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In vivo nanotoxicity testing using the zebrafish embryo assay

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Nanoparticles are increasingly used for biomedical purposes. Many different diagnostic and therapeutic applications are envisioned for nanoparticles, but there are often also serious concerns regarding their safety. Given the fact that numerous new nanomaterials are being developed every day, and that not much is known about the long-term toxicological impact of exposure to nanoparticles, there is an urgent need to establish efficient methods for nanotoxicity testing. The zebrafish (*Danio rerio*) embryo assay has recently emerged as an interesting 'intermediate' method for *in vivo* nanotoxicity screening, enabling (semi-) high-throughput analyses in a system significantly more complex than cultured cells, but at the same time also less 'invasive' and less expensive than large-scale biocompatibility studies in mice or rats. The zebrafish embryo assay is relatively well-established in the environmental sciences, but has not yet gained wide notice in the nanomedicine field. Using prototypic polymeric drug carriers, gold-based nanodiagnostics and nanotherapeutics, and iron oxide-based nanodiagnostics, we here show that toxicity testing using zebrafish embryos is easy, efficient and informative, and faithfully reflects, yet significantly extends, cell-based toxicity testing. We therefore expect that the zebrafish embryo assay will become a popular future tool for *in vivo* nanotoxicity screening.

Introduction

Nanomaterials hold significant potential for biomedical applications.[1,2] Proper assessment of the toxicological aspects of nanomaterials is extremely important, especially if intending to administer them to patients.[3,4] *In vitro* cytotoxicity testing in cell lines and/or isolated primary human cells (*e.g.* macrophages), as well as comparative analyses *versus* established or clinically approved reference materials, is generally implemented to obtain initial information on nanoparticle toxicity. As opposed to large scale *in vivo* analyses, which are laborious, expensive and to some extent also unethical, *in vitro* nanotoxicity testing is rapid, simple and cheap. It does suffer, however, from several obvious shortcomings, such as over-simplified systems and setups, moderately informative and conclusive results, and limited translational values. In addition to this, the *in vitro–in vivo* correlation (IVIVC) of nanoparticle toxicity in cells *vs.* animals (and patients) is known to be at best moderate, since the *in vivo* situation is known to be much more complex. Factors such as route of administration, biodistribution, biodegradability, long-term disposition, induction of developmental defects, and activation of the compliment and/or immune system all are major issues in determining *in vivo* nanotoxicity, and cannot be properly addressed using *in vitro* experimental setups.

To enable simple, efficient and high-throughput toxicity testing in systems significantly more complex than cultured cells, and at the same time to reduce the potential suffering of 'higher' organisms, such as mice or rats, *in vivo* experiments involving *Danio rerio* zebrafish (and their embryos) are becoming increasingly popular.[5,6] The zebrafish embryo assay is relatively well-established in the environmental sciences, to assess both the acute toxic effects and the 'long-term' developmental defects resulting from exposure to environmental chemicals and (nano-) particles. In the nanomedicine and drug delivery field, however, in which toxicity is a highly important issue, this assay has not yet gained much attention. As outlined in Fig. 1, there are several reasons why this assay might be

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highly useful for 'intermediate' nanomedicine toxicity testing, after completing initial experiments in cells, and before turning to *in vivo* experiments in rodents.

In the present study, the toxicity of three different classes of commercially available and/or previously extensively characterized nanomedicine materials was evaluated in cells and zebrafish embryos. Charged and neutral polymers, routinely employed for gene and drug delivery, were used in the initial set of experiments, followed by experiments involving gold and iron oxide nanoparticles (Fig. 2). Polymers included the well-known cationic transfection agents pEI (*i.e.* poly(ethylene imine)) and pDMAEMA (*i.e.* poly(2-(dimethylamino) ethylmethacrylate)),[7–9] as well as the uncharged macromolecular drug carrier material pHPMA (*i.e.* poly(*N*-2-hydroxypropyl) methacrylamide), which has been extensively used for improving the delivery of chemotherapeutic agents to tumors.[10–12] Gold nanoparticles encompassed the well-tolerated computed tomography (CT) contrast agent Aurovist (1.9 nm), as well as slightly smaller triphenylphosphane monosulfonate (TPPMS)-capped gold nanoparticles (Au1.4MS; 1.4 nm), previously shown to be highly efficient in killing (cancer) cells.[13,14] Finally, three different ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles were evaluated, including standard uncoated USPIO, flavin mononucleotide-coated fluorescent USPIO (*i.e.* FLUSPIO), and the dextran- and sodium-citrate-coated formulation Sinerem, which are clinically used for iron replacement therapy in anemia.[15–18] USPIO are widely used in nanodiagnostics, and can be used for liver and macrophage imaging, as well as for lymph node and (stem) cell tracking. In addition, they have also been more and more used for drug delivery and theranostic purposes.[19,20]

Due to the ever-increasing interest in using such polymer- and particle-based nanomaterials for biomedical purposes, tools to properly evaluate their *in vitro* and *in vivo* biocompatibility are highly needed. We here show that *in vivo* nanotoxicity testing using the zebrafish embryo assay is easy, efficient and

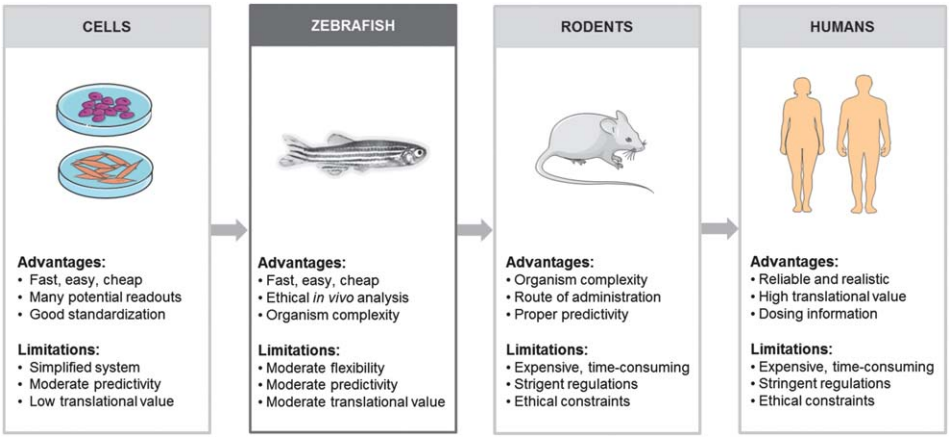

Fig. 1 Suggested workflow for translational toxicity testing of nanomedicine formulations. In general, toxicity studies commence with *in vitro* experiments in several different cells lines, at several different nanoparticle concentrations. Before going into patients, the material is then tested in several animal models, in particular in mice and rats. Before going into such 'higher' animal models, acute and chronic toxicity testing can be performed – at relatively high-throughput – using the zebrafish embryo assay. Assessing both acute and chronic effects in fish embryos which might be somewhat less informative than standard *in vivo* nanotoxicity screening, but it is relatively easy, more economic and arguably also more ethical. Consequently, we expect this *in vivo* nanotoxicity testing tool to become increasingly popular in the nanomedicine field. Figures are partially based on Servier Medical Art (http://www.servier.com).

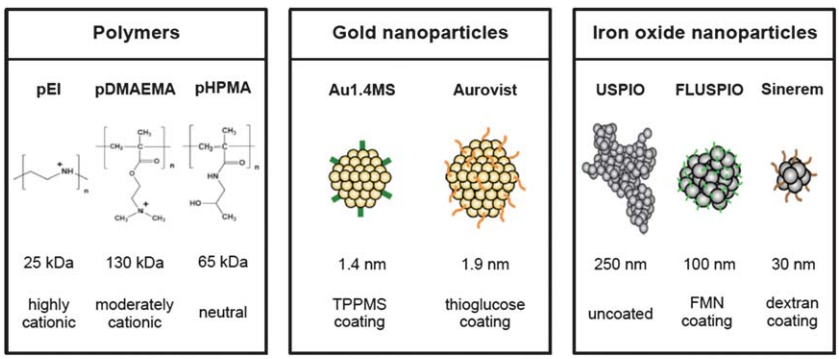

Fig. 2 Schematic depiction of the prototypic nanomaterials evaluated. Three different polymeric carrier materials and iron oxide nanoparticles, as well as two different gold nanoparticles, were used. Polymers differed in size and charge, and gold and iron oxide nanoparticles in size and surface coating. Routinely used, commercially available and/or previously extensively characterized materials were used. More details on the synthesis, properties and physicochemical characteristics of these materials can be found in ref. 7–22.

informative, and we therefore propose to use this assay as an intermediate between cell-based toxicity screening and nanotoxicity testing in rodents.

Experimental

Polymeric carrier materials

Poly(ethylenimine) (pEI; 25 kDa) was purchased from Polysciences Inc. Poly(*N*-2-hydroxypropyl) methacrylamide (pHPMA; 65 kDa) and poly(2-(dimethylamino) ethyl-methacrylate) (pDMAEMA; 130 kDa) were synthesized as described in ref. 7–9, 21 and 22. All polymers had a PDI $\leq$ 2. The transfection agents pEI and pDMAEMA contain positively charged side chains, while pHPMA is a neutrally charged macromolecular drug carrier.

Gold nanoparticles

Au1.4MS gold particles (overall chemical formula: $Au_{55}[(C_6H_5)_2P(C_6H_4SO_3Na)]_{12}Cl_6$; core size: 1.4 nm) were synthesized as described by Pan *et al.*,[13] using the Schmid method.[23] Their synthesis was based on a ligand exchange reaction of Au1.4TPP nanoparticles ($Au_{55}[(C_6H_5)_3P]_{12}Cl_6$) with TPPMS, in which Au1.4TPP (the non-sulfonate ligand derivative $P(C_6H_5)_3$), dissolved in methylene chloride, was transferred into the aqueous phase. During phase transfer, TPP was replaced TPPMS present in the water phase. The two-phase system was stirred for 3 days, the solvent was removed and the precipitate was washed, before being filtered and stored in a solid form. Aurovist®, a 1.9 nm sized commercially available gold nanoparticle formulation with a thioglucose coating, was purchased from Nanoprobes Inc.

Iron oxide nanoparticles

Three different ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles were used. Uncoated USPIO nanoparticles ($\sim$5 nm core size in TEM; $\sim$252 nm aggregates in PBS) and FLUSPIO ($\sim$100 nm hydrodynamic diameter in DLS) were prepared as described in ref. 17. FLUSPIO particles were synthesized using USPIO cores and coating them with flavin mononucleotide (FMN) and guanosine monophosphate (GMP). Sinerem (Combidex®; $\sim$30 nm hydrodynamic diameter in DLS) was kindly provided by Guerbet SA (France).

Cell lines

HeLa (human cervical carcinoma), HUVEC (human umbilical vein endothelial) and SMC (ovine smooth muscle) cells were obtained from ATCC, and were used for *in vitro* cytotoxicity analysis. HUVEC were cultivated in Endopan 3 Medium (Pan Biotech, Germany), and HeLa and SMC cells in DMEM (Gibco, Germany), supplemented with 1% Pen/Strep and 10% FBS. Cell lines were maintained at 95% relative humidity, 5% CO_2 and 37 °C.

Cytotoxicity assays

Cells were exposed to different concentrations of the polymers and nanoparticles for 3–48 h. Upon incubation, cells were washed, MTT solution (Roche, Switzerland) was added, and plates were incubated with MTT for 4 h. After this, the MTT solution was discarded and DMSO was added to dissolve the formed formazan crystals. Solubilization of the crystals was performed overnight. Supernatant absorbance was measured using a TECAN reader at 570 nm, with a reference wavelength of 690 nm. Based on this, IC_{50} values (representing the concentration of a drug or (nano-) chemical material inhibiting *in vitro* cellular proliferation by 50% as compared to untreated controls) were calculated and compared.

Zebrafish assay

The *in vivo* toxicity of the polymers and nanoparticles was evaluated using the zebrafish embryo assay. Fertilized eggs were transferred into 96-well plates (16-cell stadium, 1 egg per well), and different polymer and nanoparticle concentrations were added. Polymers were tested at 0.0001–1.0 mg ml^{-1}, and gold and iron oxide nanoparticles at concentrations of 0.01–10 mg ml^{-1}. Gold concentrations correspond to x (mg ml^{-1})/55 000 (M_w of cluster in gram) $\times$ 55 (number of Au atoms per cluster) mM Au. Concentrations were chosen on the basis of *in vitro* toxicity profiles, and were diluted in E3 zebrafish embryo medium. The development of the zebrafish embryos was evaluated using a Leica DMI 6000B inverted microscope from the moment of nanoparticle addition onwards (which lasted for maximally 72 h), and was longitudinally monitored at 24, 72 and 168 (upon transferring the animals to normal fish tanks) hours of post-fertilization, to assess toxicity and potential developmental defects.

Results and discussion

Polymeric drug carriers

Assessment of the toxicity profile of the three polymeric carrier materials demonstrated that cationic pEI (25 kDa; and pDMAEMA) showed a much higher degree of *in vitro* cell killing than did neutral pHPMA. In line with previous experiments, the latter was found to be non-toxic up to a concentration of 1 mg ml^{-1}, whereas the former two had IC_{50} values of $\sim$0.03 and 0.3 mg ml^{-1}, respectively (Fig. 3A). These *in vitro* toxicological profiles correlated very well with the observed *in vivo* toxicity in zebrafish embryos. As shown in Fig. 3C, at 24 h after adding the polymers to the embryos, pEI had prevented them from properly developing already at concentrations as low as 0.01 mg ml^{-1}, while pDMAEMA prevented proper embryo development from concentrations of 0.5 mg ml^{-1} onwards. For pHPMA, embryo development was shown to be affected only at a concentration of 1 mg ml^{-1}. For pEI and pDMAEMA, similar trends were observed over time. At later time points, also for pHPMA, abnormalities regarding embryo development were observed, and also upon 72 h of exposure to 0.5 mg ml^{-1}, some indications for teratogenicity could be obtained (Fig. 3D).

Overall, *in vitro* and *in vivo* toxicity correlated very well for these three polymer-based carrier materials. The findings in Fig. 3D, however, indicate that also the prolonged exposure of zebrafish embryos to relatively high concentrations of polymers believed to be non-toxic on the basis of *in vitro* results, *i.e.* pHPMA, might lead to – at least some – growth retardation and/

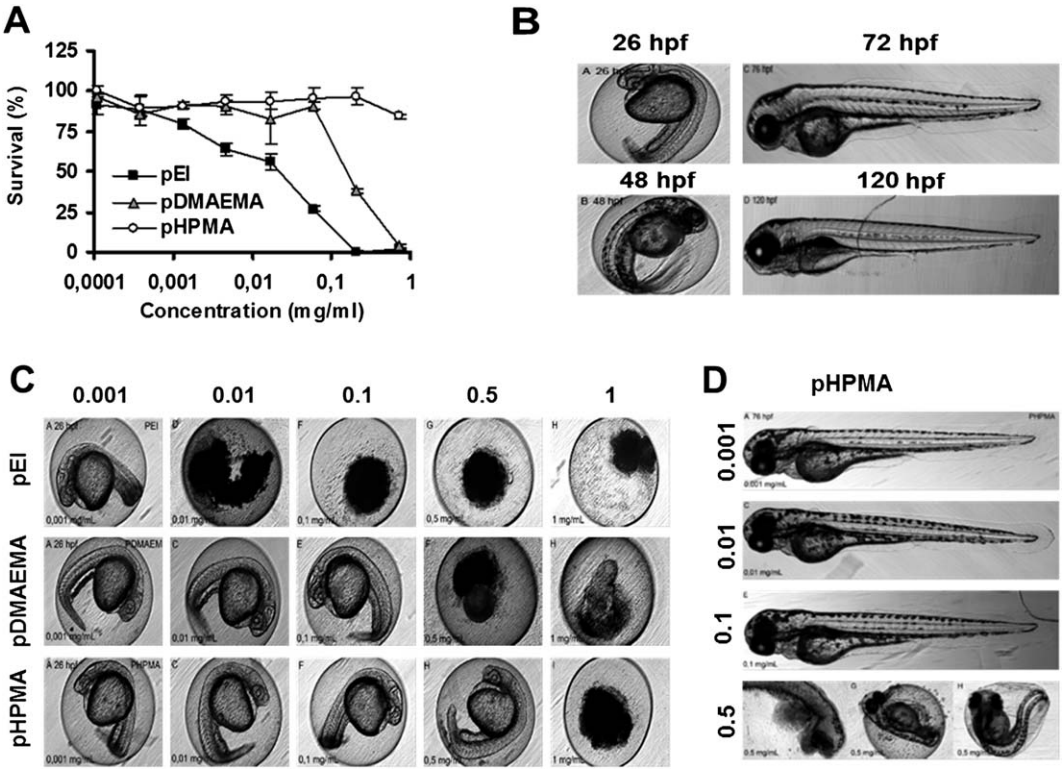

Fig. 3 Toxicity induced by cationic and neutral polymeric carrier materials. (A) *In vitro* survival of HeLa cells, as determined by MTT, upon exposure to highly cationic pEI, moderately cationic pDMAEMA, and neutral pHPMA. (B) Zebrafish embryo development under standard/healthy conditions (hpf = hours post-fertilization). (C) Zebrafish embryo development after 24 h of incubation with the indicated polymeric carrier materials (in mg ml^{-1}), showing good correlation with the effects observed *in vitro*. For pHPMA, only at the highest dose, significant mortality could be observed. (D) Upon prolonged exposure (72 h) to 0.5 mg ml^{-1} of pHPMA, intermediate developmental defects (*i.e.* delayed hatching) were observed.

or developmental defects. In line with previous studies, these findings therefore suggest that there might be a discrepancy between the results obtained purely on the basis of *in vitro* findings and those observed *in vivo*. With regard to this, the *in vivo* zebrafish embryo assay seems to be more sensitive for picking up potential adverse effects than *in vitro* experiments in cells. Consequently, the zebrafish embryo assay might prove to be an interesting and important 'intermediate' tool for facilitating efficient and ethical *in vivo* nanotoxicity testing.

Gold nanoparticles

In vitro cytotoxicity analyses clearly demonstrated that 'therapeutic' Au1.4Ms nanoparticles were much more toxic than 'diagnostic' Aurovist nanoparticles (Fig. 4A). HUVEC and SMC showed an evident decrease in viability at a dose of 1 and 10 mg ml^{-1} of Au1.4MS, while no toxicity was observed for Aurovist particles, at all of the tested concentrations. These results are largely in line with the results observed using the *in vivo* zebrafish embryo assay, which also clearly demonstrated that at 24 h after particle addition, the toxicity of Au1.4MS nanoparticles was much higher than that of Aurovist nanoparticles (Fig. 4B). These results were well-reflected at later time points, though with somewhat higher sensitivity, showing also toxicity and developmental defects at lower concentrations. As exemplified by Fig. 3C, longer exposure to lower doses (*i.e.* 72 h at 0.1 and 1 mg ml^{-1}) of Au1.4MS particles also had effects on the embryos, revealing delayed embryonic development (*i.e.* embryos still in the egg at 72 and 168 hours of post-fertilization (hpf)).

The observed higher toxicity of Au1.4MS nanoparticles in comparison to Aurovist is in accordance with previously published results,[13,24,25] suggesting that gold nanoparticle toxicity depends both on size and surface chemistry.[26] The thioglucose coating, as present in the commercially available product Aurovist, is likely responsible for the significantly reduced toxicity profile of these particles. Furthermore, gold nanoparticles do not present aggregation patterns, which means that due to their small size (1.4 nm for Au1.4MS and 1.9 nm for Aurovist), they can be easily internalized by cells *via* endocytosis, and enter the embryos *via* chorion pores. As shown by Pan *et al.*,[25] both Au1.4MS and Aurovist nanoparticles demonstrate similar internalization rates, providing further evidence that the thioglucose coating in Aurovist conveys protection towards both cells and zebrafish embryos.

Iron oxide nanoparticles

In vitro MTT analyses did not show any difference in cytotoxicity between standard USPIO, FMN-coated FLUSPIO and Sinerem. At concentrations up to 10 mg ml^{-1}, none of the three iron oxide nanoparticles caused a significant decrease in viability, neither

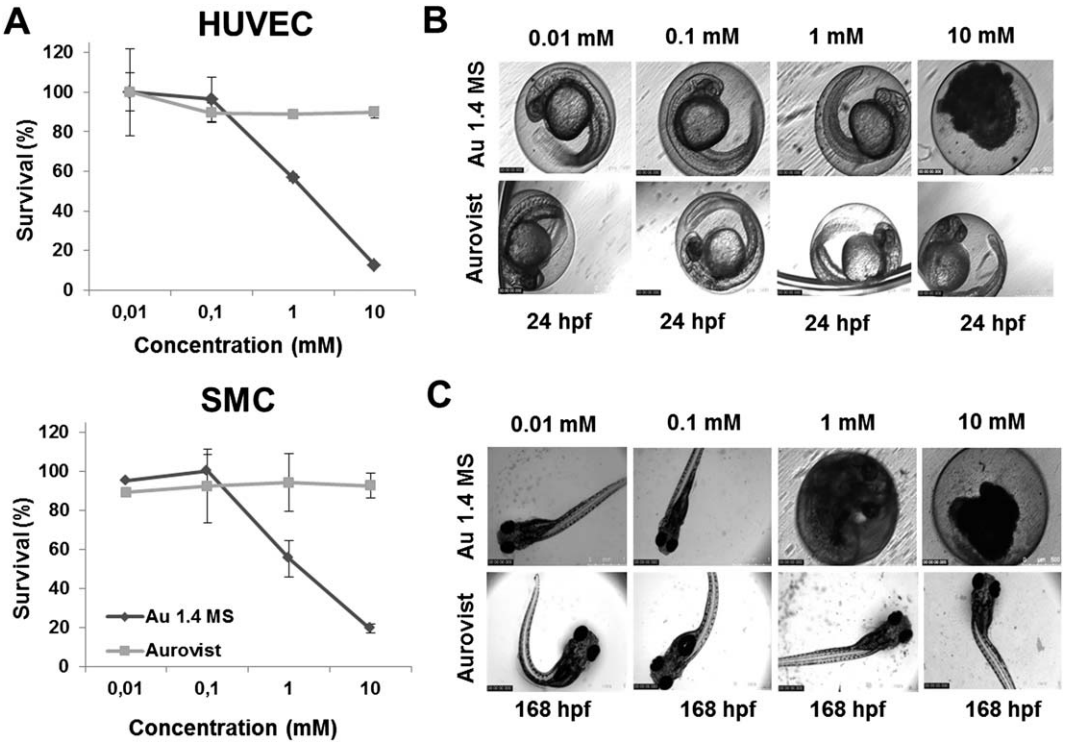

Fig. 4 Toxicity induced by gold nanoparticles. (A) *In vitro* survival of HUVEC and SMC exposed to increasing amounts of 'therapeutic' 1.4 nm (Au1.4MS) and 'diagnostic' 1.9 nm (Aurovist) gold nanoparticles. (B and C) Short- and long-term impact of gold nanoparticle exposure on zebrafish embryo development, showing very good correlation with *in vitro* findings. While Au1.4MS particles induced both acute (at 24 h; continuous exposure; at 10 mM) and delayed (at 168 h; 72 h exposure; at 1 mM) toxicity, Aurovist did not demonstrate any toxic effects, not even at high doses.

in HUVEC, nor in SMC (Fig. 5A). Conversely, however, *in vivo* toxicity analyses using zebrafish embryos rendered different results, hinting towards a higher level of toxicity for uncoated USPIO as compared to FLUSPIO and Sinerem (Fig. 5B). For the former, clear evidence for embryonic lethality was obtained at a dose of 10 mg ml^{-1}, killing all embryos already at 24 hpf. For the latter two, no signs of toxicity or developmental abnormalities were observed at this dose, not even upon nanoparticle exposure for 72 h and follow-up until 168 h (Fig. 5C).

These results can be partially explained by nanoparticle aggregation, which in zebrafish embryo medium (*i.e.* NaCl, KCl, $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$; pH 7.2) occurred predominantly for USPIO. As already demonstrated by Jayapaul *et al.*,[17] USPIO particles are quite unstable at pH = 7 in both PBS and saline solution (0.9% NaCl), and tend to aggregate, while FLUSPIO and Sinerem tend to be much more stable. This is exemplified by the higher background darkness of the USPIO images in Fig. 5B and C, in particular at higher concentrations, which is the result of nanoparticle aggregation and sedimentation. Properly dispersed (coated) iron oxide nanoparticles tend to have hydrodynamic diameters of 10–100 nm, and therefore are small enough to pass through the egg chorion pores (which are 500–700 nm).[27] Aggregates, on the other hand, which can have sizes of up to several microns, might get trapped in the chorion pores, and as suggested by Cheng *et al.*[28] and Bai *et al.*,[29] might thereby block oxygen transport from the medium into the embryo. This notion is expected to contribute, at least to some extent, to the higher toxicity caused by uncoated USPIO nanoparticles.

The fact that no toxicity was observed *in vitro*, but that contrasting results were found in zebrafish embryos, might be explained on the basis of uncoated USPIO aggregation in PBS and saline solutions, which tends to be avoided when uncoated USPIO are diluted in cell culture medium (most likely because of colloidal stabilization of iron oxide particles by serum proteins).[17] Further and more detailed studies on nanoparticle aggregation, rate of particle internalization by both cells and embryos, and hypoxia levels in the embryos will likely provide more insights into the toxicity mechanism(s) of these iron oxide-based nanoparticles.

In vitro vs. in vivo toxicity screening

The present toxicity analyses with three different classes of representative nanomedicine materials highlight the importance of critically evaluating the correlation between *in vitro vs. in vivo* nanotoxicity testing. The assessment of polymers, gold and iron oxide nanoparticles towards both cells and zebrafish embryos clearly demonstrated that not always a correlation between the two techniques can be observed. Our study revealed an agreeable correlation between *in vitro vs. in vivo* findings for both polymeric drug carriers and gold nanoparticles. These nanosystems presented good correspondence between *in vitro* toxicity evaluations

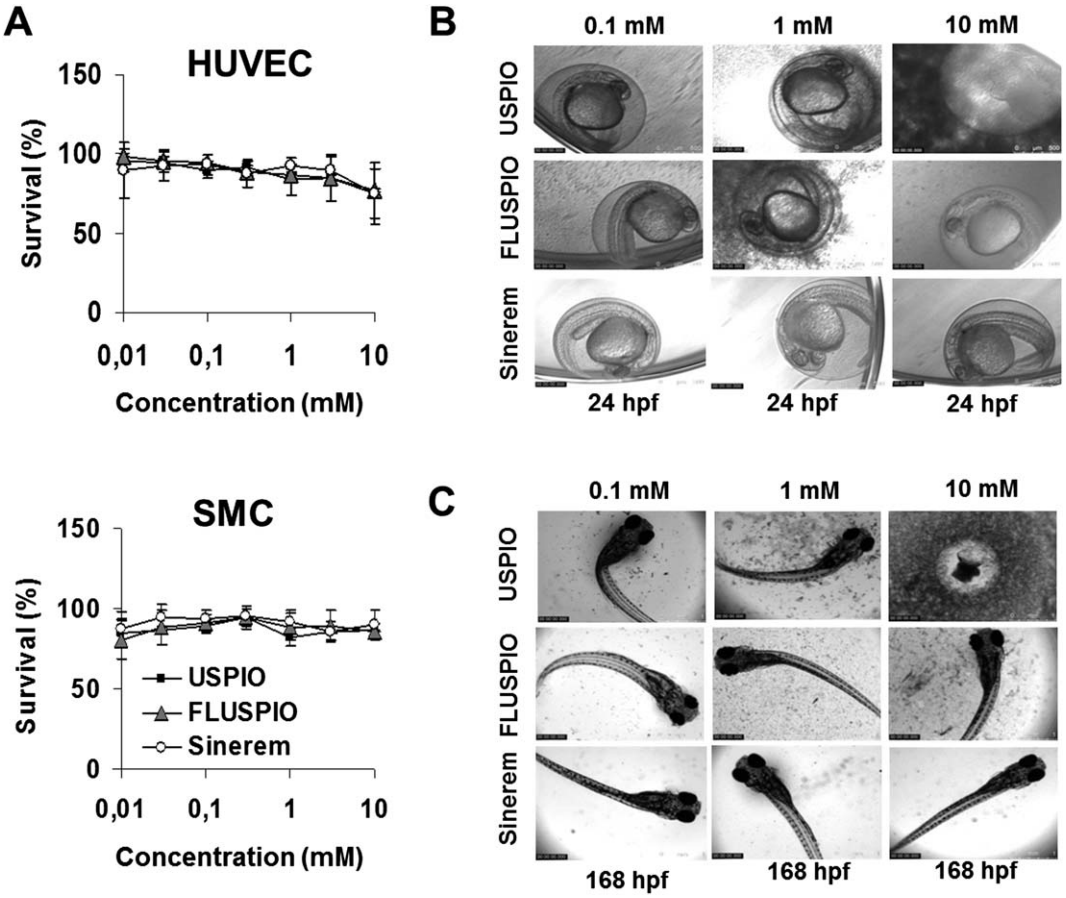

Fig. 5 Toxicity induced by iron oxide nanoparticles. (A) *In vitro* survival of HUVEC and SMC exposed to increasing amounts of uncoated USPIO, FMN-coated USPIO (FLUSPIO), and dextran- and sodium citrate-coated USPIO (Sinerem). (B and C) Short- and long-term impact of iron oxide nanoparticle exposure on zebrafish embryo development, showing a partially very good, and a partially discordant correlation with *in vitro* findings. *In vitro*, neither of the three formulations demonstrated toxicity at doses up to 10 mM. *In vivo*, on the other hand, uncoated USPIO were found to be toxic at a dose of 10 mM.

and their dose-dependent effects on embryonic development in zebrafish. Iron oxide nanoparticles, on the other hand, revealed contrasting results: although no toxicity was observed in cells (at least not in the tested concentrations; which were maximally concentrated), evident particle-related effects were observed in the zebrafish embryo assay. Particle aggregation of uncoated USPIO is thought to be, at least to some extent, involved in the toxicity observed in the embryos, as aggregates trapped in the chorion pores might obstruct oxygen transport between the medium and the embryos. Coating the particles, and thereby preventing them from aggregation, seems to have a beneficial impact on their biocompatibility, as neither FLUSPIO nor Sinerem turned out to be toxic *in vivo*.

For the particles that did show a toxic effect in the embryos (Au1.4MS and USPIO particles), this effect seemed to be more pronounced at 24 hpf, when the first impact on the embryos was already evident. This observation is in agreement with the findings reported by Tyl *et al.*,[30] which point out that the vulnerability to teratogenic malformations is highly dependent on the embryonic stage. The most critical and sensitive stage of development is indeed suggested to be 1 day after fertilization (*i.e.* 24 hpf), when organogenesis takes place. At this phase, rapid differentiation of organs occurs and the extensive rate of cell proliferation makes them particularly prone to teratogenic factors leading to most of the structural malformations.

According to guidelines from the European Centre for Validation of Alternative Methods, studies on the toxicity of chemicals and (nano-) particles in zebrafish embryos reveal a fairly acceptable level of toxicity predictivity, ranging from "sufficient" (65–75%) to "good" (75–85%) predictivity. This discrepancy is mainly related to non-standardized methodologies among the different research institutions, as well as to a lack of systematic definition of parameters to allow head-to-head comparison and validation of results.[31]

Taken together, the zebrafish embryo assay is emerging as a rapid, easy and efficient method to assess nanoparticle toxicity. It provides the benefit of *in vivo* toxicity evaluation in a complex vertebrate organism, much more complex than (over-) simplified setups in cells. The assay is shown to be a simple way to assess the overall fish viability towards different types of nanoparticles, longitudinal monitoring of embryonic fish development and also the potential of detecting toxicity-related teratogenic effects. Although not a truly representative replacement for traditional

in vivo analyses in mice and rats, the zebrafish embryo assay is shown to be highly suitable as an intermediate screening tool, between preliminary toxicity evaluation in cells, and more conclusive and more translationally relevant follow-up toxicity assessment in 'higher' organisms. Future efforts regarding this *in vivo* nanotoxicity screening tool will also encompass the possibility of analyzing nanoparticle-mediated stress induction, using HSP70-GFP-transgenic zebrafish embryos, and correlating *in vitro* stress induction (*via* ROS assays) with *in vivo* HSP70 expression (*via* the activation of the 70 kDa heat shock protein, which is coupled to GFP expression in transgenic fish embryos). Initial studies in this regard have already been undertaken for cytotoxic Au1.4MS gold nanoparticles,[13,25] and will be extended to several other types of diagnostic, therapeutic and theranostic nanomaterials in the years to come.

Conclusions

We here demonstrate that *in vivo* nanotoxicity testing using the zebrafish embryo assay can be used to validate and complement findings obtained *in vitro*. Polymeric carrier materials and gold nanoparticles presented with a good *in vitro vs. in vivo* correlation regarding toxicity parameters. It should be noted, however, that upon longer-term exposure to relatively high doses of neutral and *in vitro* non-toxic pHPMA polymers, some developmental abnormalities were observed in the zebrafish embryo assay, illustrating its potential for more sensitive, more detailed and more informative nanotoxicity testing. In line with this, non-coated iron oxide-based nanoparticles were found to be non-toxic *in vitro*, but presented with significant toxicity towards zebrafish embryos *in vivo*. Based on these notions, the zebrafish embryo assay seems to be an easy, efficient and ethical method to assess both the acute effects of exposure to nanoparticles, as well as their long-term impact on embryonic development, providing important information on whole organism teratogenicity, and a clear benefit in comparison to (over-) simplified *in vitro* toxicity parameters. This *in vitro vs. in vivo* toxicity correlation, as well as the elucidation of potential discrepancies, has important implications for facilitating the translation of nanomedicine materials into clinical trials.

Acknowledgements

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