An early developmental vertebrate model to assess nanomaterial safety: Bridging cell-based and mammalian nanoparticle toxicity assessment.

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Abstract

Nanomaterials represent one of the most rapidly growing areas for drug development and as such nanotherapeutics are being produced for an ever-increasing number of diseases. With this rise however, comes an urgent need to efficiently and effectively assess the toxicity of new nanomaterials in biological systems. To address this need, we have developed an integrated tool for pre-clinical nanotoxicity assessment. Specifically we propose a nanoparticle hazard assessment protocol, combining mammalian cytotoxicity data with embryonic vertebrate abnormality scoring (using frog embryos), to determine an overall toxicity index as a pre-clinical nanotoxicity assessment tool. To validate this tool we have phenotypically anchored low-toxicity scoring to mammalian models, to demonstrate how the protocol could be used as a predictive tool for nanomaterial safety in a pre-clinical setting. Overall, we have shown the potential of Xenopus analysis to be integrated in a fast screening approach for nanotoxicity assessment, which could be introduced as a standardized protocol for the routine testing of candidate nanomaterials in biomedical applications in the future. Furthermore, this robust nanotoxicity assessment protocol could be applied early-on in the development of novel nanomedicines to reduce the dependency on laborious and slow mammalian testing as a screening tool. Thus, only formulations with low risk-to-benefit ratios will continue through to testing in rodent models.

KEYWORDS. Nanotoxicity, nanomaterials, *Xenopus laevis*, nanomedicine, cytotoxicity, *in vitro-in vivo* correlations.

1. Introduction

Objects at nanoscale (<100 nm in length), take on novel characteristics due to their small size and extensive surface area, providing markedly different properties to develop reagents for biomedicine. As such, nanomaterials are being developed for an ever-increasing number of diseases including; infectious diseases, hepatitis, cardiac and vascular disorders, immune and degenerative diseases, and cancer [1]. However, their high surface area to volume ratio renders nanoparticles (NPs) highly-reactive, which can result in toxicity within biological systems [1-3]. Currently a dedicated regulatory framework for testing nanomedicines does not yet exist and cell-based assays are the existing method of choice despite providing poor *in vitro-in vivo* correlations of nanomaterial toxicity [4]. The increase in number of publications in nanomedicine as well as in nanotoxicology has been exponential in the past 5-10 years [5, 6], but it is often difficult to obtain conclusive results on the toxicity of engineered nanomaterials both because of poor physical chemical characterization of the material and a distinct lack of standardized protocols [7], which is seriously hampering the transfer of many promising nanomedicines to the clinic. Given the ever-increasing number of examples in the literature of engineered nanomaterials designed for biomedical applications, it is also expected that the number of *in vivo* studies in mammals necessary to test their toxicity will also rise concomitantly.

Safety assessment is a necessary step in the process to develop a novel nanomedicine and should involve further refinement at early stages in NP development to guarantee appropriate safety. Learning from the development of first generation nanomedicines that have been used in clinical procedures for years [8] and that have been tested using the standard procedures for the evaluation of all drugs (http://www.ich.org) it is desirable to develop methods that are tailored to evaluating

specific toxicities associated with nanomaterials. Recently, much effort from the nano-community has been directed to optimize protocols that assess the safety of nanomaterials. This concept is known as ‘safety by design’ [5] and is particularly true for the production of nanomedicines, for which engineering at the nanoscale can cause unpredictable biological responses [9]. A compromise has to be met in order to produce nanomaterials with the desired properties (which for targeted treatments are often multifaceted, complex formulations), with acceptable toxicity and thus there is a need for robust screening methodologies that evaluate the toxicity of nanomedicines at an early stage in their development. Currently the field requires a system that can bridge the safety assessment of NPs in cell-based assays with the reliable data generated from mammalian *in vivo* toxicity assessment, to provide stronger correlations between the two toxicological models at early stages in the development of novel nanomaterials.

A pre-clinical screening tool for testing the toxicity of nanomedicines will enormously reduce the number needed to be tested in mammals, while also improving the success rates of novel nanomaterials to pass mammalian toxicity assessment models. In fact, most of the new generation nanomedicines are multi-component systems with complex chemical structures joining the properties of different materials in one unique engineered object, often involving the use of non-FDA approved materials and therefore there is a clear need to test the toxicity of the engineered nano-object at different stages of its production. Thus, one consequence of generating multi-component NPs for biomedical purposes is the potential of having to use a larger number of mammals for safety assessment than for the development of conventional drugs. It would advantageous to be able to test NP toxicity using a system that can bridge the gap in consistency between data generated from *in vitro*

cell based assays and mammalian models of toxicity assessment [10, 11]. Early developmental, vertebrate models such as *Danio rerio* (zebrafish) and *Xenopus laevis* (the African clawed frog) provide rapid and relatively inexpensive systems for NP toxicity assessment, compared to adult mammalian models [10, 12-14], *X. laevis* produces embryos in large quantities (adaptable for high-throughput screening) that are relatively large (approximately 1 mm in diameter even at the earliest stages of embryonic development), making them highly suitable *in vivo* systems for the assessment of toxicants. Indeed *X. laevis* has a long-running history in toxicity testing, such as the frog teratogenesis assay-*Xenopus* (FETAX), which provides high prediction of teratogens in mammals [15]. Further advantages of using *X. laevis* as an *in vivo* toxicity model include external development of the embryo to allow for temporal control over the exposure to NPs, facilitating investigation into toxic effects at critical stages of embryogenesis, such as gastrulation and neuralation. Furthermore, the *X. laevis* developmental stages are well characterized making it an ideal model for phenotypic assays [16, 17]. The sensitivity of embryos during development can be exploited to identify varying degrees of toxicity by a range of phenotypic abnormalities and give an indication into safe dosages, information that historically is not accurately known before assessment in mammalian models.

In this study we propose to use *X. laevis* embryos in combination with *in vitro* testing in mammalian cell lines to provide a multi-tiered, rapid and low cost approach to assess NP toxicity prior to *in vivo* mammalian testing. We have developed a methodology that assesses the toxicity of nanomaterials at an early stage in their production, before further assessment for translational applications (Fig. 1). The methodology combines a full physico-chemical characterization of the nanomaterial

in the biological environment with quick and inexpensive *in vitro* cytotoxicity assays, and analysis in *X. laevis* embryos at different developmental stages, prior to mammalian testing. This has been tested using four different NPs; CdSe NPs (QDs), pegylated magnetite NPs (Fe_3O_4-PEG), amine-functionalized magnetite NPs (Fe_3O_4-NH_2), and fluorescent carboxylated polystyrene NPs (PS-COOH), selected for: a) a putative highly toxic NP as a positive control (QDs); b) two iron-oxide NPs (possible MRI contrast agents), with different surface functionalization and physical chemical stability to test the ability of the protocol to detect possible related toxicity effects [18, 19]; and c) a standard negative control NP (PS-COOH) [20], being part of the first group of materials to be evaluated for safety at the nanoscale.

2. Materials and methods

NP sources and synthesis. NH_2-Fe_3O_4 NPs were purchased from Chemicell Gmbh (Berlin, Germany) (FluidMAG-Amine), stated as size of 50 nm (hydrodynamic diameter). PS-COOH (FluoSphere fluorescent carboxylated-modified microspheres yellow-green; λ_{ex}, 505 nm; λ_{em}, 515 nm) were purchased from Molecular Probes (Eugene, OR) at 20 nm. CdSe QDs were synthesized by microwave heating an aqueous solution of 0.1 M cadmium perchlorate with 0.01 M N, N-dimethyl selenourea in the presence of a stabilizer, 0.1% (w/v) sodium citrate as previously described [21]. This resulted in uncoated 10:1 CdSe QDs. Monodispersed Fe_3O_4 were synthesized by high-temperature phase reaction of Fe(acac)3 (2 mmol), 1,2-tetradecanediol (10 mmol), benzyl ether (20 mL), in the presence of oleic acid (2 mmol), and oleylamine (2 mmol) as described by Sun et al., [22]. The resulting hydrophobic Fe_3O_4 underwent a phase transfer by addition of an amphiphilic polymer, Poly(maleic anhydride -alt -1- octadecene) (PMAO), to the surface of the NP [23]

before PEGylation with Jeffamine M-1000 polyetheramine. PEG-Fe_3O_4 were purified by dialyzing against PBS pH 7.4, (10 mM NaCl) 0.31 osmol using a dialysis membrane with Mw cut-off (MWCO) of 100 kDa. Further purification was achieved by ultracentrifugation through a 2 - 66% sucrose gradient as described previously described [23], before a final dialysis with MWCO of 2 kDa. All particles were sonicated before use.

NP Characterization. The concentration of in-house NPs was detected by ICP-AES analysis of Fe. Hydrodynamic diameter, size distribution, and ζ-potential of each NP was analyzed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Core NP size was visualized using Tecnai 20 transmission electron microscope (TEM) with AMT cameras, operating at an accelerating voltage of 200 kV. NPs were dried on carbon-coated 300–mesh copper grids (Agar Scientific) and counterstained with 2 % uranyl acetate. Representative images were analyzed using Fuji Image J software and 100 of each NP were used for statistical analysis of particle size.

Cell Culture. The melanoma SK-MEL-28 cell line was a kind gift from Prof. A. Chien & Prof. R. T. Moon, University of Washington, Seattle. The immortalized kidney MDCK cell line was a kind gift from M. Mogensen, University of East Anglia, Norwich and the lung carcinoma A549 cell line was a kind gift from D. Sexton, Liverpool John Moores University, Liverpool. All lines used were regularly confirmed as mycoplasma free using a standard protocol [24, 25]. Cells were sub-cultured as previously described [26]. All cells were incubated in 37°C, 5% CO_2/95% air. A549 and the SK-MEL-28 cell lines were cultured in RPMI-1640 (Life Technologies, Carlsbad, CA) and the MDCK cell line cultured in DMEM (Life

Technologies, Carlsbad, CA). Both media types were supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, all purchased Life Technologies, Carlsbad, CA. Cells were sub-cultured when they reached 70-80 % confluency and were never cultured beyond passage 30.

Trypan blue exclusion assay. A549 and SK-MEL-28 were seeded at 26550 cells per ml and MDCK at 20000 cells per ml in a 24 well-plate for trypan blue exclusion assay. After attachment overnight, cells were exposed to different concentrations of NPs for 72 h. Experiments were done in a minimum of triplicates.

MTT assay. To assess cell viability after exposure to NPs at a range of concentrations the MTT assay was performed [27]. Cells were seeded in 96-well plates at an initial density of 4500 cells/well and allowed to attach overnight before exposure to NPs for 72 h . Percentage viability of the cells was calculated as a ratio of mean absorbance from quadruplicate readings with respect to the control wells of non-stimulated cells. Experiments were repeated a minimum of 3 times.

Immunoblotting. Cells were lysed in 50 mM Tris-HCl (pH 7.5), 1 % triton X-100, 150 mM NaCl, and spun at 16000 x g for 30 min at 4^{o}C. Immunoblotting was performed as previously described [28] with 1:1000 Anti-PARP-1 (Santa Cruz Biotechnology) and 1:5000 Anti-Rabbit (Cell Signaling), in 5% BSA.

***X. laevis* phenotypic assay.** All experiments were performed in compliance with the relevant laws and institutional guidelines at the University of East Anglia. The

research has been approved by the local ethical review committee according to UK Home Office regulations. *X. laevis* embryos were obtained as previously described [29, 30]. Embryos were staged using the Nieuwkoop and Faber (NF) normal table of *Xenopus* development [31]. Briefly, embryos were left to develop at 18^{o}C until they reached the required stage. Dead embryos were removed. Embryos at the required stage were placed in a 24-well plate with varying concentrations of NPs at 5 embryos/well and incubated at 18^{o}C. Once at the required stage embryos were washed twice with PBS before fixing in MEMFA (3.7% formaldehyde 1 x MEM salts, MQW [1:1:8]) overnight at 4^{o}C. A further two PBS washes of the embryos was performed before embryos were scored for phenotypic abnormalities.

***X.laevis* whole mount florescence microscopy.** NF stage 38 *X. laevis* embryos were exposed to 10^{15} NP/ml PS-COOH until NF stage 45 and anaesthetized with 0.6 mg/ml ethyl 3–aminobenzoate methanesulfonate salt (Sigma Aldrich). PS-COOH particles were imaged in the bloodstream of anaesthetized embryos using Nikon Eclipse 600 with a CCD digital camera using an emission filter of 509–547 nm**.**

***X.laevis* TEM imaging.** Embryos exposed to $10^{15.3}$ NP/ml PEG-Fe_3O_4 during NF stage 38 to NF stage 45 were euthanized, fixed, embedded, and sectioned as previously described [32]. Sections were observed using a Tecnai 20 TEM with AMT cameras, operating at an acceleration voltage of 200 kV.

Murine Procedures. Experiments involving mice and their care were conducted in conformity with the institutional guidelines at the IRCCS — Institute for Pharmacological Research “Mario Negri” in compliance with national (Decreto

Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 8th edition, 2011). This work has been reviewed by IRCCS-IRFMN Animal Care and Use Committee (IACUC) and then approved by the Italian "Istituto Superiore di Sanità" (code: 17/01 D Appl 3). Fifteen 3-month-old NFR mice (females) were bred and maintained under pathogen-free conditions in the Institute's Animal Care Facilities. Mice received food and water *ad libitum* and were regularly checked by a certified veterinarian who is responsible for animal welfare supervision, and experimental protocol revision. The animals were randomly divided in three experimental groups as follows: PBS-treated mice (n = 3), Fe_3O_4-NH_2-NP (n = 6) and Fe_3O_4-PEG-NP-treated mice (n = 6). Both PBS and the solution containing NPs were injected as a single dose in the tail vein (125 μg Fe_3O_4/mL solution, 150 μL).

Magnetic Resonance Imaging (MRI). For all animals, MRI analysis was carried out before NP or PBS injection. MRI experiments were performed on a 7 T/30 cm horizontal bore magnet (Bruker-Biospin, Ettlingen, Germany) equipped with a 12-cm gradient set capable of supplying up to 400 mT/m. T2w images were obtained using a spin-echo MRI sequence (TR/TE = 4000/36 ms, 78 μm2 in-plane resolution, FOV 2x2 cm3, matrix size =256x256, slice thickness=1 mm). During MRI, the animals were anesthetized by breathing a mixture of 1% isoflurane, 30% O_2, 70% NO_2. The animal temperature was kept at 36 +/-1°C using a warmed cradle. To verify the possible NPs accumulation in liver and kidneys the scanning was focused on the peritoneal region. Serial coronal sections were generated and Regions of interest (ROIs) were manually defined to determine the degree of intensity of the signal with

ImageJ software (http://rsbweb.nih.gov/ij/). The signal from dorsal muscles (a region in which NP do not accumulate) was utilized to ensure that the level of signal was homogenous for all scanning. To measure the mean level of intensity for each mouse, the grey value of four different squares (having the same area) were considered for each sections (three serial sections for each organ were processed). Grey levels from liver and kidneys were also measured by using the same approach. Three animals for each group were enrolled, MRI was performed 24 hr and 120 hr after NP (or PBS) administration. All animals were sacrificed with an overdose of ketamine (75 mg/kg)-medetotimine (1mg/kg) one hour after the last MRI scan.

Immuohistochemistry. Liver and kidneys were collected from NP, and control treated animals following MRI analysis. The organs were rapidly post-fixed in 4% paraformaldehyde for 24 hours and samples were processed for histology and paraffin embedded using routine protocols. 5 μm sections were obtained from paraffin blocks and stained with hematoxylin and eosin (HE) for light microscopic examination. To highlight iron accumulation, Perl's staining was performed in seriate microtomic sections of kidney and liver following the manufacturer's instructions (Diapath Perls staining Kit).

Statistical analysis. MTT and Trypan blue data expressed with ± S.E.M, all other data expressed as mean ± S.D. The mean values from vehicle-treated mice were normalized to 1. Student's t-test was used to compare the grey values between PBS- and NP-treated mice (at the two different time-points) in kidneys and liver. *P*-values of ≤ 0.05 were considered significant. All statistical analyses were done using the GraphPad Prism version 6.00 for Windows (Graph-Pad Software, San Diego, CA, USA).

3. Results

Nanoparticle Synthesis and Characterization. Both QD and Fe_3O_4-PEG NPs were synthesized and purified in-house as explained in the material and methods section, while PS-COOH and Fe_3O_4-NH_2 NPs were bought from specific suppliers and further characterized. NP characterization is an essential first step in our protocol design (Fig. 1).

TEM and mean core sizes for the NPs are reported in Table 1, while statistical analysis of the size together with representative images are shown in Fig. S1 in the Supplementary data section. NPs were also characterized by DLS and ζ-potential in aqueous dispersions except for the Fe_3O_4-PEG NPs that were dispersed in PBS, where hydrodynamic diameters were generally larger than TEM core sizes and where hydrodynamic sizes for QD and Fe_3O_4-NH_2 NPs indicated the formation of NP clusters to some extent (Fig. S2). NP stability and aggregation behaviors were studied in the embryo (0.1X MMR) and cell growth media (RPMI+10% FBS) over 72 hours, where the results at the starting time for the highest concentration measured are reported in Table 1. PS-COOH and Fe_3O_4-PEG NPs were stable, whilst QD and Fe_3O_4-NH_2 showed extensive aggregation at this concentration, however aggregation was less extensive at lower concentrations for both NPs, so we studied the biological response of all the chosen NPs. NP hydrodynamic sizes in the different environments were monitored over the experimental time for biological analysis and remained consistent as shown in Fig. S2.

Cytotoxicity assessment analysis. We used a diverse array of cell types for cytotoxicity analysis (A549, SK-MEL-28 and MDCK cells), which were exposed to varying concentrations of NPs over 72 hours. After NP stimulation, trypan blue

exclusion assay, MTT assay, and protein expression of full and cleaved poly (ADP-ribose) polymerase-1 (PARP-1) were performed to evaluate cytotoxic effects. Fig. 2 shows the cytotoxic effects the NP panel in the three cell types as a function of exposure concentration expressed as NP/ml (for clarity analogue graphs are reported versus concentration with different metrics [w/v and surface area/v] shown in Fig. S3, with representative images of the treated cells at the highest concentration of NP treatment shown in Fig. S4. QDs, as expected, were highly toxic in all cell types for concentrations $>10^{14}$ NP/ml, which corresponds to a weight concentration of about 0.1 μg/ml. The MTT results showed that exposure to the highest concentrations of Fe_3O_4-NH_2 and PS-COOH NPs on MDCK resulted in a 40% reduction in cell viability when compared to the other lines, whilst trypan blue results also showed reduced cell viability for the highest concentration of Fe_3O_4-NH_2. PARP-1, activated by the production of reactive oxygen species (ROS), is a substrate for active cysteinyl-aspartate protease cleavage and is a marker of apoptotic cell death [33]. Previous studies have suggested an increase in ROS production after exposure to nanomaterials [34-36]. We investigated PARP-1 cleavage in the cells in response to NP treatment and found that only QDs induced apoptosis in cells at a comparable concentration to that of the cytotoxic drug, cisplatin, whilst no cleaved PARP-1 was observed in cells incubated with the other NPs (Fig. 2c). Taken together these *in vitro* findings show that the QDs induced high levels of cytotoxicity in mammalian cells compared to the other NPs tested.

***X. laevis* as a multiparametric nanotoxicity assessment tool.** *X. laevis* embryos at different stages in their development were exposed to varying concentrations of NPs and morphological scoring of an array of phenotypic abnormalities was carried out

(Fig. 3a). Gastrulation occurs at an early phase of vertebrate development (occurring in *X. laevis* at NF stage 10) and is a highly sensitive time to environmental changes during embryonic development, so to assess NP effects during this phase of development, embryos were exposed to the NPs at NF stage 4 and left to develop until NF stage 38 (Fig. 3c). Another critical developmental process is neurulation, which occurs before organogenesis and gives rise to precursor tissues of the spinal cord, brain and neural crest. To assess NP effects during neurulation and before organogenesis, embryos were exposed to the NPs at NF stage 15 and left to develop until NF stage 38 (Fig. 3d). Finally, for *in vivo* embryonic systems that can represent an adult model, embryos were exposed to NPs at tadpole stages during organogenesis, specifically at NF stage 38 through to stage 45 (Fig. 3b and e). QDs showed severe toxicity in *X. laevis* embryos at all stages, which is in good agreement with the literature [37], and the observed cytotoxicity data (Fig. 2). The other NPs showed limited toxicity, with the Fe_3O_4-NH_2 and PS-COOH NPs only showing toxicity effects in the embryos at higher concentrations, whilst the Fe_3O_4-PEG NPs, synthesized in our laboratory as potential theranostic NPs, showed no toxicity at all the stages before and after organogenesis, also in accordance with our *in vitro* findings.

We wanted to check that the NPs exhibiting lower toxicity in *X. laevis* embryos (iron oxide NPs and PS-COOH NPs) were taken up into the embryos following exposure. Embryo uptake by the iron oxide NPs and PS-COOH NPs was monitored by TEM imaging of embryo sections at NF stage 45 (Fig. S5) and fluorescence microscopy (FM) of live embryos at NF stage 45 (Fig. S6), respectively. TEM analysis demonstrated the presence of the NPs in the embryo tissues for both iron oxide NPs (Fig. S5). FM of intact embryos treated with PS-COOH NPs permitted us to detect their presence within the embryos and NP uptake seemed to be proportional

to the NP concentrations as shown by FM images of treated embryos at different NP doses (Fig. S6). Moreover, we made videos (Movie) of anesthetized embryos where PS-COOH NPs (or NP agglomerates) have been observed moving through the inter-somatic blood vessels of developing embryos from which time frame stills over 0.70 s have been extracted and are shown in Fig. S6b. These data show that the NPs used in this study are readily taken up by the *X. laevis* embryos.

Comparison of cytotoxicity and X. *laevis* data for hazard ranking of NPs. We developed a logical ranking system for comparing cytotoxicity and *X. laevis* results and to provide an overall hazard score from each assay for the investigated NPs (Table 2a). The hazard score from the cytotoxicity assays was conducted on the results obtained from the cytotoxicity data for one chosen NP concentration (the highest one); whilst for the *X. laevis* analysis we only took into account the results generated from the tadpole stage analysis (Fig. 3b - NF 38-45; when the embryo has fully formed organs and therefore the evaluation of nanotoxicity is more physiologically relevant to adult tissues). The obtained hazard indices are reported in Table 2a, which evaluate if there is a correlation between the *in vitro* and *in vivo* results. The assays fully agreed on the evaluation of the toxicity for the highly toxic QDs that scored severe effects in all assays, as well as for the two iron oxide NPs that showed no toxicity. However, PS-COOH NPs did not affect cell viability by the *in vitro* assays (Fig. 2), yet exhibited severe toxicity effects at high concentrations and moderate toxicity effects at lower concentrations (Fig. 3). This suggests that the introduction of a *X. laevis* sampling test early on in NP development could help in identifying false negatives produced by cell-based assays.

Assessment of NP safety in a mammalian system. In our protocol (Fig. 1) if developmental defects are observed in the *X. laevis* model, the NP will not move on to further assessment, rather only those nanoformulations that give no-to-low toxicity will progress to evaluation in small rodent models (in our data this was the two iron oxide core NPs; Fig. 3 and Table 2). To validate the low-to-no toxic prediction of the iron oxide NPs, we administered these two NPs in mice to test for possible toxicity effects on the tissues where they accumulated (the target organs), which could be easily assessed by MRI analysis and showed that in T2 weighed MRI coronal slices from epigastric and mesogastric regions, both iron oxide NPs accumulated in the liver and kidneys of the NP-treated animals compared to vehicle-treated animals (Fig. 4a and 4b). Importantly, post-mortem histological sections from the tissues where these NPs accumulated (the liver and kidneys) demonstrated not only that the iron distribution (as assessed by Perl's staining) corroborated the MRI findings, but that there was no gross morphological tissue toxicity observed (Fig. 4c). These findings suggest that *X. laevis* embryos are a useful tool to predict NP safety prior to administration and further testing in mammalian models.

4. Discussion

By 2015 nanotechnology has been predicted to become a market worth a $1 trillion [38], highlighting the rapid development of nanomaterials for a range of uses including biomedical applications. With this growth comes an ever increasing need to effectively assess how these nano-sized therapeutics can effect biological systems and in particular systemic toxicity mechanisms. As such there has emerged a need for models that can produce effective toxicity data as a transition between 2D *in vitro* cell culture assays and expensive, often time-consuming *in vivo* mammalian models. Here we have bridged this gap using an early vertebrate developmental model as an

assessment tool of NP safety, for use early on in nanotherapeutic design and production.

This need has been recognized by identifying that *in vitro* and *in vivo* nanotoxicity data can often be conflicting, highlighting a need to bridge this gap, whilst also recognising a need to reduce the number of animals used for *in vivo* toxicity testing. One approach has been to develop 3D and co-culture *in vitro* cell-based models that better represent the organization of tissues compared to conventional 2D monocultures [39]. These organotypic cell cultures offer models that more closely resemble the *in vivo* condition, better mimicking the biological behavior of cells in tissues. Several groups have been using such organotypic cell culture techniques for nanomaterial toxicity assessment, based on a variety of tissue types [40-44]. However, these tissue mimetic models are not without their limitations too, not least of all because they fail to recapitulate the complete systemic interactions present between organ systems *in vivo*, as they tend to focus on the development of individual, tissue-specific models. To overcome this we are proposing an alternative approach, using early vertebrate developmental models that can provide a complete *in vivo* model system, but with the added major advantage of being a rapid toxicity assessment tool thus reducing time and costs, whilst still remaining a relevant model for nanotoxicity. Specifically we have proposed the use of *X. laevis* as a readily available laboratory model to develop such a nanotoxicity screening tool (Fig. 1), although the protocol does not need to be limited to the exclusive use of *Xenopus* embryos, but rather can be easily extended to other commonly used developmental models such as the zebrafish model, which has also been used for NP toxicity testing [10, 45-48]. The specific advantages of *Xenopus* as a model include, permeable skin, that they are tetrapods (therefore developing lungs and limbs), possessing all

additional organs/tissues most commonly affected in human diseases (skin, sensory organs, lymphatic, nervous systems, kidneys, gastrointestinal tract, cardiovascular, hepatic and circulatory systems), they are the highest evolutionary order providing free-living embryos to permit screening in multi-well formats, and in earlier life stages prior to the onset of independent feeding (before NF 45) they are described under the EU directive as not protected as non-human vertebrate models, thus making them highly amenable for toxicity testing procedures, as previously reviewed [17].

In this work we have established an integrated, fast screening methodology to assess NP toxicity with the added advantage of reducing the requirement for large numbers of nanomaterials to be tested in mammalian models. This screening methodology envisages a standardized experimental protocol (Fig. 1) to be routinely applied to candidate nanomaterials for biomedical applications. This approach produces a reliable score of the nanomaterial toxicity (Table 2). However, we do not consider this methodology a replacement of the toxicity testing on mammalian models, but rather a robust screening procedure to use early on in nanotherapy development to refine and reduce the number of nanomaterials to be tested in rodent models.

In this study we chose four putative NPs that spanned a variety of materials and presented different toxicity indices to test the suitability of this as a nanotoxicity protocol. Fe_3O_4-NH_2 NPs were found, according to our methodology, as having low-to-no-toxicity and further tested in mice with no evidence of any major acute toxicity effect. For the most part we observed good agreement between *in vitro* and *in vivo* analysis for the nanomaterials tested. The reputed toxic QD NPs and the iron oxide core NPs were ranked as toxic and low-to-no-toxicity, respectively, which is in agreement with data reported by others for similar NPs [49, 50]

It is imperative that the physicochemical properties of the NPs are rigorously tested in the relevant biological fluids prior to toxicity assessment, so that the behaviour of the NPs are well understood before proceeding to testing, which is a crucial step that we have incorporated into our protocol (Fig. 1). This allows us to analyse the behaviour of specific NP types in relevant medias, prior to application to the biological system enabling us to infer whether or not toxicity profiles are in fact dependent on the nanomaterial itself or potential aggregation of the NPs. For example, the high salt concentration in the *X. laevis* embryo MMR buffer, strongly affected the stability of electrostatically stabilized NPs such as QD and Fe_3O_4-NH_2 (Table 1), whilst the presence of proteins with further formation of protein-NP complexes [51] reduces aggregation in such NPs, suggesting that once *in vivo*, following initial exposure to aggregating NPs, this aggregation may be reduced in biological fluids.

Another crucial step in our protocol is the comparison between the *in vitro* cytotoxicity data and the *in vivo* findings from the *Xenopus* studies (Fig. 1). A significant dichotomy in the ranking of the PS-COOH NPs was found between *in vitro* and *in vivo* results. Specifically, cytotoxicity was not detected in the investigated concentration range by any of the assays used, while mortality and abnormalities were scored for the embryos at the higher PS-COOH NP concentrations, thus highlighting the importance of a fully integrated cell line and *X. laevis* approach to accurately assess nanomaterial safety and prevent the identification of false negatives. The physical characterization of PS-COOH NPs in the biological fluids did not show any agglomeration effects that could promote toxicity. However, there is experimental evidence in the literature of toxicity effects due to carboxylic surface functionalization of polystyrene NPs is related to interaction with the blood plasma proteins. Clancy et

al., have shown that both carboxylated polystyrene fluorospheres and QDs had great propensity to aggregate in blood vessels of diameter <60 μm in chicken embryo chorioallantoic membrane, while analogue NPs with no carboxylic groups did not form aggregates [52]. Recently, Oslakovic et al. [53] reported on the ability of carboxylated polystyrene NPs to activate the intrinsic pathway of coagulation in plasma, in contrast to amine-modified NPs. In our case, FM on live embryos (Movie) has confirmed the presence of high concentrations of PS-COOH NPs in the *X. laevis* tissues. However, further investigations and advanced mathematical analysis (beyond the scope of this present study) are required to relate the observed toxicity to possible aggregation events.

Moreover, another toxicity aspect was tested as the two chosen iron oxide NPs, bearing specific surface functionalizations, showed different stability in the biological fluids mostly at the highest concentration, where the Fe_3O_4-NH_2 NPs aggregated extensively. This concentration-dependent agglomeration was more pronounced in the MMR buffer than in cell growth media, promoting mortality of the embryos at the highest Fe_3O_4-NH_2 NP concentration, whereas no toxicity was observed for the lower concentrations at tadpole stages in the embryos (NF 38-45). This indicates that such a toxic effect was potentially related to the colloidal instability of the NPs rather than to the material composition of the NP itself. Instead, *in vitro* analysis provided mixed results depending on the cell line and of the assay that was used. Some toxicity was detected in the trypan blue exclusion assay at the highest concentration, which was not readily detectable in the MTT assay (Fig. 2a and b). Of note, PARP-1 cleavage assay (Fig. 2c) showed unambiguously no apoptosis for all the treated cell lines at the highest NP concentration, highlighting that numerous cytotoxicity assays are required in combination to effectively assess NP toxicity.

For the *X. laevis* studies, phenotypic abnormalities were scored and used to assess the NP effect on the development of 30 embryos at all three of the selected stages. Common abnormalities recorded included; edema, blistering, eye deformities, loss of melanocytes, tail loss, stunted growth, axial defects such as bent spines, degradation of tissue, and developmental delay (Fig. 3a and b). Based on these abnormalities embryos were scored collectively as non-abnormal, abnormal, or dead. This facilitated simplified scoring of the embryos as having no abnormalities, abnormalities (any of those listed above) or as dead, allowing us to easily and rapidly assess NP-mediated toxicity in *Xenopus* embryos at differing developmental stages (Fig. 3c-e).

We found that embryos exposed to PS-COOH NPs showed some toxicity at all stages at the higher concentrations, in particular at the earliest stage (NF 4-38) where toxicity was observed from concentrations $>10^{14}$ NP/ml. Embryos exposed to Fe_3O_4-NH_2 NPs showed toxicity at the earliest stage from concentrations $>10^{14}$ NP/ml, while only at the highest NP concentration was 100% mortality observed at the other two stages of development. The severe toxicity observed at the highest concentration of Fe_3O_4-NH_2 NPs was related to the extensive aggregation of these NPs in the MMR medium, which resulted in a large precipitation that blanketed the embryos at high concentrations. However, at the latest stage (NF 38-45), for which the embryo has developed most of the organs, no toxicity was observed at all the other concentrations for Fe_3O_4-NH_2 NPs. Generally, we can state that where the embryos developed in the presence of NPs, NF stage 4 to NF stage 38 showed more phenotypic abnormalities and death at lower NP concentrations than embryos exposed at NF stages 15 and 38. It is of note that between NF 38-45 the gills and mouth are open providing, in addition to the porous skin, possible respiratory and oral tract routes of exposure as well [54].

NPs were more toxic to embryos in early development (4-38 and 15-38 NF stages, Fig. 3), in particular at the earliest stage, although it is difficult to say if this is connected to the different phase of development, or due to the longer exposure time to the NPs in these animals. Whilst the first two stages might not be adaptable as prognostic model for toxicity in mammals, they can be used to evaluate teratogenesis, for example to predict specific toxicity effects on pregnant women. A study on *X. laevis* embryos, in which NPs were microinjected between NF stage 1 and 3, has shown that nanodiamond (ND) NPs with carboxylic surface groups promoted teratogenesis at both gastrulation and neurulation stages [55]. Other studies on *X. laevis* of metal oxide NPs (CuO, ZnO, etc.) have evaluated teratogenesis using the FETAX assay and reported major toxicity effects in the digestive system [13, 32, 56]. This demonstrates that early vertebrate models not only represent cheap, fast, high-throughput systems to screen nanomaterials at the different stages of their development (safety by design) for evaluating potential toxicity effects, but can also be used to investigate the mechanisms inducing this toxicity. In fact, the use of advanced confocal and electronic microscopy techniques allows tracking of the NPs *in vivo* highlighting eventual lesions to specific organs and providing important biodistribution data about novel nanomaterials. Importantly, we were able to use these techniques to demonstrate that the embryos had indeed taken up the iron core and PS-COOH NPs tested. The severe developmental abnormalities noted for the QD NPs, suggested that these NPs are also readily taken up by the *X. laevis* embryos to induce such toxic effects.

Our protocol predicts that the combined cytotoxicity and *in vivo Xenopus* scores will facilitate optimal prediction for toxicity in mammalian systems (Fig. 1). Both iron core NPs gave a low hazard ranking for the cell culture and *Xenopus*

embryo assessment protocols (Table 2.b). For this reason we decided to evaluate both NPs in rodent models to confirm low toxicity. The other two NPs tested (PS-COOH and QD NPs) caused toxicity *in vivo* and therefore through our protocol (Fig. 1) would not progress to rodent model testing.

We chose to intravenously inject the iron core NPs into mice and monitor where they accumulated in the animals using MRI to firstly identify those organs exposed to the NP treatment. MRI measurements were performed after 24 hr and 120 hr post-administration. This analysis showed that 24 hours post NP administration, a marked lowering of brightness in the liver occurred (Fig. 4). This reduction was observed for both NPs, even if it appears stronger in Fe_3O_4-NH_2 NP-treated mice. This trend was similar for all mice and statistical analyses of grey values confirmed this qualitative evaluation. Conversely, the overall signal from kidneys did not show significant differences between NP and vehicle-treated mice 24 hr after i.v. administration. Quantitative measurements confirmed this suggestion, as no significant differences were found between the three experimental groups for the kidneys at this time-point (Fig. 4b). The hypo-intensity of signal, due to the presence of iron oxide NPs in the parenchyma of the kidneys and liver, was still present in both groups of NP-treated mice five days following NP administration. The quantification of grey values confirmed the MRI scanning and suggests a prolonged permanence of NPs in liver.

We used histochemical staining of the liver and kidneys from these mice to monitor iron distribution (which was consistent with the MRI results), but crucially also to monitor potentially histopathologies that may have been induced by prolonged NP exposure to these organs. Positive Perl's staining was evident in liver of both Fe_3O_4-PEG and Fe_3O_4-NH_2 treated animals, which was detected as a cytoplasmic granular blue staining of scattered interstitial cells. In renal tissue of both Fe_3O_4-PEG and

Fe_3O_4-NH_2 treated animals, positive Perl's staining was multi-focally evident as a finely granular blue staining within tubular epithelial cells (Fig. 4c). In vehicle-treated animals, positive Perl's staining in the liver was restricted to occasional single scattered interstitial cells and within kidney parenchyma as finely granular intracytoplasmic staining of scattered convolute tubules. Neither tissue presented with gross histopathological defects, suggesting there was no cytotoxic effects of the iron core NPs in either of the NP exposed organs. Crucially these results validate our findings from the integrated cell line and *X. laevis* toxicity assessment protocol suggesting the iron core NPs are non-toxic in mammalian models. Thus, our integrated approach in identifying non-toxic NPs for biomedical applications was validated by i.v administration of the iron oxide NPs in mice, which demonstrated that these NPs are well tolerated and cleared by filter organs.

5. Conclusions

We have developed a powerful integrative tool for screening nanomaterials that can work as bridge between *in vitro* and *in vivo* analysis for nanotoxicity. To do this we have a used a strategy that incorporates cytotoxicity analysis in a panel of mammalian lines coupled with the scoring of developmental abnormalities in *X. laevis* embryos. Our data demonstrate the flexibility of this multimodal pre-clinical assessment tool in the hazard assessment of nanomaterials, during the early stages of their production for biomedical applications. As such, functional modifications can be made to nanotherapeutics with an aim to enhance their efficacy, which can be quickly analyzed using the protocol developed here so that any potential nanotoxicity effects identified, can be addressed at an early stage in development of the nanotherapy, prior to mammalian testing. This allows the rapid and cheap identification of possible NP-

induced toxicities that may not be detectable through conventional cytotoxicity assays at an early stage of the development of nanotherapeutics.

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