive, then this sex should be
88 used for testing. When the test is conducted in males, adequate justification should be
89 provided. Nevertheless, when knowing the maximum tolerated dose is required it is
90 very common to use other species such as rabbits, dogs and apes.

91 In acute studies it is necessary to keep the animals in an identical environment for at
92 least 5 days before performing the testing procedure. In the unusual circumstance that
93 administering a full single dose is not possible, the dose may be given in smaller
94 fractions over a period not exceeding 24 hours. After the xenobiotic administration,
95 animals must be observed individually at least once during the initial 30 minutes,
96 periodically during the first 24 hours, with more frequent observations during the first 4
97 hours, and daily thereafter, for a total of 14 days, except where they need to be removed
98 from the study and humanely euthanized for animal welfare reasons or are found dead.
99 Observations should include: body weight and food/water consumption; hematological
100 tests; clinical biochemistry and histopathological studies. Additional observations might
101 be necessary if the animals continue to display signs of toxicity ³.

102 In general, acute toxicity tests are used for initial *in vivo* testing looking for information
103 on whether a “go” or “non-go” decision can be made about either the drug or the
104 nanoparticle. A significant number of articles studying acute toxicity for different types
105 of nanoparticles have been published. Some recent examples for different types of
106 nanoparticles including a polydopamine (PDA) nanoparticle loading ionic liquids
107 (ILs/PDA) as microwave susceptible agent for enhancing the selectivity and targeting of
108 tumor microwave thermal therapy that showed good biocompatibility and lack of
109 toxicity in acute studies ⁴. Other NPs such as poly (β -L-malic acid) (PMLA)
110 nanoparticles have been shown to be safe in acute toxicity testing ⁵. Moreover, the
111 influence of surface functionalization and size on PAMAM dendrimers (G3.5-COOH,
112 G4-NH₂, G4-OH, G6.5-COOH, G7- NH₂ and G7-OH) acute toxicity was evaluated.

Dendrimers were administered *per os* to CD-1 mice and the animals followed for 10 days. No toxicity was observed for all dendrimers tested but G7-OH and G7- NH₂⁶. Studies of acute toxicity help to establish a correlation between the magnitude of the toxic effect and the administered dose. In addition, these studies also provide statistical information about the dose causing death in 50% of treated animals (LD₅₀). For instance, it has been reported that PAMAM-NH₂ dendrimers have antibacterial activities⁷ and this actions might be useful for treating septicemia induced by resistant bacteria. To distinguish therapeutic from toxic doses, BALB/c mice were intraperitoneally injected with G2.0 PAMAM-NH₂ at increasing doses to determine nanoparticle LD₅₀. This acute toxicity assay revealed that LD₅₀ for G2.0 PAMAM- NH₂ was 80 mg/kg while G2.0 PAMAM-NH₂ at 10 mg/kg was effective against bacteria with relatively low toxicity⁸). Finally, acute toxicity studies can be used to set up the basis for the dose range to be used in longer toxicity studies.

b) **Subchronic toxicity studies.**

The objective of this kind of studies consists in recognizing and evaluating the toxic effects of a xenobiotic that appear after continuous exposure to the chemical compound. Subchronic toxicity studies range from 28 days to 90 days and provide information about: i) the main toxic effects of compound; ii) the target organs involved; iii) kinetics of biotransformation of the xenobiotic; iv) the accumulative nature of toxic effects, v) the possible reversibility of observed effects; and vi) the dose to be used in chronic toxicity studies⁹. There are not many examples of sub-chronic studies on toxicity of nanoparticles since these studies are expensive and time-consuming, being carried out for nanoparticles with a clear potential for reaching the clinical stage. Thus, monomethoxy(polyethyleneglycol)-poly (D, L-lactic-co-glycolic acid)-monomethoxy (PELGE) nanoparticles were studied for subchronic toxicity in rats. No clear signs of subchronic toxicity were observed following 28 days of continuous intravenous administration based on clinical observation, body weight, hematology parameters and histopathology analysis. However, PELGE nanoparticles were associated with histopathological changes in the spleen, increased serum IgM and IgG levels, changes in blood lymphocyte subpopulations and enhanced expression of proinflammatory cytokines. Therefore, these results showed that PELGE nanoparticles produced low subchronic toxicity but substantial immunotoxicity,¹⁰. Subchronic toxicity in mice of graphene quantum dots (GQD) has been also studied. Mice were intravenously (i.v.) or

147 intraperitoneally (i.p.) injected with 20 mg/kg of pegylated GQD (GQD-PEG) every
148 other day for 14 days, and then monitored for up to 40 days. GQD-PEG did not show
149 any toxicity in hematological, biochemical, and histopathology studies. On the contrary,
150 the liver and the spleen of treated mice looked darker than controls, consistent with
151 aggregation of the nanoparticle, and suggesting chronic damage due to slow clearance
152 of the nanoparticle ¹¹. Oral subchronic exposure to silver nanoparticles (AgNPs) in rats
153 daily treated by gavage during 90 days did not produce any toxicity ¹².

154 c) **Chronic toxicity studies.**

155 The objective of this type of study is to identify toxic effects that appear following daily
156 administration of the xenobiotic for a long time. Tests used in chronic toxicity studies
157 are designed to identify those toxic effects with long latency time, such as carcinogenic
158 effects or those produced by accumulative mechanisms caused by the xenobiotic
159 compound. For this purpose, they are conducted using a minimum of one rodent and
160 one non-rodent species being the use of the later considered when available data suggest
161 that the data obtained in the later specie are more relevant for the prediction of toxic
162 effects on human health. The preferred rodent species is rat, although other rodent
163 species, e.g., mouse, may be used. Although the use of mice in carcinogenicity testing
164 may have limited utility, under some current regulatory programs carcinogenicity
165 testing in mice is still required. Rats and mice have been the preferred experimental
166 models because of their relatively short life span, their widespread use in
167 pharmacological and toxicological studies, their susceptibility to tumor induction, and
168 the availability of sufficiently characterized strains.

169 Chronic toxicity study design consists of two parallel phases, a chronic phase and a
170 carcinogenicity phase. The xenobiotic is generally administered orally although testing
171 by inhalation or through the dermis may also be appropriate. For the chronic phase, the
172 compound is administered daily at different doses to several groups of test animals, one
173 dose per group, generally for a 12 months period, although longer or shorter times may
174 also be chosen depending on regulatory requirements. In the majority of cases, one or
175 more doses are tested, between 25% and 50% of the Maximum Tolerated Dose (MTD)
176 which is the highest dose that can be tolerated without significant lethality from causes
177 other than tumors. The length of the toxicological study is chosen to ensure sufficient
178 time to allow any effect of cumulative toxicity to become manifest, without the

179 confounding effects of changes associated to aging. The study design may also include
180 one or more interim animal sacrifice, e.g. at 3 and 6 months, and additional groups of
181 animals may be included to accommodate this experimental design. For the
182 carcinogenicity phase, the test substance is administered daily to several groups of test
183 animals during a major portion of their life span. The animals in both phases are
184 observed closely for signs of toxicity and for the development of tumoral lesions.
185 Animals which die or are euthanized during the test are necropsied and, at the
186 conclusion of the test, surviving animals are euthanized and necropsied to look for
187 signs of tumors.

188 Chronic toxicity studies determine the possible carcinogenicity, genotoxicity, or
189 embryotoxicity of the xenobiotic. In addition, different parameters of hematological and
190 clinical biochemistry, phototoxicity, allergic reactions, as well as behavior studies and
191 weight control of treated animals are also determined. Histopathological tests of main
192 target organs are also performed being the localization and distribution of toxics or its
193 metabolites evaluated in those samples. These studies also provide information about
194 toxic effects latency, possible reversibility of observed effects and threshold dose,
195 meaning the maximum dose that not produce toxic effects. The threshold dose is very
196 useful as it provides the bases for identifying important parameters like daily admissible
197 dose and tolerance limits ¹³.

198 Although some chronic toxicity studies for nanoparticles have been described in the
199 literature,^{14,15} they do not fulfil strictly the above criteria to be considered chronic
200 studies since those studies do not cover the 12 month study time established in the
201 OECD and ICH guidelines for chronic studies. Therefore, they have been considered in
202 this review as subchronic toxicity studies.

203

204 **2. Methods used in *in vivo* toxicological testing of xenobiotics**

205 The most commonly used tests to identify possible *in vivo* toxic actions of xenobiotics
206 are described in the following paragraphs.

207 2.1 Pyrogenic test

208 Pyrogens are substances that cause fever. An important initial step in the preclinical
209 characterization of nanoparticles is testing for sterility and pyrogenicity. Unites States

Food and Drug Administration (FDA) recommends two methods for pyrogenicity evaluation: the lumulus ameocyte lysate (LAL)-based assay and the rabbit pyrogen test¹⁶. However, it has been described that certain nanoparticles may interfere with one or more formats of this test (i.e PMLA, PAMAM NDs, gold colloids)¹⁷. So, the *in vivo* rabbit pyrogen test might not be used with these type of nanoparticles^{5,18}.

To perform the *in vivo* rabbit pyrogen test the USP 151 protocol is generally followed¹⁹. This test involves measuring the rise in temperature of rabbits following the intravenous injection of a xenobiotic test solution. The procedure is the following: within 30 minutes prior to the injection of the test dose, the temperature for each rabbit is determined. This will be considered the baseline for determining any temperature increase resulting from the injection of the test solution. Every animal group must be very homogeneous and the intragroup variation should not exceed one degree Celsius and no animal should exceed a temperature of 39.8°C. Unless otherwise specified, 10 mL of the test solution containing the xenobiotic (at the test concentration) per kg of body weight are injected into an ear vein of each of three rabbits, completing each injection within 10 minutes. The solution containing the xenobiotic is warmed to 37 ± 2 °C before the injection and the animal temperature is recorded every 30 minutes between one and 3 hours after the injection. If no rabbit shows a rise in temperature of 0.5 °C or more above its respective baseline, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5°C or more, further testing on 5 additional rabbits should be performed. Then, if no more than three of the eight rabbits show individual rises in temperature of 0.5°C or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°C, the material under examination meets the requirements for the absence of pyrogens. The procedure is represented in Figure 2.

Pyrogenic test is designed to predict whether the parenteral administration of a xenobiotic will have an acceptable risk level of a febrile reaction in the future patient.. Although fever, within reasonable limits, is not considered a serious side-effect, pyrogenic tests might provide a warning on other possible side-effects, that might appear during wide spread use of a drug, including in this category nanoparticles. The test is very simple to perform and it should be done, at an early stage, as part of the toxicity studies for any nanoparticle aimed to reach clinical setting.

243 2.2 Genotoxicity studies

244 Genotoxicity studies are essential to evaluate effects of a xenobiotic on genetic material.
245 These type of studies are important because the induction and accumulation of genetic
246 damage can cause genomic instability leading to cancer^{20,21}. There are several *in vitro*
247 and *in vivo* tests that explore the possible genotoxic potential of a compound. Before
248 explaining the different tests used to study *in vivo* genotoxicity it is necessary to define
249 two basic concepts: a) clastogenic agent is the physical or chemical agent able to
250 produce chromosome breaks, and b) aneugenic agent is the chemical compound that
251 affects cell division and the components involved in the cellular mitosis producing gain
252 or loss of a full chromosome leading to aneuploidy²².

253 The mechanisms of NP-induced genotoxicity are still not well understood and it is often
254 difficult to identify whether a toxic action on DNA is specific for the presence of the
255 nanoparticle. It has been described that genotoxicity may be produced by the direct
256 interaction of NPs with the genetic material (DNA)²³⁻²⁵. However, NPs can potentially
257 interact not only with DNA but also with proteins involved in DNA replication,
258 transcription or repair (e.g C60 fullerene binds to human DNA topoisomerase II α in the
259 ATP binding domain, which might inhibit the enzyme activity)²⁶. Moreover, NPs can
260 also cause indirect DNA damage by NP-induced reactive oxygen species (ROS), or by
261 toxic ions released from soluble NPs^{21,23,27,28}.

262 The most used *in vivo* genotoxicity tests are described in the following paragraphs.

263 *2.2.1 Mammalian in vivo chromosomal aberrations assay (CA)*

264 The mammalian *in vivo* chromosome aberration test is used for the detection of
265 structural chromosome aberrations induced by xenobiotics in bone marrow cells
266 obtained from animals, usually rodents. Structural chromosomal aberrations may affect
267 two structures: chromosomes or chromatids. While the majority of genotoxic chemical-
268 induced aberrations affect chromatids, chromosome aberrations might also occur.
269 Chromosomal damage and related events are the cause of many human genetic diseases
270 and there is substantial evidence that, when these lesions and related events cause
271 alterations in oncogenes and tumor suppressor genes, they are involved in the genesis of
272 cancer both in humans and animals. This test is performed following the standardized
273 protocol OECD 475²⁹. The general procedure is shown in Figure 3.

274 Animals are exposed to the xenobiotic using the proper route of exposure and are
275 euthanized. Prior to sacrifice, animals are injected intraperitoneally with an appropriate
276 dose of metaphase arresting agent (e.g. colchicine). Immediately after sacrifice, bone
277 marrow is obtained, exposed to hypotonic solution and fixed. Cells are then spread on
278 slides and stained. Cells in metaphase are analyzed to detect chromosomal aberrations.
279 Both treated and control groups must include at least 5 animals per sex. Test substances
280 should be preferably administered as a single dose. At least 3 subtoxic concentrations of
281 the compound, with corresponding positive and negative controls should be used. The
282 mitotic index, taken as a marker for cytotoxicity, should be determined as a measure of
283 cytotoxicity in at least 1,000 cells per each animal including positive controls, and
284 untreated or vehicle/solvent-treated animals taken as negative controls. Since slide
285 preparation procedures often result in breakage of a proportion of metaphases with the
286 resulting loss of chromosomes, to be considered a valid test the cell score should,
287 therefore, contain a number of centromeres not less than $2n \pm 2$, where n is the haploid
288 number of chromosomes for that species.

289 There are several criteria for considering a result positive for this test such as: i) a dose-
290 related increase in the relative number of cells with chromosome aberrations; ii) a clear
291 increase in the number of cells with aberrations in a single dose group at a single
292 sampling time; iii) an increase in polyploidy that may indicate that the test substance
293 has the potential to induce numerical chromosome aberrations; and iv) an increase in
294 endoreduplication that may indicate that the test substance has the potential to inhibit
295 cell cycle progression. For more in-depth information on the possible genotoxic actions
296 of the xenobiotic, other techniques such as, fluorescence in situ hybridization (FISH)
297 might be used. FISH uses specific molecular probes for specific chromosomal regions
298 to facilitate the detection of break points or fragile regions within that chromosome.
299 However, when the objective consists only in detecting possible clastogenic activity, it
300 is not necessary to apply a technique like FISH.

301 As mentioned above, genotoxicity may be induced by ROS generation. ZnO-NP has
302 immense potential and application in the health care sector. *In vivo* results demonstrated
303 that ZnO-NP induced ROS production and genotoxic response in mice bone marrow.
304 The CAs scored were mainly found to be chromatid breaks and the number of breaks
305 per cell to be significantly higher than the control. About 10-fold increase in CAs was
306 observed at the highest dose administered (25 mg/kg body weight)³⁰.

307 2.2.2 The Micronucleus Test

308 The micronucleus test *in vivo* is a method devised primarily for screening chemicals
309 with potentials to cause chromosome abnormalities. These abnormalities can be
310 structural or numeric. This test is performed in rodents and, therefore, like other *in vivo*
311 tests, it has the advantage of taking into account the pharmacokinetic properties of the
312 xenobiotic ³¹.

313 Micronucleus is the name given to the small nucleus that is formed when a chromosome
314 or a fragment of a chromosome is not incorporated into one of the daughter nuclei
315 during cell division. Micronuclei are generated during anaphase from lagging acentric
316 chromosome or chromatid fragments caused by incorrectly repaired or unrepaired DNA
317 breaks or by nondisjunction of chromosomes. This incorrect segregation of
318 chromosomes may result from hypomethylation of repeat sequences present in
319 pericentromeric DNA, irregularities in kinetochore proteins or their assembly,
320 dysfunctional spindle apparatus, or flawed anaphase checkpoint genes ³². Micronuclei
321 can be formed in all kind of cells from any proliferative tissue, but it can be detected
322 easily in those cells without nucleus, such as erythrocytes. A restricted analysis to
323 nucleated bone-marrow cells is rather tedious and unreliable if the xenobiotic toxic
324 effects are small. For this reason, micronucleus test is performed most often in rat or
325 mouse bone-marrow erythrocytes, although it is possible to analyze others tissues.
326 Specifically, immature erythrocytes are used in this method, when they expel their
327 nucleus but still preserve stain properties different from older, more mature, forms. In
328 addition, the number of polychromatic erythrocytes is almost unlimited and the
329 spontaneous rate of micronucleated cells is low and consistent. The test is very sensitive
330 since it can identify the effect of very low dosages of standard chromosome-breaking
331 mutagens ³².

332 This test is performed following the standardized procedure indicated in protocol
333 OECD 474 ³³. When compounds of unknown mutagenic activity are tested, a subacute
334 treatment over 30 hours should be chosen. The intention is to expose a large proportion
335 of the cell population to the compound to be tested during two cell cycles. The dose
336 administered should always cover a wide range, from the likely therapeutic dose up to
337 the highest tolerable dose. The route of administration should be the same that it is
338 anticipated to be used in humans. Animals are euthanized between 24 and 48 hours after
339 treatment. In this test, animals are not treated previously with metaphase arresting

agents. Then cells are stained and are analyzed by microscopy. A perfect morphology of the nucleated cells serves as criterion of good quality for the procedure, even though the nucleated cells are not evaluated in the test. Their staining has to be vigorous, red in mature erythrocytes and with a strong bluish tint in the immature forms (polychromatic erythrocytes). Generally, around 1,000 polychromatic erythrocytes are evaluated per animal and the total number of mature micronucleated erythrocytes is used as control. The result is considered positive when in a xenobiotic-treated group shows a statistically significant increase in the number of micronucleus number respect to a negative control.

This testing procedure has a number of important advantages over the analysis of bone-marrow metaphase chromosomes since it is simpler and faster than chromosome analysis for the same material, but not at the expense of accuracy. The test is, at least, as sensitive as the metaphase method for monitoring chromosome breakage. In addition, it allows identification of toxic effects on the spindle apparatus. All these properties render this method highly suitable for routine toxicological screening³¹.

This test has been used to test nanoparticle genotoxicity. Thus, single-wall carbon nanotubes (SWCNTs) genotoxicity was evaluated using a bone marrow micronucleus test. No effect of SWCNTs on the number of micronuclei in the immature erythrocytes of mice was observed³⁴. The genotoxicity of gold nanoparticles (AuNPs) of different sizes (2, 20 and 200 nm) was also studied in the lungs of rats and no toxicity was found³⁵. However, in other study AuNPs (5, 20, 50 nm) were evaluated in mice. While the standard *in vivo* micronucleus test, showed no toxicity after 4 days exposure to NPs, exposure for 14 days, indicated that the 5 nm AuNPs presented significant clastogenic damage, with a dose-dependent increase in the number of micronuclei observed³⁶.

2.2.3 Comet assay

Comet assay identifies DNA chain breaks in eukaryotic cells. This method can be used to identify possible mutagens and carcinogens for humans. In addition, it has high sensibility and reproducibility³⁷. This test is performed following the normalized procedure OECD 489³⁸.

After xenobiotic administration, cells suspensions are obtained from different organs by incubation with collagenase or trypsin. Then, cells are placed in agarose on a slide and finally are lysed. Only a few cells per visual field are typically used since higher cell

densities might result in a significant proportion of overlapping comets. After the agarose gel has solidified, slides are placed, generally for at least one hour, in a lysis solution containing high salt concentration and detergents. There is a minimal time needed to appropriately release the DNA and this time might vary depending on the cell type. At the next stage, prior to electrophoresis, the slides are incubated in a strong alkaline (pH > 13) electrophoresis buffer to produce single stranded DNA. After DNA unwinding, the single-stranded DNA is electrophoresed under alkaline conditions to produce comets. After electrophoresis, the alkaline buffer in the gels is neutralized by rinsing the slides with a suitable buffer. After neutralization, slides can be stained and the comets scored. The tail intensity of the comet compared to their head intensity is proportional to the number of DNA chain breaks^{38,39}. This test has been used to study genotoxicity of AgNPs with a size ranging from 90 to 180 nm in Swiss Albino male mice. Single intraperitoneal administration of AgNPs gave a significant increase in tail DNA intensity at doses of 10 mg/kg body weight⁴⁰. Moreover, cerium oxide nanoparticles have also shown toxicity in comet assays following oral administration to Wistar rats⁴¹.

388

389 2.3. Carcinogenetic studies

Currently, the available methods for evaluating *in vivo* the carcinogenic potential for different xenobiotics are limited. There are both *in vitro* and *in vivo* tests available to evaluate carcinogenesis. In *in vitro* tests, the ability of xenobiotics to transform healthy cells into tumoral cells is observed. However, it must be noted that this type of test is able to detect carcinogenicity, but not genotoxicity. In addition, *in vitro* tests overlook the chemical biotransformation of the xenobiotic that takes place in the organism and, for this reason, the conclusions drawn from them, are somehow limited.

In vivo, the most commonly used carcinogenic test is based on the observation of the xenobiotic effect in rodents for two years. Since carrying on this test might involve the life span of the animals, it is expensive and complex. This test is performed following normalized protocol OECD 451⁴². The maximum tolerated dose is administrated chronically to the animals. So, it is possible to evaluate the tumoral incidence for each organ. To perform this test, there are different groups of animals: control group, test group and satellite group being this last group used for determining whether the

xenobiotic effects are reversible or not. In this type of study, food intake and the weight loss is thoroughly controlled, as well as different parameters of hematological and clinical biochemistry. In addition, when the animals are euthanized, histopathological studies of the main target organs are performed¹³. Positron emission tomography (PET) might be used to identify the appearance of early abnormal growth. PET imaging has unique advantages combining high sensitivity and the ability to conduct quantitative analysis of whole-body imaging.⁴³

However, despite the importance of conducting this type of studies, due to the long time and the high costs, no studies of carcinogenesis for nanoparticles have been described so far.

2.4. Embryotoxicity studies

Knowledge of xenobiotic effects on embryonic development is very important in the field of public health and critical for new drug approval. After thalidomide disaster, reproduction toxicity testing has been routinely performed prior to a drug being released into the market¹. According to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)⁴⁴ and normalized protocol OECD 422⁴⁵, most commonly used tests are: i) fertility and reproduction test, evaluating reproductive ability in males and females (gametes development, fertility, pre- and post-implantation viability, delivery and lactation); ii) teratogenicity test evaluating morphology and viability (external, visceral and skeletal) of fetus before birth; and iii) perinatal test to evaluate postnatal survival, external morphology and development⁴⁴. There is not *a priori* preferred animal model to study the risk of the use of a xenobiotic on human embryology. Accordingly, different species such as rats, mice, guinea pigs, cats, dogs and monkeys have been used for this purpose.

A significant number of embryotoxicity studies have been performed using different types of NPs. Covering all of them is beyond the scope of this short review. Here, we present some examples on rodents and also several embryotoxicity studies in zebra fish, an animal model that has revolutionized initial screening for embryological toxicity studies due to the short developmental period and the easy observation of possible embryological defects in this animal. The biodistribution and potential toxic effects of AuNPs of different size (ranging from 1.5 to 70 nm in diameter) was studied in non-

pregnant and pregnant mice at different gestational ages (E5.5, 7.5, 9.5, 11.5, and 13.5). No apparent toxic effects were observed in pregnant mice⁴⁶. On the other hand, the possible embryotoxic effect of a single low (10 mg/kg) or high (100 mg/kg) dose of positively-charged polyethyleneimine-Fe₂O₃-NPs (PEI-NPs), or negatively-charged poly (acrylic acid)- Fe₂O₃-NPs (PAA-NPs) during critical windows of organogenesis (gestational day 8 to 10) was studied. While the low dose of NPs, regardless of charge, did not induce toxicity, the larger dose led to charge-dependent fetal loss as well as to morphological alterations in the uterus (caused by both positively and negatively charged NPs) and testes (only produced by positively charged NPs) of surviving offspring. In addition, positively-charged PEI-NPs given later in organogenesis resulted in a combination of short-term fetal loss (42%) and long-term alterations in reproduction, including increased fetal loss for second generation offspring (mice exposed in utero)⁴⁷. In addition, CdTe quantum dots enter and accumulate in mouse testis causing dose-dependent toxicity⁴⁸.

Recently, zebrafish embryos (*Danio rerio*) have been used for *in vivo* development security tests because they have several advantages over other species such as: i) it is possible to generate and maintain large numbers of them in an animal house due to its small size; ii) they have a quick embryonic development (72 hours post-fecundation) markedly shortening the times to identify potential effects on embryonic development; iii) they only need three months to reach sexual maturity; iv) they produce large amounts of eggs (300-500 per female); and v) the embryos are transparent allowing a real time study of the toxic effects as well as recording images of *in vivo* pathological phenotypes without requiring sacrifice of the animal. In addition, sequencing of the zebrafish genome has revealed a similarity of about 70% with humans suggesting that many of the molecular and cellular mechanisms relevant for pathogenesis of certain human diseases might be present in the zebrafish⁴⁹⁻⁵¹.

Embriotoxicity tests in zebrafish are performed according to the normalized protocol (OECD 236)⁵². The procedure is schematically presented in Figure 4. Newly fertilized zebrafish eggs are exposed to the xenobiotic for a period of 96 hours. Every 24 hours, the eggs are observed for indicators of lethality such as: i) coagulation of fertilized eggs, ii) lack of somite formation, iii) lack of detachment of the tail-bud from the yolk sac, and iv) lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on positive scores in any of the four observations recorded, and the LD50 is

469 calculated. Concerning substances that may be metabolically activated, there is evidence
470 that zebrafish embryos do have biotransformation capacities. However, the metabolic
471 capacity of embryonic fish is not always similar to that of juvenile or adult fish.
472 Therefore, if there are any indications that metabolites or other transformation products
473 may be more toxic than the parent compound, it is also recommended to perform the
474 test with those metabolites/transformation products and taking also into account those
475 results when drawing conclusions from the toxicity tests. Zebrafish embryos are not
476 expected to be sensitive to substances with a very bulky molecular structure or to
477 substances causing delayed hatch which might preclude or reduce the post-hatch
478 exposure due of limited bioavailability of the substance. Under these conditions, other
479 toxicity tests might be more appropriate.

480 Zebrafish is becoming a very popular model for NP toxicity testing. Toxicity of silica
481 nanoparticles with diameters 20, 50 and 80nm was evaluated using an *in vivo* zebrafish
482 platform that analyzes multiple endpoints related to developmental, cardio-, hepato-,
483 and neurotoxicity. Results showed that except for an acceleration in hatching time and
484 alterations in the behavior of zebrafish embryos/larvae, silica NPs did not elicit any
485 developmental defects, or any cardiac or hepatotoxicity⁵³. On the other hand,
486 embryotoxicity studies in zebrafish of Poly (ethylene glycol) methyl ether-block-poly
487 (lactide-co-glycolide) (mPEGPLGA) nanoparticles carrying acetyltanshinone IIA
488 (ATA), a novel anti-breast cancer agent, displayed much lower toxicity than free ATA
489⁵⁴.

490 PAMAM dendrimer embryotoxicity has been evaluated at 24 and 120 hours
491 postfecundation (hpf) on embryonic zebrafish mortality, development, and
492 malformations. Neutral thiophosphoryl dendrimers did not produce malformations at
493 any dose throughout the exposure. However, exposure to 50 ppm cationic PAMAM
494 dendrimers G3-amine, G4-amine, G5-amine, and G6-amine caused 100% mortality by
495 24 hpf. Cationic PAMAM G4-amine and neutral PAMAM G6-amidoethanol caused
496 significant cardiac effects (pericardial edema) at 10 ppm. However, negatively charged
497 succinamic acid G5 and G6 dendrimers did not show any significant adverse effects
498 even at the highest concentration tested. These results indicated that surface charge may
499 be the best indicator of dendrimer embryotoxicity. In addition, dendrimer class and
500 generation are other potential contributors to dendrimer embryotoxicity⁵⁵. One relevant
501 application of dendrimers is their use as drug carriers to improve water solubility of

some drugs to improve their bioavailability. In this direction, PAMAM dendrimer G4.5 (-COOH) was used as drug carrier for the antipsychotic drug risperidone and its toxicity tested on zebrafish by exposing embryos four days post fertilization (dpf) to increasing concentrations of risperidone and DG4.5-risperidone for 24h. The compounds produced a cellular disorganization in the raphe neuronal population being this effect larger in animals treated with free risperidone than those receiving the complex (DG4.5-risperidone). Therefore, in this case, the dendrimer decreased drug embryotoxicity ⁵⁶.

Embriotoxicity studies represent a key part of data required for a new drug approval. The regulatory requirements on this subject are rather strict and are covered by both International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) ⁴⁴. In this direction, nanoparticles, although specific regulatory requirements for them is under development, should be subjected to the same strict requirements as small drugs. Initial testing on possible embryotoxic effects has been significantly accelerated by the use of animal models such as zebrafish where developmental processes require little time as compared to mammals and where possible developmental defects can be easily observed. However, data obtained in fish, and also in rodents, on possible embryotoxic effects of nanoparticles (and small drugs) cannot exclude embryotoxicity in humans as thalidomide dramatically showed. Thus, a continuous pharmacovigilance surveillance should be maintained in the case that some nanoparticles reach a clinical setting.

522

523 2.5. Immunotoxicity studies

Xenobiotics might produce significant effects on the immune system. In general, cationic (positively charged) particles are more likely to induce inflammatory reactions than anionic (negatively charged) and neutral species ⁵⁷. Therefore, it is important to evaluate immunotoxicity caused by nanoparticles. According to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Safety Guideline (ICH S8) ⁵⁸ the evaluation of xenobiotic effects on immune system can be made in two stages: a) in the first stage, a general immunotoxicity study is performed to analyze different immune parameters. In this type of study, animals are not exposed to antigens and are not infected with bacteria or parasites, meaning that, at this stage, functional studies are not performed; and b) in a

534 second stage, and depending on the results of the first stage, more specific and
535 functional tests on immune system functionality can be performed.

536 General immunotoxicity tests have been developed to detect possible effects on the
537 immune system, and these tests analyze the following parameters: i) blood cells count
538 and formula; ii) plasma levels of IgM, IgG and IgA immunoglobulins; iii) weight and
539 histopathological study of main lymphoid organs and tissues (thymus, spleen, lymph
540 nodes, lymphoid tissue, and gut-associated lymphoid tissue); iv) bone-marrow
541 morphological examination; and v) flow cytometry or immunohistochemistry studies to
542 detect the possible variation of immune cells subpopulation. If the obtained results
543 suggest an effect on the immune system, a functional test to determine humoral
544 acquired immunity through colony-forming unit (CFU) analysis should be performed.
545 This test determines host ability to trigger antibodies reaction against some specific
546 antigens. This last process requires the coordinated action of several immunity cells
547 such as macrophages, T cells and B cells. Thus, any problem in these cells can have an
548 impact on B cells antibodies production ability. The in vivo CFU test is performed using
549 serum obtained from immunized mice⁵⁸

550 Nanoparticle effect on immune parameters might constitute a problem due to two
551 possible pathways: a) immune reaction induced by surface components of the
552 nanoparticles as it could be the case when nanoparticles are decorated with peptides to
553 facilitate targeting or biological barriers (i.e. blood brain barrier) crossing or, more
554 commonly, b) a direct effect of nanoparticles lacking peptide components on the
555 immune system as is the case for metal-based nanoparticles. In this case, both immune
556 system inhibition and a inflammatory response have been reported. For instance, silver
557 nanoparticles (Ag-NPs) administered using a 28-day i.v. repeated-dose administration
558 pattern, caused a strong reduction in NK cell activity, and reduced IFN- γ production
559 suggesting suppression of the functionality of the immune system⁵⁹. Similarly,
560 dextran-stabilized iron oxide nanoparticle showed significant suppression of
561 proliferation of cultured T-lymphocytes stimulated with the mitogen
562 phytohemagglutinin (PHA)⁶⁰. On the other hand, mesoporous nanoparticles (MPS NPs
563) induced an inflammatory response characterized by an increase in liver and spleen
564 weight and splenocyte proliferation in mice. In addition, mice treated with MPS NPs
565 showed altered lymphocyte populations (CD3(+), CD45(+), CD4(+), and CD8(+)) in
566 the spleen, and increased serum IgG and IgM levels⁶¹,

It is important to note that it is estimated that about 20 % of approved drugs withdrawal from market is due to immunological problems meaning either immune system blockade leading to deficient response to infection or immune stimulation leading to inflammation. Nanoparticles, as indicated above, reproduce such pattern in preclinical studies which indicates that nanoparticle immunotoxicity should be studied for nanoparticles aimed to reach clinical setting following the International Conference on Harmonization Safety Guidelines procedures⁵⁸. However, it should be kept in mind that it is possible that, due to the complexity of nanoparticles, when used as carriers for different drugs or genetic material, the actual guidelines might fall short to identify all immunotoxic effects of such as compounds since the final compound, aimed to be therapeutic, might incorporate the mixed effects of nanoparticle, active pharmacological component, and NP coating.

579

580 2. 6. Hematological studies.

For evaluation of xenobiotic toxic effects on blood tissue the following test are generally used: i) red blood cells count per blood volume (RBC); ii) hemoglobin concentration (Hb); iii) mean corpuscular hemoglobin (MCH) or hemoglobin amount in each red cell; iv) mean corpuscular hemoglobin concentration (MCHC) which is the hemoglobin concentration (not the amount) in each red cell; v) hematocrit value that measures the volume of red blood cells compared to total blood volume (red blood cells and plasma); vi) white blood cells count (WBC); vii) platelet count; viii) neutrophil count; ix) lymphocytes count; x) Coagulation tests such as prothrombin time and activated partial thromboplastin time; xi) reticulocytes count expressed in percentage respect to total mature red blood cells; and xii) complement activation that has been studied for PAMAM dendrimers (G5 PAMAM-NH₂; G5 PAMAM-OH; G4.5 PAMAM-COOH) showing that PAMAM dendrimers with amine, hydroxyl or carboxyl terminal groups at doses ranging from 0.001 to 0.1 mg/mL inhibited C3a production⁶².

An additional test, hemolysis test, has been widely used when studying preliminary toxicity of nanoparticles. In this test, blood is obtained from healthy rodents or humans and it is anticoagulated with sodium heparin. Erythrocytes are separated from blood plasma by centrifugation at 4°C, then washed and finally resuspended in physiological saline. The RBC suspension is incubated with different concentrations of the xenobiotic for 2 hours at 37°C under agitation. As negative control, RBC suspension is incubated

with physiological saline. As positive control the RBC suspension is incubated with Triton X-100 (10%, v/v) to obtain complete hemolysis. After the incubation period, the RBC suspension is centrifuged at $1,000\times g$ for 10 minutes. The amount of released hemoglobin in the supernatants is determined spectrophotometrically at 540 nm. The degree of hemolysis is determined by the following equation:

$$\% \text{ Hemolysis} = 100 \times (Abs - Abs_0) / (Abs_{100} - Abs_0)$$

Where Abs, Abs₀, and Abs₁₀₀ are the absorbances of test samples, the suspension treated with physiological saline, and the suspension treated with Triton X-100 (10%, v/v), respectively ⁶³.

Some NPs like polyethylenimine (PEI) enter systemic circulation and interact with various blood components such as RBC membrane structure and blood coagulation process ⁶⁴. On the other hand, graphene quantum dots did not modify standard hematological markers, such as white and red blood cell count, hematocrit, mean corpuscular volume, total hemoglobin, platelet count, and mean corpuscular hemoglobin indicating that chemical composition of the NOPs plays a key role in hematological toxicity ¹¹.

2.7. Biochemical parameters.

Biochemical parameters, both in urine and plasma, represent markers for internal organ dysfunction and so, represent early signs of possible xenobiotic toxicity. Generally, biochemical markers are grouped in profiles each one related to a major organ or pathological entity. The most relevant profiles used are the following

2.7.1 General profile

The plasma concentration of different electrolytes such as calcium, potassium, chloride, sodium as well as glucose concentration are determined ⁶⁵.

2.7.2 Lipid profile

This profile is related to identify a xenobiotic effect on lipid metabolism and related atherosclerosis. For that purpose, the levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, total lipids and triglycerides are determined in plasma ⁶⁶.

629 2.7.3 Renal profile

630 Urea and creatinine plasma levels are good markers for a decrease in glomerular
631 filtration rate (GFR). However, these markers only increase when there are GFR
632 reductions in the range of 50 to 70%. For this reason, blood urea nitrogen is usually
633 preferred as a marker for renal dysfunction⁶⁷. Changes in normal urine composition can
634 reveal toxic renal effects caused by a xenobiotic. The most commonly used urine
635 parameters to assess correct function of kidneys are: i) volume and osmolality; ii) the
636 presence of proteins like Beta 2-Microglobulin suggesting a possible problem in tubules
637 and albumin that might indicate a glomerular problem; and iii) glycosuria.

638 2.7.4 Hepatic profile

639 Alanine transaminase and aspartate transaminase are mainly distributed in liver cells,
640 and their levels rise during liver cells necrosis. Levels of these enzymes correlate very
641 well with the extent of liver cell damage, and are commonly used as indicators of liver
642 function⁶⁷. Iron oxide magnetic nanoparticles (IOMNPs) have a great potential in a
643 wide variety of biomedical applications such as contrast enhancement of magnetic
644 resonance imaging, targeted drug delivery or gene delivery and tissue engineering⁶⁸.
645 IOMNPs potential side-effects on liver, spleen and kidneys were evaluated in mice by
646 monitoring various serum biochemical markers. The results showed no obvious hepatic
647 and renal toxicity in the animals after IOMN treatment⁶⁹.

648 Biochemical parameters represent a good index for correct function of different internal
649 organs. Modification of these parameters by xenobiotics, including nanoparticles, are
650 good indicators of arising toxicity that appears, generally, well in advance of severe
651 organ damage. Thus, it is very important that this type of test will be performed during
652 preclinical testing of a nanoparticle. Since biochemical determinations of the different
653 profiles indicated above only involve determination of plasma levels of ions (calcium,
654 potassium, etc.) or chemical compounds (cholesterol, glucose, creatinine, etc.) or
655 enzyme activity (transaminases), the situation does not differ significantly from that for
656 small drugs and similar procedures might be followed.

657

658

659

660 2.8. Histopathological studies

661 If a possible damage pattern is observed by using one of the above methods,
662 histopathological studies of the affected organs should be performed by using optic and
663 electronic microscopy to determine the place and nature of the injuries^{49,70}.

664 Histopathological studies have been widely used to asses NP toxicity. Thus, a
665 bioconjugate between duplex oligodeoxynucleotides (dODNs) and a PAMAM-
666 succinamic acid dendrimer G4 used as a drug delivery system for the anticancer drug
667 doxorubicine showed no severe tissue histological changes⁷¹. Moreover, a G5 PAMAM
668 dendrimer, modified with folic acid to increase targeting to tumor cells, was injected
669 intravenously and heart, liver, spleen, lung, kidney, and brain tissues were collected and
670 analyzed with no sign of tissue damage observed⁷². In addition to dendrimers, other
671 NPs have been studied for their tissue toxicity. Thus, O-Carboxymethyl chitosan
672 encapsulated metformin (O-CMC) did not produce any structural alteration to organs
673 such as brain, lung, heart, liver, spleen and kidney⁷³. Moreover, citrate-or
674 polyvinylpyrrolidone-coated Ag NPs injected in mice did not cause any relevant change
675 in the examined organs⁷⁴.

676 In conclusion, detailed histopathological studies are not routinely performed to
677 determine nanoparticle toxicity. However, they must be performed whenever a possible
678 macroscopic organ damage or clinical symptoms suggesting organ involvement are
679 observed in order to identify the structural damage at cellular level. Generally,
680 histological damaged will be heralded by alterations in one or several of plasma
681 biochemical parameters indicated in section 2.7.

682

683 2.9. Behavioral studies

684 Many toxics can produce behavioral changes before histological damage can be
685 observed, so behavioural studies provide a very efficient screening test for xenobiotic
686 toxicity For this kind of studies, rodents, followed by apes, are generally chosen as the
687 animal species,. Irwin test is the most commonly used test to determine adverse
688 behavioural effects to a xenobiotic and to evaluate its possible neurotoxicity in rodents.
689 In this test, behavioral profile is evaluated determining several parameters: i) alert level
690 or stupor; ii) motor activity; iii) central excitation to determine neurological profile; iv)
691 motor incoordination; v) muscle tone; vi) reflexes and; vii) autonomic profile though

692 ocular signs, secretions and general signs such as piloerection, hypothermia or colour
693 skin ^{75,76}.

694 Behavioral studies have been performed in mice for different NPs including
695 recombinant human serum albumin with incorporated hydrophilic (NH₄)₂Ce (IV)
696 (NO₃)₆·γFe₂O₃ particles for medical imaging . without adverse side effects suggesting a
697 possible use as MRI contrasting agent ⁷⁷. Lipid nanocapsules have been also tested for
698 behavioral abnormalities in mice without signs of toxicity ⁷⁸.

699

700 3. Conclusions

701 It is generally accepted that nanoparticle-based medicinal products will play a relevant
702 role in the therapeutic armamentarium in a near future. In fact, several nanoparticle-
703 based medicines including liposomes, lipid suspensions or nanocrystals have been
704 already authorized by the European Union to reach the market under the present
705 regulatory framework. The inexistence of specific guides for nanoparticle toxicity
706 testing makes that nanoparticles are subject to the same regulatory processes as all
707 other therapeutics. However, nanoparticles represent a clear challenge since they are
708 much more complex than small drugs and , so, more specialized tests and experts to
709 perform them will be likely needed as it has been already recognized by the European
710 Medicine Agency (EMA).

711 The different procedures described in this review allow to obtain valuable information
712 about the possible toxic effects *in vivo* for a xenobiotic, including nanoparticles, with a
713 possible therapeutic application under current regulatory rules. However, a negative
714 result in these toxic tests do not provide absolute certainty that xenobiotic-induced
715 human toxicity will not appear in the future when a more wide use of the compound in
716 therapeutics will occur. For this reason, it is important to understand the key role that
717 this kind studies play to minimize the possible risks for patients once the compound has
718 been approved for therapeutic use.

719

720

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725 **Figure legends**

726

727 **Figure 1. Types of nanoparticles.**

728 **Figure 2: General scheme of the pyrogenic test.** See the text for explanation.

729 **Figure 3: General scheme of in vivo chromosomal aberrations assay.** See the text
730 for explanation.

731 **Figure 4: In vivo Zebrafish embryotoxicity studies.**

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References

1. Shanks, N.; Greek, R.; Greek, J. Are animal models predictive for humans? *Philos. Ethics Humanit. Med.* **2009**, *4*, 2.
2. Fielden, M. R.; Kolaja, K. L. The role of early in vivo toxicity testing in drug discovery toxicology. *Expert. Opin. Drug Saf.* **2008**, *7* (2), 107-110.
3. OECD 423 . OECD Guideline For Testing Of Chemicals. Acute Oral Toxicity - Acute Toxic Class Method. Acute oral toxicity. 2001. 2001.
4. Tan, L.; Tang, W.; Liu, T.; Ren, X.; Fu, C.; Liu, B.; Ren, J.; Meng, X. Biocompatible Hollow Polydopamine Nanoparticles Loaded Ionic Liquid Enhanced Tumor Microwave Thermal Ablation in Vivo. *ACS Appl. Mater. Interfaces.* **2016**, *8* (18), 11237-11245.
5. Ljubimova, J. Y.; Portilla-Arias, J.; Patil, R.; Ding, H.; Inoue, S.; Markman, J. L.; Rekechenetskiy, A.; Konda, B.; Gangalum, P. R.; Chesnokova, A.; Ljubimov, A. V.; Black, K. L.; Holler, E. Toxicity and efficacy evaluation of multiple targeted polymalic acid conjugates for triple-negative breast cancer treatment. *J. Drug Target* **2013**, *21* (10), 956-967.
6. Thiagarajan, G.; Greish, K.; Ghandehari, H. Charge affects the oral toxicity of poly(amidoamine) dendrimers. *Eur. J. Pharm. Biopharm.* **2013**, *84* (2), 330-334.
7. Wu, L. P.; Ficker, M.; Christensen, J. B.; Trohopoulos, P. N.; Moghimi, S. M. Dendrimers in Medicine: Therapeutic Concepts and Pharmaceutical Challenges. *Bioconjug. Chem.* **2015**, *26* (7), 1198-1211.
8. Xue, X.; Chen, X.; Mao, X.; Hou, Z.; Zhou, Y.; Bai, H.; Meng, J.; Da, F.; Sang, G.; Wang, Y.; Luo, X. Amino-terminated generation 2 poly(amidoamine) dendrimer as a potential broad-spectrum, nonresistance-inducing antibacterial agent. *AAPS. J.* **2013**, *15* (1), 132-142.
9. OECD 408 . OECD Guideline For Testing of Chemicals. Repeated Dose 90-Day Oral Toxicity Study in Rodents. **1998**.
10. Liao, L.; Zhang, M.; Liu, H.; Zhang, X.; Xie, Z.; Zhang, Z.; Gong, T.; Sun, X. Subchronic toxicity and immunotoxicity of MeO-PEG-poly(D,L-lactic-co-glycolic acid)-PEG-OMe triblock copolymer nanoparticles delivered intravenously into rats. *Nanotechnology.* **2014**, *25* (24), 245705.
11. Chong, Y.; Ma, Y.; Shen, H.; Tu, X.; Zhou, X.; Xu, J.; Dai, J.; Fan, S.; Zhang, Z. The in vitro and in vivo toxicity of graphene quantum dots. *Biomaterials* **2014**, *35* (19), 5041-5048.

- 771 12. Garcia, T.; Lafuente, D.; Blanco, J.; Sanchez, D. J.; Sirvent, J. J.; Domingo, J. L.;
772 Gomez, M. Oral subchronic exposure to silver nanoparticles in rats. *Food*
773 *Chem. Toxicol.* **2016**, *92*, 177-187.
- 774 13. OECD 453 . OECD Guideline for testing Chemicals. Combined chronic
775 toxicity/carcinogenicity studies. **1981**.
776
- 777 14. Wen, R.; Yang, X.; Hu, L.; Sun, C.; Zhou, Q.; Jiang, G. Brain-targeted
778 distribution and high retention of silver by chronic intranasal instillation of
779 silver nanoparticles and ions in Sprague-Dawley rats. *J. Appl. Toxicol.*
780 **2016**, *36* (3), 445-453.
- 781 15. Fasehee, H.; Dinarvand, R.; Ghavamzadeh, A.; Esfandiyari-Manesh, M.;
782 Moradian, H.; Faghihi, S.; Ghaffari, S. H. Delivery of disulfiram into
783 breast cancer cells using folate-receptor-targeted PLGA-PEG
784 nanoparticles: in vitro and in vivo investigations. *J. Nanobiotechnology.*
785 **2016**, *14*, 32.
- 786 16. Hall, J. B.; Dobrovolskaia, M. A.; Patri, A. K.; McNeil, S. E. Characterization of
787 nanoparticles for therapeutics. *Nanomedicine. (Lond)* **2007**, *2* (6), 789-803.
- 788 17. Dobrovolskaia, M. A.; Neun, B. W.; Clogston, J. D.; Grossman, J. H.; McNeil, S.
789 E. Choice of method for endotoxin detection depends on nanoformulation.
790 *Nanomedicine (Lond)* **2014**, *9* (12), 1847-1856.
- 791 18. Dobrovolskaia, M. A.; Neun, B. W.; Clogston, J. D.; Ding, H.; Ljubimova, J.;
792 McNeil, S. E. Ambiguities in applying traditional Limulus amoebocyte
793 lysate tests to quantify endotoxin in nanoparticle formulations.
794 *Nanomedicine. (Lond)* **2010**, *5* (4), 555-562.
- 795 19. USP29 . 151 Pyrogen test. **2010**.
796
- 797 20. Kang, S. H.; Kwon, J. Y.; Lee, J. K.; Seo, Y. R. Recent advances in in vivo
798 genotoxicity testing: prediction of carcinogenic potential using comet and
799 micronucleus assay in animal models. *J. Cancer Prev.* **2013**, *18* (4), 277-
800 288.
- 801 21. Kwon, J. Y.; Lee, S. Y.; Koedrith, P.; Lee, J. Y.; Kim, K. M.; Oh, J. M.; Yang, S.
802 I.; Kim, M. K.; Lee, J. K.; Jeong, J.; Maeng, E. H.; Lee, B. J.; Seo, Y. R.
803 Lack of genotoxic potential of ZnO nanoparticles in in vitro and in vivo
804 tests. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2014**, *761*, 1-9.
- 805 22. International Conference on Harmonisation of Technical Requirements for
806 Registration of Pharmaceuticals for Human Use (ICH) . Genotoxicity
807 Testing and Data Interpretation for Pharmaceuticals Intended for Human
808 Use (S2R1). **2012**.
809

- 810 23. Magdolenova, Z.; Collins, A.; Kumar, A.; Dhawan, A.; Stone, V.; Dusinska, M.
811 Mechanisms of genotoxicity. A review of in vitro and in vivo studies with
812 engineered nanoparticles. *Nanotoxicology*. **2014**, 8 (3), 233-278.
- 813 24. Li, K.; Du, S.; Van, G. S.; Chen, Y. Atomic force microscopy study of the
814 interaction of DNA and nanoparticles. *Adv. Exp. Med. Biol.* **2014**, 811, 93-
815 109.
- 816 25. Kwon, J. Y.; Koedrith, P.; Seo, Y. R. Current investigations into the genotoxicity
817 of zinc oxide and silica nanoparticles in mammalian models in vitro and in
818 vivo: carcinogenic/genotoxic potential, relevant mechanisms and
819 biomarkers, artifacts, and limitations. *Int. J. Nanomedicine*. **2014**, 9 Suppl
820 2, 271-286.
- 821 26. Baweja, L.; Gurbani, D.; Shanker, R.; Pandey, A. K.; Subramanian, V.; Dhawan,
822 A. C60-fullerene binds with the ATP binding domain of human DNA
823 topoisomerase II alpha. *J. Biomed. Nanotechnol.* **2011**, 7 (1), 177-178.
- 824 27. Manke, A.; Wang, L.; Rojanasakul, Y. Mechanisms of nanoparticle-induced
825 oxidative stress and toxicity. *Biomed. Res. Int.* **2013**, 2013, 942916.
- 826 28. Annangi, B.; Rubio, L.; Alaraby, M.; Bach, J.; Marcos, R.; Hernandez, A. Acute
827 and long-term in vitro effects of zinc oxide nanoparticles. *Arch. Toxicol.*
828 **2016**, 90 (9), 2201-2213.
- 829 29. OECD 475 . OECD Guideline For Testing of Chemicals. Mammalian Bone
830 Marrow Chromosomal Aberration Test. **1997**.
831
- 832 30. Ghosh, M.; Sinha, S.; Jothiramajayam, M.; Jana, A.; Nag, A.; Mukherjee, A.
833 Cyto-genotoxicity and oxidative stress induced by zinc oxide nanoparticle
834 in human lymphocyte cells in vitro and Swiss albino male mice in vivo.
835 *Food Chem. Toxicol.* **2016**, 97, 286-296.
- 836 31. Schmid, W. The micronucleus test. *Mutat. Res.* **1975**, 31 (1), 9-15.
- 837 32. Kumar, A.; Dhawan, A. Genotoxic and carcinogenic potential of engineered
838 nanoparticles: an update. *Arch. Toxicol.* **2013**, 87 (11), 1883-1900.
- 839 33. OECD 474 . OECD Guideline for Testing of Chemicals. Mammalian Erythrocyte
840 Micronucleus Test. **2016**.
841
- 842 34. Naya, M.; Kobayashi, N.; Mizuno, K.; Matsumoto, K.; Ema, M.; Nakanishi, J.
843 Evaluation of the genotoxic potential of single-wall carbon nanotubes by
844 using a battery of in vitro and in vivo genotoxicity assays. *Regul. Toxicol.*
845 *Pharmacol.* **2011**, 61 (2), 192-198.
- 846 35. Schulz, M.; Ma-Hock, L.; Brill, S.; Strauss, V.; Treumann, S.; Groters, S.; van, R.
847 B.; Landsiedel, R. Investigation on the genotoxicity of different sizes of

- 848 gold nanoparticles administered to the lungs of rats. *Mutat. Res.* **2012**, 745
849 (1-2), 51-57.
- 850 36. Xia, Q.; Li, H.; Liu, Y.; Zhang, S.; Feng, Q.; Xiao, K. The effect of particle size
851 on the genotoxicity of gold nanoparticles. *J. Biomed. Mater. Res. A* **2017**,
852 105 (3), 710-719.
- 853 37. Karlsson, H. L. The comet assay in nanotoxicology research. *Anal. Bioanal.*
854 *Chem.* **2010**, 398 (2), 651-666.
- 855 38. OECD 489 . OECD Guideline for Testing Chemicals. In Vivo Mammalian
856 Alkaline Comet Assay. **2016**.
- 857
- 858 39. Gleis, M.; Schneider, T.; Schlormann, W. Comet assay: an essential tool in
859 toxicological research. *Arch. Toxicol.* **2016**, 90 (10), 2315-2336.
- 860 40. Ghosh, M.; J M; Sinha, S.; Chakraborty, A.; Mallick, S. K.; Bandyopadhyay, M.;
861 Mukherjee, A. In vitro and in vivo genotoxicity of silver nanoparticles.
862 *Mutat. Res.* **2012**, 749 (1-2), 60-69.
- 863 41. Kumari, M.; Kumari, S. I.; Grover, P. Genotoxicity analysis of cerium oxide
864 micro and nanoparticles in Wistar rats after 28 days of repeated oral
865 administration. *Mutagenesis* **2014**, 29 (6), 467-479.
- 866 42. OECD 451 . OECD Guideline for testing Chemicals. Carcinogenicity Studies.
867 **2009**.
- 868
- 869 43. Sun, X.; Cai, W.; Chen, X. Positron emission tomography imaging using
870 radiolabeled inorganic nanomaterials. *Acc. Chem. Res.* **2015**, 48 (2), 286-
871 294.
- 872 44. International Conference on Harmonisation of Technical Requirements for
873 Registration of Pharmaceuticals for Human Use (ICH) . Detection of
874 Toxicity to Reproduction for Medicinal products & toxicity to male
875 fertility S5(R2). **2005**.
- 876
- 877 45. OECD 422 . OECD Guideline for testing Chemicals. Combined Repeated Dose
878 Toxicity Study with the Reproduction/Developmental Toxicity Screening
879 Test. **2016**.
- 880
- 881 46. Yang, H.; Du, L.; Tian, X.; Fan, Z.; Sun, C.; Liu, Y.; Keelan, J. A.; Nie, G. Effects
882 of nanoparticle size and gestational age on maternal biodistribution and
883 toxicity of gold nanoparticles in pregnant mice. *Toxicol. Lett.* **2014**, 230
884 (1), 10-18.
- 885 47. Di Bona, K. R.; Xu, Y.; Gray, M.; Fair, D.; Hayles, H.; Milad, L.; Montes, A.;
886 Sherwood, J.; Bao, Y.; Rasco, J. F. Short- and Long-Term Effects of
887 Prenatal Exposure to Iron Oxide Nanoparticles: Influence of Surface

- 888 Charge and Dose on Developmental and Reproductive Toxicity. *Int. J.*
889 *Mol. Sci.* **2015**, *16* (12), 30251-30268.
- 890 48. Li, X.; Yang, X.; Yuwen, L.; Yang, W.; Weng, L.; Teng, Z.; Wang, L. Evaluation
891 of toxic effects of CdTe quantum dots on the reproductive system in adult
892 male mice. *Biomaterials* **2016**, *96*, 24-32.
- 893 49. Wang, M.; Wang, J.; Sun, H.; Han, S.; Feng, S.; Shi, L.; Meng, P.; Li, J.; Huang,
894 P.; Sun, Z. Time-dependent toxicity of cadmium telluride quantum dots on
895 liver and kidneys in mice: histopathological changes with elevated free
896 cadmium ions and hydroxyl radicals. *Int. J. Nanomedicine*. **2016**, *11*,
897 2319-2328.
- 898 50. Asmonaite, G.; Boyer, S.; Souza, K. B.; Wassmur, B.; Sturve, J. Behavioural
899 toxicity assessment of silver ions and nanoparticles on zebrafish using a
900 locomotion profiling approach. *Aquat. Toxicol.* **2016**, *173*, 143-153.
- 901 51. Ramachandran, R.; Krishnaraj, C.; Sivakumar, A. S.; Prasannakumar, P.; Abhay
902 Kumar, V. K.; Shim, K. S.; Song, C. G.; Yun, S. I. Anticancer activity of
903 biologically synthesized silver and gold nanoparticles on mouse myoblast
904 cancer cells and their toxicity against embryonic zebrafish. *Mater. Sci. Eng*
905 *C. Mater. Biol. Appl.* **2017**, *73*, 674-683.
- 906 52. OECD 236 . OECD Guideline for testing Chemicals. Fish Embryo Acute Toxicity
907 (FET) Test. **2013**.
- 908
- 909 53. Pham, D. H.; De, R. B.; Nguyen, X. B.; Vervaele, M.; Kecskes, A.; Ny, A.;
910 Copmans, D.; Vriens, H.; Locquet, J. P.; Hoet, P.; de Witte, P. A. Use of
911 Zebrafish Larvae as a Multi-Endpoint Platform to Characterize the
912 Toxicity Profile of Silica Nanoparticles. *Sci. Rep.* **2016**, *6*, 37145.
- 913 54. Wang, Q.; Wei, N.; Liu, X.; Chang, A.; Qian, L. K. Enhancement of the
914 bioavailability of a novel anticancer compound (acetyltanshinone IIA) by
915 encapsulation within mPEG-PLGA nanoparticles: a study of formulation
916 optimization, toxicity, and pharmacokinetics. *Oncotarget*. **2017**. *8*(7),
917 12013-12030.
- 918 55. Pryor, J. B.; Harper, B. J.; Harper, S. L. Comparative toxicological assessment of
919 PAMAM and thiophosphoryl dendrimers using embryonic zebrafish. *Int.*
920 *J. Nanomedicine*. **2014**, *9*, 1947-1956.
- 921 56. Prieto, M. J.; del Rio Zabala, N. E.; Marotta, C. H.; Carreno, G. H.; Arevalo, A.
922 R.; Chiaramoni, N. S.; del Valle, A. S. Optimization and in vivo toxicity
923 evaluation of G4.5 PAMAM dendrimer-risperidone complexes. *PLoS.*
924 *ONE*. **2014**, *9* (2), e90393.
- 925 57. Ayyappan, J. P.; Sami, H.; Rajalekshmi, D. C.; Sivakumar, S.; Abraham, A.
926 Immunocompatibility and toxicity studies of poly-L-lysine nanocapsules in
927 sprague-dawley rats for drug-delivery applications. *Chem. Biol. Drug Des*
928 **2014**, *84* (3), 292-299.

- 929 58. International Conference on Harmonisation of Technical Requirements for
930 Registration of Pharmaceuticals for Human Use (ICH) . Immunotoxicity
931 studies for Human Pharmaceuticals (S8). **2005**.
932
- 933 59. Vandebriel, R. J.; Tonk, E. C.; de la Fonteyne-Blankestijn LJ; Gremmer, E. R.;
934 Verharen, H. W.; van der Ven, L. T.; van, L. H.; de Jong, W. H.
935 Immunotoxicity of silver nanoparticles in an intravenous 28-day repeated-
936 dose toxicity study in rats. *Part Fibre. Toxicol.* **2014**, *11*, 21.
- 937 60. Easo, S. L.; Mohanan, P. V. In vitro hematological and in vivo immunotoxicity
938 assessment of dextran stabilized iron oxide nanoparticles. *Colloids Surf. B*
939 *Biointerfaces.* **2015**, *134*, 122-130.
- 940 61. Lee, S.; Kim, M. S.; Lee, D.; Kwon, T. K.; Khang, D.; Yun, H. S.; Kim, S. H. The
941 comparative immunotoxicity of mesoporous silica nanoparticles and
942 colloidal silica nanoparticles in mice. *Int. J. Nanomedicine* **2013**, *8*, 147-
943 158.
- 944 62. Lin, J.; Hua, W.; Zhang, Y.; Li, C.; Xue, W.; Yin, J.; Liu, Z.; Qiu, X. Effect of
945 poly(amidoamine) dendrimers on the structure and activity of immune
946 molecules. *Biochim. Biophys. Acta* **2015**, *1850* (2), 419-425.
- 947 63. Zhou, Y.; Li, J.; Lu, F.; Deng, J.; Zhang, J.; Fang, P.; Peng, X.; Zhou, S. F. A
948 study on the hemocompatibility of dendronized chitosan derivatives in red
949 blood cells. *Drug Des Devel. Ther.* **2015**, *9*, 2635-2645.
- 950 64. Zhong, D.; Jiao, Y.; Zhang, Y.; Zhang, W.; Li, N.; Zuo, Q.; Wang, Q.; Xue, W.;
951 Liu, Z. Effects of the gene carrier polyethyleneimines on structure and
952 function of blood components. *Biomaterials* **2013**, *34* (1), 294-305.
- 953 65. Siddique, M. I.; Katas, H.; Amin, M. C.; Ng, S. F.; Zulfakar, M. H.; Jamil, A. In-
954 vivo dermal pharmacokinetics, efficacy, and safety of skin targeting
955 nanoparticles for corticosteroid treatment of atopic dermatitis. *Int. J.*
956 *Pharm.* **2016**, *507* (1-2), 72-82.
- 957 66. Kanakia, S.; Toussaint, J. D.; Mullick, C. S.; Tembulkar, T.; Lee, S.; Jiang, Y. P.;
958 Lin, R. Z.; Shroyer, K. R.; Moore, W.; Sitharaman, B. Dose ranging,
959 expanded acute toxicity and safety pharmacology studies for intravenously
960 administered functionalized graphene nanoparticle formulations.
961 *Biomaterials* **2014**, *35* (25), 7022-7031.
- 962 67. Zhang, X. D.; Wu, D.; Shen, X.; Liu, P. X.; Yang, N.; Zhao, B.; Zhang, H.; Sun,
963 Y. M.; Zhang, L. A.; Fan, F. Y. Size-dependent in vivo toxicity of PEG-
964 coated gold nanoparticles. *Int. J. Nanomedicine.* **2011**, *6*, 2071-2081.
- 965 68. Ling, D.; Lee, N.; Hyeon, T. Chemical synthesis and assembly of uniformly sized
966 iron oxide nanoparticles for medical applications. *Acc. Chem. Res.* **2015**,
967 *48* (5), 1276-1285.

- 968 69. Yang, L.; Kuang, H.; Zhang, W.; Aguilar, Z. P.; Xiong, Y.; Lai, W.; Xu, H.; Wei,
969 H. Size dependent biodistribution and toxicokinetics of iron oxide
970 magnetic nanoparticles in mice. *Nanoscale*. **2015**, 7 (2), 625-636.
- 971 70. Greaves P. Histopathology of preclinical toxicity studies: interpretation and
972 relevance in drug safety evaluation. Fourth Edition. Elsevier. Amsterdam.
973 **2012**.
974
- 975 71. Lee, I. H.; Yu, M. K.; Kim, I. H.; Lee, J. H.; Park, T. G.; Jon, S. A duplex
976 oligodeoxynucleotide-dendrimer bioconjugate as a novel delivery vehicle
977 for doxorubicin in in vivo cancer therapy. *J. Control Release* **2011**, 155
978 (1), 88-95.
- 979 72. Xu, X.; Li, J.; Han, S.; Tao, C.; Fang, L.; Sun, Y.; Zhu, J.; Liang, Z.; Li, F. A
980 novel doxorubicin loaded folic acid conjugated PAMAM modified with
981 borneol, a nature dual-functional product of reducing PAMAM toxicity
982 and boosting BBB penetration. *Eur. J. Pharm. Sci.* **2016**, 88, 178-190.
- 983 73. Snima, K. S.; Jayakumar, R.; Lakshmanan, V. K. In vitro and in vivo biological
984 evaluation of O-carboxymethyl chitosan encapsulated metformin
985 nanoparticles for pancreatic cancer therapy. *Pharm. Res.* **2014**, 31 (12),
986 3361-3370.
- 987 74. Recordati, C.; De, M. M.; Bianchessi, S.; Argenti, S.; Cella, C.; Mattiello, S.;
988 Cubadda, F.; Aureli, F.; D'Amato, M.; Raggi, A.; Lenardi, C.; Milani, P.;
989 Scanziani, E. Tissue distribution and acute toxicity of silver after single
990 intravenous administration in mice: nano-specific and size-dependent
991 effects. *Part Fibre. Toxicol.* **2016**, 13, 12.
- 992 75. Venkatasubbu, G. D.; Ramasamy, S.; Gaddam, P. R.; Kumar, J. Acute and
993 subchronic toxicity analysis of surface modified paclitaxel attached
994 hydroxyapatite and titanium dioxide nanoparticles. *Int. J. Nanomedicine*.
995 **2015**, 10 Suppl 1, 137-148.
- 996 76. Irwin S. Comprehensive observational assessment: Ia. A systematic, quantitative
997 procedure for assessing the behavioral and physiologic state of the mouse.
998 *Psychopharmacologia*. **1968**, 13(3), 222-257.
999
- 1000 77. Rosenberger, I.; Schmithals, C.; Vandooren, J.; Bianchessi, S.; Milani, P.;
1001 Locatelli, E.; Israel, L. L.; Hubner, F.; Matteoli, M.; Lellouche, J. P.;
1002 Franchini, M. C.; Passoni, L.; Scanziani, E.; Opdenakker, G.; Piiper, A.;
1003 Kreuter, J. Physico-chemical and toxicological characterization of iron-
1004 containing albumin nanoparticles as platforms for medical imaging. *J.*
1005 *Control Release* **2014**, 194, 130-137.
- 1006 78. Hureauux, J.; Lagarce, F.; Gagnadoux, F.; Rousselet, M. C.; Moal, V.; Urban, T.;
1007 Benoit, J. P. Toxicological study and efficacy of blank and paclitaxel-
1008 loaded lipid nanocapsules after i.v. administration in mice. *Pharm. Res.*
1009 **2010**, 27 (3), 421-430.

Figure 1

Figure 2

Figure 3

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Figure 4

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