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REVIEW

Relation between biophysical properties of nanostructures and their toxicity on zebrafish

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Abstract In recent years, the use of commercial nanoparticles in different industry and health fields has increased exponentially. However, the uncontrolled application of nanoparticles might present a potential risk to the environment and health. Toxicity of these nanoparticles is usually evaluated by a fast screening assay in zebrafish (*Danio rerio*). The use of this vertebrate animal model has grown due to its small size, great adaptability, high fertilization rate and fast external development of transparent embryos. In this review, we describe the toxicity of different micro- and nanoparticles (carbon nanotubes, dendrimers, emulsions, liposomes, metal nanoparticles, and solid lipid nanoparticles) associated to their biophysical properties using this model. The main biophysical properties studied are size, charge and surface potential due to their impact on the environment and health effects. The review also discusses the correlation of the effects of the different nanoparticles on zebrafish. Special focus is made on morphological abnormalities, altered development and abnormal behavior. The last part of the review debates changes that should be made in future directions in order to improve the use of the zebrafish model to assess nanotoxicity.

Keywords Biophysical properties · Nanostructures · Np · Toxicity · Zebrafish

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Abbreviations

dpf	Days post-fecundation
hpf	Hours post-fecundation
NPs	Nanoparticles

Introduction

Nanoparticles (NPs), nanotechnological products of increasing importance due to their unique properties, have been used in several applications such as cosmetics, cleaning materials, electronic components, food additives, medicines, sports equipment and surface coatings (Mahmoudi et al. 2011). However, the properties of NPs are different from those exhibited by the same material at macroscale and may cause undesired toxicity. It has been reported that their size and great surface area related to total volume may affect the way an organism responds to, distributes and eliminates NPs (Lanone and Boczkowski 2006). In addition, to increase their systemic circulation, make them more biocompatible, and be able to use them as delivery systems, several NPs are functionalized on their surface. These added functional groups might interact with biological components, alter their functions and allow the passage of NPs to certain cells in which they would not be absorbed normally (Fischer and Chan 2007). Moreover, it is important to take into account NPs degradation, because this is an important issue in acute toxicity and long-term toxicity; non-degraded NPs can accumulate in organs and cause detrimental effects, while biodegradable materials can generate products that cause unexpected toxicity (Aillon et al. 2009; Fischer and Chan 2007; Papp et al. 2008). Based on the aforementioned, in order to reach a safe usage and handling of nanoparticles, it is critical to study the effects of exposure to nanomaterials.

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The zebrafish is a widely used model system for developmental and environmental toxicology studies due to its small size, high fertilization rate and rapid external development of transparent embryos (Frederiksen et al. 2003). The cardiovascular, nervous and digestive systems of zebrafish are similar to those of mammals (Aillon et al. 2009). Indeed, the zebrafish genome shares a high degree of homology with the human genome (Müller et al. 2002). Despite the increasing number of reports on emergent NPs in recent years, little is known about the biological impact of NPs.

The goal of this review is to present an overview of the available toxicity assessments of NPs (carbon nanotubes, dendrimers, emulsions, liposomes, metal nanoparticles, and solid lipid nanoparticles) in zebrafish and the possible relationships with their biophysical properties.

Nanoparticles

Carbon nanotubes

Carbon nanotubes (CNTs) are nanomaterials composed of graphite sheets. Depending on the number of sheets, these tubes are called single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) (Fig. 1). CNTs display novel properties as great mechanical strength, electrical conductivity, thermal conductivity and chemical stability (He et al. 2013). In aqueous environments, pristine CNTs tend to aggregate. Thus, much research has been carried out to functionalize their surface with attached groups to improve their dispersion and increase their biocompatibility (Fu and Sun 2003). It is noteworthy that, due to their nanosize, CNTs can be great drug delivery systems since they can travel inside the body, easily pass through cellular barriers and transport active compounds either on their surface or inside their structure (Faraji and Wipf 2009; Pastorin et al. 2006; Prato et al. 2008; Sharma et al. 2016). CNT applications are continuously increasing and currently cover electronics, cosmetics, cleaning materials, coatings, food packaging, building materials (de Paula et al. 2014) and medicines (Nyilasi et al. 2008).

Since a wide variety of CNTs are being introduced in the nanobiotechnology field, the consequences of their cumulative presence in the environment and increasing contact with humans must be carefully analyzed. CNTs are able to accumulate in the liver, spleen, kidney, lung, muscle, skin and bones (Deng et al. 2007; Wang et al. 2004). Although the first studies on the effects of CNTs were performed in the lungs (Fröhlich 2012; Nagel 2001; Yostawonkul et al. 2017), several organs and mechanisms of damage are currently being studied to reach a complete toxicological profile of these nanomaterials (Asharani et al. 2008; Cheng and Cheng 2012; Usenko et al. 2007). The toxicological studies of CNTs in zebrafish carried out so far have revealed differential toxicity according to the CNT dimension, functionalization and impurities attached resulting from the treatment. Table 1 describes the different functionalization and toxicological assays carried out in each CNT. Cheng et al. (2007) described a delay in the hatching rate of zebrafish embryos incubated with SWCNTs and MWCNTs, with more marked effects by SWCNTs. Wang et al. (2016) found that bioaccumulation of MWCNTs was greater than that of SWCNTs and that embryos treated with SWCNTs presented severe malformations whereas embryos treated with MWCNTs presented normal development. In addition, Cheng et al. (2007) and (Girardi et al. 2017) reported a delay in the hatching rate of zebrafish embryos incubated with SWCNTs in a concentration-dependent manner. In contrast, working with a wide CNT concentration range that included the concentrations used by Cheng and Girardi, Bayat et al. (2015) found no effects. Wang et al. (2016) described that, although embryos accumulated SWCNTs in a dose-dependent manner, their toxicity was low. On the other hand, Asharani et al. (2008) reported mortality of zebrafish embryos when they were incubated with 60 ppm of MWCNTs, while Liu et al. (2014) observed no induced mortality although WMCNTs were used in concentrations of up to 100 ppm. In addition, Asharani et al. (2008)

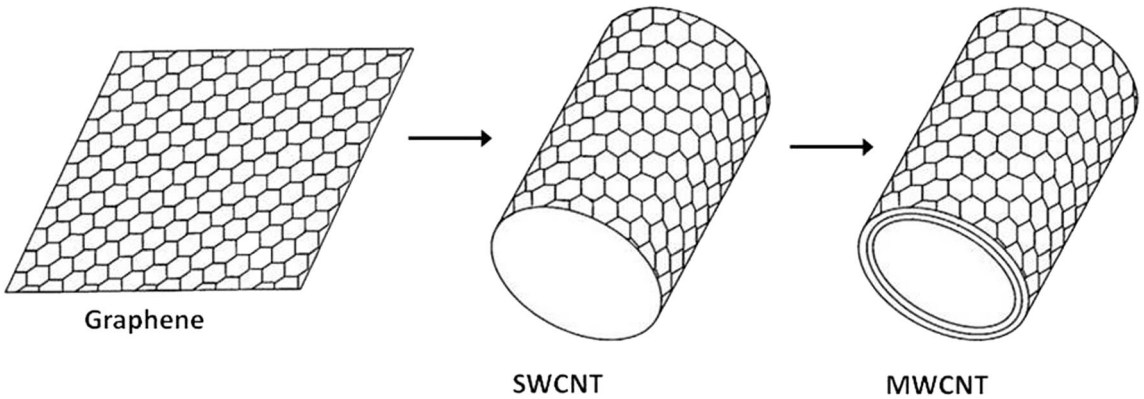

Fig. 1 Carbon nanotubes (CNTs) are structures of rolled graphene sheets, composed of a single wall (SWCNT) or multi-walls (MWCNT)

Table 1 Studies on the toxicity of carbon nanotubes in zebrafish embryos

CNT type	Treatment of CNT	Stage	Assays	Critical biophysical parameters	Reference
SWCNT	Pristine	Embryos/larvae (0–96 hpf)	Hatching rate Heart rate Length Mortality Spontaneous movement	The effects of SWCNTs on hatching rates, mortality and morphology might be due to the concentration of CTNs but most probably to metal contamination.	Girardi et al. 2017
	Pristine	Embryos/larvae (0–120 hpf)	Hatching rate Malformations Mortality	SWCNTs instigates mortality but no significant malformations.	Bayat et al. 2015
	Pristine	Embryos/larvae (4-96hpf)	Hatching rate	SWCNTs affects hatching rate more than MWCNTs. The delay is concentration-dependent.	Cheng et al. 2007
	Nitric acid	Embryos/larvae (0–96 hpf)	Hatching rate Respond to touch Length Bent spine Mortality Spontaneous movement	Functionalized SWCNTs do not cause any change, at any concentration assased.	Ong et al. 2013
	Pristine/sulfuric acid/surfactant PF108	Embryos/larvae (8–120 hpf)	Bioaccumulation Development Mortality	Pristine SWNTs causes severe malformations while functionalized SWCNTs do not. Bioaccumulation of SWCNTs is smaller than MWCNTs.	Wang et al. 2016
	Pristine	Adults	Oxidative stress	SWCNTs reduce the total antioxidant capacity.	da Rocha et al. 2013
	Polyethylene glycol	Adults	Bioaccumulation	SWNTs modified with short-chain PEGs leads to the accumulation of the material, tissue damage andalteration of the behavior.	Weber et al. 2014
MWCNT	Pristine	Embryos/larvae (4-96hpf)	Hatching rate	SWCNTs affects hatching rate more than MWCNTs. The delay is concentration-dependent.	Cheng et al. 2007
	Pristine	Embryos/larvae (0–98 hpf)	Hatching rate Heart rate Length Malformations Mortality Spontaneous movement	MWCNTs do not induce mortality, morphological malformation or changes in hatching rate, even at high concentrations, but causes changes in the heart rate and larval length.	Liu et al. 2014
	Pristine	Embryos/larvae (1–72 hpf)	Hatching rate Heart rate lLength Bent spine Mortality	Depending on the concentration, MWCNTs can cause changes in hatching rate and heart rate, bent spine and even mortailty.	Asharani et al. 2008
	Nitric acid	Embryos/larvae (0–80 hpf)	Malformatios	SWCNTs affects hatching rate more than MWCNTs. The delay is concentration-dependent.	Cheng and Cheng 2012
	Sulfuric-nitric acid	Embryos/larvae (120 hpf)	Mortality	Among the physicochemical properties studied -surface charge, surface oxygen, aggregate size and morphology, and electrochemical activity-surface charge has the closest influence on mortality.	Gilbertson et al. 2014
	Pristine/carboxilated - surfactant PF108	Embryos/larvae (8–120 hpf)	Bioaccumulation Development Mortality	Pristine and carboxilated MWCNTs causes little effect on embryo viability and development.	Wang et al. 2016
	Surfactant PF127	Adults	Tissue histology	Agglomeration of MWCNTs causes a reduction in CNT reactivity	Filho Jde et al. 2014

observed changes in the hatching rate, bent spine and heart rate in zebrafish embryos incubated with MWCNTs in concentrations higher than 60 ppm, while Liu et al. (2014) described changes in the heart rate and larval length with concentrations higher than 10 ppm and no changes in the hatching rate. It is noteworthy that, during recent years, the agglomeration of nanotubes has been indicated as a critical determinant of CNT toxicity. In fact, Filho Jde et al. (2014) revealed that the agglomeration of CNTs caused a reduction in CNT reactivity. As mentioned above, another problem in toxicology studies of CNTs is the presence of impurities. Girardi et al. (2017) established that the presence of metal impurities might be the principal cause of toxicity because the purification process is not able to remove them completely.

Results of the toxicity of CNTs in zebrafish are controversial. This controversy seems to be due to the fact that CNTs vary not only in their structure and composition but also in their properties, depending on the manufacturing process. CNTs can be synthesized by different methods, each of which leaves impurities that seem to have a significant effect on the biological response of zebrafish, affecting the evaluation of the toxicity of the nanomaterial (Farre et al. 2009; Nayak et al. 2010). In light of all the evidence, the critical step in evaluating the toxicology of a CNT is its physicochemical characterization, a first step indispensable to compare results between reports (Asharani et al. 2008; Cheng et al. 2009; Cheng and Cheng 2012; Cheng et al. 2007).

Dendrimers

Dendrimers are an emerging class of polymeric nanomaterials with core–shell architecture. These highly symmetrical branched polymers are characterized by a multifunctional initiator core, repeating branched units attached to the core, and terminal functional groups attached to the outermost shell (Frechet 1994; Gupta and Perumal 2014). The core is a molecule that must have reactive centers to which the dendrons (branches) can be attached. Different types of cores, like ammonia, ethylenediamine (EDA), diaminobutane (DAB), thiophosphoryl chloride (TC), triazine, polyethylene glycol (PEG) and carbosilane, have been used to synthesize dendrimers. The layers of radially repeating branches around the core are called generations (G). Succeeding generations increase the diameter and duplicate the number of terminal functional groups respect to their predecessor. Products obtained in an intermediate step of the synthesis, before completing the layer, are denoted as half-generation dendrimer (G .5) (Suarez et al. 2011; Tomalia et al. 1985). Dendrimer size ranges from 10 to 130 Å, increasing with the generation. The number and density of terminal groups on the surface of the dendrimers also increase with the generation and the molecule becomes spherical (Boas and Heegaard 2004; Svenson and Tomalia 2012; Tomalia et al. 1990). Usually, the lower-generation dendrimers (G1–G3) are characterized by an open structure and a highly asymmetric shape in comparison to the more globular and compact structure present in higher generations (>G4) (Caminati et al. 1990; Svenson and Tomalia 2012; Tomalia et al. 1990).

The unique characteristics of this class of polymeric nanomaterials allow their applications in various scientific fields, as shown in Table 2. The multivalent and multifunctional surface and the monodispersed and small size of dendrimers result in cooperative binding with different drugs and biological membranes, allowing their use as permeability

Table 2 Main characteristics and consequent applications of dendrimers

Characteristics	Reference
Well-defined, symmetric and stable molecular architecture	Tomalia et al. 1985
Monodispersed and controlled size in the range of nanometers	Tomalia et al. 1990
Multivalent, multifunctional and highly branched structure	Kesharwani et al. 2014
Controlled number, type and charge of surface groups	Madaan et al. 2015
Extremely high area/volume ratio	Tomalia et al. 1985
High chemical reactivity and water solubility	Tomalia et al. 1990
Applications	**Reference**
Controlled drug delivery and tissue targeted therapy	Kesharwani et al. 2014
Penetration enhancers	Yang et al. 2013
In vitro gene transfection and in vivo gene therapy	Yang et al. 2015
Additives in the pharmaceutical and cosmetic industries	Ammala et al. 2013
Medical imaging and early diagnostics	Qiao et al. 2015
Analytical sensors and chelating agent of metals	Hasanzadeh et al. 2014
Antimicrobial and antiviral properties	Reddy and Babu 2016
Anti-inflammatory properties	Neibert et al. 2013

enhancers. In addition, the core–shell architecture of dendrimers facilitates host–guest entrapment, allowing their use as drug delivery systems. Furthermore, their derivatizable branched architecture allows modifying them in numerous ways, to be used in the conjugation of drugs and as diagnostic agents and targeting ligands.

Different types of dendrimers have so far been reported. The most commonly used are polyamidoamine (PAMAM) and polypropyleneimine (PPI) dendrimers (Kaur et al. 2016) with cores of EDA, DAB or TC, and terminal groups like aldheyde, amine, carboxylate, succinamic acid or amidoethanol (Fig. 2).

In cell culture, PAMAM dendrimers generally display concentration-dependent, charge-dependent, and generation-dependent cytotoxicity. In particular, cationic dendrimers are more cytotoxic than anionic or neutral dendrimers and, also, the toxicity increases with the increase in dendrimer generation and concentration (Jain et al. 2010). To our knowledge, only four publications describe the use of the zebrafish model to analyze the toxicity of diverse dendrimer types (Bodewein et al. 2016; Heiden et al. 2007; Oliveira et al. 2014; Pryor et al. 2014) (Table 3). Another two papers have used the zebrafish model to test the toxicity of drug-dendrimer complexes (Prieto et al. 2013, 2014); and another one to test the biocompatibility of dendrimers with zebrafish red blood cells (Jones et al. 2012).

Heiden et al. (2007) exposed zebrafish embryos of 6 h post-fertilization (hpf) to G3.5 and G4 PAMAM dendrimers and found that G4 PAMAM dendrimers, with amine terminal groups, were toxic in lethal and sublethal parameters in a dose- and time-dependent way, and that G3.5 PAMAM dendrimers, with carboxylic terminal groups, were not toxic at any of the concentrations tested (no increase in mortality and no sublethal signs of toxicity

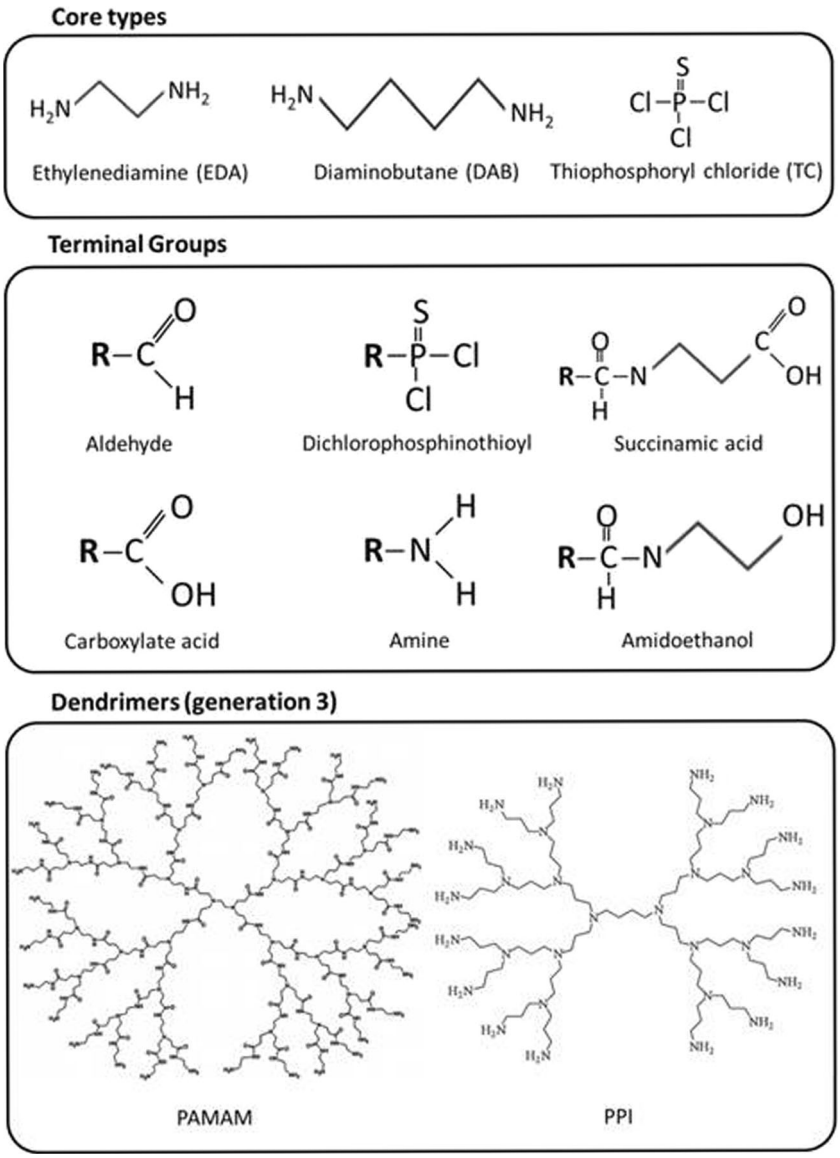

Fig. 2 Dendrimers are formed by a core, repeating branched units attached to the core and terminals functional groups attached to the outermost shell. Examples of core- and terminal group-types, and generation 3.0 dendrimers

Table 3 Studies on the toxicity of dendrimers in zebrafish embryos

Dendrimer type	Core	G	Surface group		Charge	Molecular weight	Treatment		Assays				Reference
			Type	Number			hpf	Chorion	EC50 at 24 hpf (ppm)	EC50 at 48 hpf (ppm)	EC50 at 72 hpf (ppm)	EC50 at 120 hpf (ppm)	
PAMAM	EDA	3.5	Carboxylate	64	−	12,931	6	+	>2844.8		>2844.8	>2844.8	Heiden et al. 2007
	EDA	4	Amine	64	+	14,215	6	+	14.2		8.5	5.7	
PAMAM	EDA	3	Amine	32	+	6909	1	+		1.8			Oliveira et al. 2013
	EDA	4	Amine	64	+	14,215	1	+		2.3			
Thiophosphoryl-phenoxymethyl (methylhydrazono)	TC	0.5	Aldehyde	3	0	426	8	+				>250	Pryor et al. 2014
	TC	1.5	Aldehyde	6	0	1422	8	+				>250	
	TC	2.5	Aldehyde	12	0	3417	8	+				>250	
	TC	3.5	Aldehyde	24	0	7405	8	+				>250	
	TC	5	Dichlorophos-phinothioyl	48	0	23,108	8	+				>250	
PAMAM	DAB	3	Amine	32	+	6937	8	+				2	
	DAB	4	Amine	64	+	14,242	8	+				6.4	
	DAB	5	Amine	128	+	28,853	8	+				4.5	
	DAB	6	Amine	256	+	58,074	8	+				18	
	DAB	5	Succinamic acid	128	−	41,662	8	+				>250	
	DAB	6	Succinamic acid	256	−	83,693	8	+				>250	
	DAB	6	Amidoethanol	256	0	58,326	8	+				>250	
PPI	DAB	3	Amine	16	+	1687	1	−	0.6	0.4			Bodewein et al. 2016
	DAB	4	Amine	32	+	3514	1	−	1.1	1.0			
	DAB	5	Amine	64	+	7168	1	−	4.2	2.6			
PAMAM	EDA	3	Amine	32	+	6909	1	−	2.6	1.5			
	EDA	3.5	Carboxylate	64	−	12,931	1	−	>646.5	>646.5			
	EDA	4	Amine	64	+	14,215	1	−	3.1	2.3			
	EDA	4.5	Carboxylate	128	−	26,258	1	−	>1312.9	>1312.9			
	EDA	5	Amine	128	+	28,825	1	−	47.4	16.1			

or malformations). This first work on zebrafish demonstrated that the toxicity of dendrimers strongly depends on the type and charge of their terminal groups (Heiden et al. 2007).

In another work, Oliveira et al. (2014) tested the effect of G3 and G4 PAMAM dendrimers, with amine terminal groups, on zebrafish embryos of 1 hpf and found that G3 PAMAM dendrimers showed higher toxicity than G4 dendrimers, with a lower lethal dose. At sublethal concentrations, both dendrimers affected the transcriptome of the embryos, following a pattern which correlates to bacterial infection in zebrafish, indicating the activation of the immune response. This second work adds the notion that, in addition to the type of terminal group, the generation of the dendrimers demarcates their toxicity in zebrafish.

More recently, Pryor et al. (2014) exposed zebrafish embryos of 8 hpf to G3, G4, G5 and G6 PAMAM dendrimers with amine terminal groups, G5 and G6 PAMAM dendrimers with succinamic acid terminal groups, G6 PAMAM dendrimers with amidoethanol terminal groups, G0.5, G1.5, G2.5 and G3.5 thiophosphoryl dendrimers with aldehyde terminal groups, and G5 thiophosphoryl dendrimers with dichlorophosphinothioyl terminal groups, and studied mortality, developmental progression, notochord malformation and spontaneous movement. These authors found that exposure to neutral or anionic dendrimers (Table 3) caused no significant morbidity or mortality at the concentration tested. In contrast, exposure to cationic PAMAM dendrimers caused mortality (lethal effect) and significant cardiac impacts, like pericardial edema (sublethal effect). In these dendrimers, the morbidity and mortality were increased as the generation decreased. In agreement with the previously described works, this study showed that the toxicity of the dendrimers depends on the type and charge of the terminal groups and the generation of the dendrimer. In addition, although the G4 PAMAM dendrimers with amine terminal groups had a different core (DAB or EDA), a correlation between the results was observed.

In another recent study, Bodewein et al. (2016) tested the effect of G3, G3.5, G4, G4.5 and G5 PAMAM dendrimers and G3, G4 and G5 PPI dendrimers on dechorionated zebrafish embryos of 1 hpf, and found that carboxylate-terminated PAMAM dendrimers were not toxic at the concentration tested, at lethal or sublethal levels. On the other hand, the amine-terminated PAMAM and PPI dendrimers increased their toxicity over time, with mortality as the predominant effect and reduced heartbeat and blood circulation as sublethal effects. Again, lower-generation and cationic dendrimers were the most toxic to zebrafish embryos. In addition, these authors demonstrated the protective effect of chorion on embryos, since the effective doses (EC50) were much lower in dechorionated embryos than in the embryos with chorion (compare the effect of the G4 PAMAM dendrimers in Heiden and Bodewein in Table 3). It could be hypothesized that dendrimers with neutral or anionic terminal groups do not cross the chorion and, therefore, do not enter the embryos, thus resulting in no toxic effects. Nevertheless, Bodewein et al. (2016) used dechorionated embryos, leaving the dendrimers "available" and, likewise, observed no toxicity.

In work carried out by our research group (Prieto et al. 2013, 2014), we studied the in vivo effect of complexes between risperidone (an antipsychotic drug) and DG4.5 PAMAM dendrimers in zebrafish larvae (96 hpf), by evaluating changes in locomotion activity, hearth rhythm, cellular distribution and development of dopaminergic- and motor-neurons. The results obtained suggest that DG4.5 PAMAM dendrimers have a protective effect on the toxicity induced by free risperidone. Particularly, at the concentrations tested, the complexes were not toxic at cardiac or cerebral level, and reversed the effect of free risperidone on locomotion activity. However, both free risperidone and risperidone–dendrimer complexes resulted in a larger area in the postoptic commissure and the raphe population zone, and a cellular disorganization in the latter, causing dramatic changes that persisted over time. These studies highlight the importance of assessing both lethal and sublethal effects on zebrafish, as the latter could lead to long-term toxicity. In addition, they emphasize the importance of using zebrafish larvae, which are no longer in early development and have the central nervous system almost fully developed, allowing the study of toxicity in greater depth and in different organs.

Finally, Jones et al. (2012) demonstrated that G7 PAMAM dendrimers, with amine terminal groups, induce fibrinogen aggregation after injection in zebrafish larvae of 96 hpf, which may contribute to a disseminated intravascular coagulation-like phenomenon. In this way, once again, the results obtained by these authors emphasize the importance of studying non-lethal side effects of positively charged dendrimers.

Taken together, all the results detailed above suggest that the surface charge and the type of terminal groups are determinant in the toxic effect of dendrimers in zebrafish embryos. Neutral or negatively charged dendrimers presented no lethal effects at the concentrations tested in any of the published studies. In contrast, lethal doses were determined and sublethal effects were observed for all the positively charged dendrimers tested.

Other factors determining the toxicity of dendrimers are their generation and size. In the case of positively charged dendrimers, a generational dependence was observed: the lower the generation and size, the greater the toxicity. These results are in opposition to those observed in cell culture, where there is a directly proportional relationship between the generation and the toxicity of the dendrimer. The observed difference could be due to the fact that, in cell culture, the cationic molecules bind to the cell membrane that has negative charge and destabilize it, leading to cell lysis (Lee and Larson 2008; Tajarobi et al. 2001). It has been shown that positively

charged dendrimers form pores in the cell membrane and the efficiency of this depends on the size of the nanoparticle, which explains that the higher the generation, the higher the toxicity (Lee and Larson 2008). On the other hand, zebrafish embryos are organisms with greater complexity, and the mechanism by which dendrimers can be incorporated is still unknown. It can be hypothesized that lower-generation dendrimers can be captured more efficiently, leading to a high effective concentration, and could interfere with critical signaling cascades during the early stages of development.

Further studies are needed to fully understand the mechanism by which dendrimers can cause toxicity in zebrafish embryos. In addition, studies in larval stages are required to understand and evaluate long-term and organ-specific effects of dendrimers. Also, studies in later stages of development are needed to compare the effects on the innate (< 28 dpf) and adaptive (> 28 dpf) immune systems of zebrafish (Lam et al. 2004).

Emulsions

Emulsions are colloidal systems consisting of small particles dispersed in a continuous phase (McClements 2012). According to their size, they can be classified in macroemulsions, microemulsions and nanoemulsions (Bouyer et al. 2012; McClements 2015; Piorkowski and McClements 2014). These systems are composed of three regions with different physicochemical properties: the drop inside, the continuous phase and the interphase positioned between them. Emulsions can also be classified according to their composition complexity. Simple emulsions can be defined in oil in water (o/w) or water in oil (w/o) emulsions, while multiple ones are more sophisticated, and can be defined in oil in water in oil (o/w/o) or water in oil in water (w/o/w) (McClements 2015) (Fig. 3).

Over the last decades, emulsions have been widely used to encapsulate, solubilize, entrap and control bioactive compounds, which can be administered through different routes, such as the oral, parenteral or topical ones (Bouyer et al. 2012; Zhao et al. 2008). As delivery systems, emulsions should increase drug solubility and permeability, prevent drug hydrolysis or early enzymatic degradation, deliver the drug in a controlled manner and facilitate its transport (Araya et al. 2005; Ganta et al. 2010). Compounds with different solubility can be encapsulated and their distribution in one of the three regions of the emulsion will depend on their concentration and polarity (McClements 2015).

Emulsions, precisely nano ones, have been used as dye-loaded lipid nanodroplets as an alternative to inorganic nanoparticles and non-toxic biodegradable materials. Klymchenko et al. (2012) and (Kilin et al. 2014) reported that highly lipophilic fluorescent components can be encapsulated in the lipophilic core of stable nanoemulsion droplets at high concentrations, to be used in diagnostic imaging for the visualization of vasculature.

Studies suggest new molecular design strategies to obtain bright nanodroplets without dye leakage and their use as efficient and stable optical contrast agents both in vitro and in vivo (Klymchenko et al. 2012). Because the zebrafish has been considered a potential model for several human diseases (Feas et al. 2017; Sloman et al. 2003) and these new molecular strategies could be applied in humans in a near future, it could be interesting to deeply evaluate nanotoxicity of these nanoemulsions in this animal model as a pre-clinical study.

The bibliography available about toxicity of emulsions in zebrafish is limited; reports are listed in Table 4. Souza et al. (2016) evaluated the acute toxicological effects of perillyl

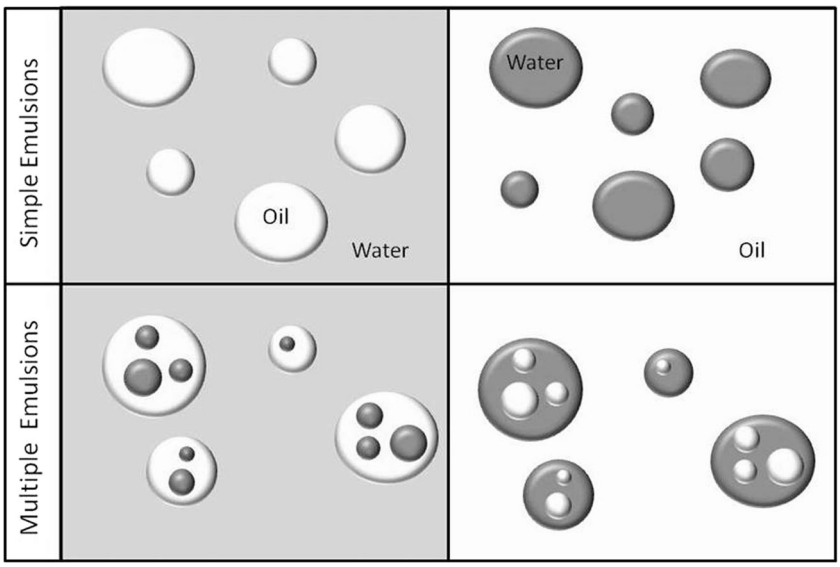

Fig. 3 Schematic representation of the different types of emulsions where the drop inside, the continuous phase and the interphase positioned between them are represented

Table 4 Studies on the toxicity of emulsions in zebrafish embryos

Nanoemulsion type	Size (nm)	Stage	Assays	Critical biophysical parameters	Reference
3-alkoxyflavone (F888) and Nile Red (NR668) lipid nano-droplets	20–40	Embryos (0–72 hpf)	Single particle tracking	Nano-droplets containing 0.1 wt.% of Nile Red and 1 wt.% of NR668 did not cause toxicity due to their injection. Loaded nano-droplets remain in the blood circulation.	Klymchenko et al. 2012
Cyanine dye lipid nano-droplets	30–90	Embryos (0–72 hpf)	Single particle tracking	8 wt% concentration did not cause toxicity due to its injection. Loaded nano-droplets remain in the blood circulation.	Kilin et al. 2014
ZnO NPs	20–30	Embryos (24–120 hpf)	Behavior Malformations Mortality	20-nm spherical ZnO NPs causes a higher mortality and malformations when compared to the formulation without NPs	Niyaghi et al. 2014
Perillyl alcohol (NPOH)	130–160	Adults	Behavior Histology LC50 Mortality	Mortality depends on nanoformulations' concentration. At higher concentrations (> 50 μg/L) 100% of mortality is achieved.	Souza et al. 2016

alcohol-nanoemulsion (NPOH) in adult zebrafish. POH is an isoprenylation inhibitor of the Ras protein, which acts in the control of cell proliferation, in the activation of post-apoptotic pathways, and in the blockage of the cell cycle of different tumor cells in vitro (Souza et al. 2016). The nanoemulsions studied by Souza et al. (2016) had a particle size between 130 and 160 nm. Exposure of zebrafish to different concentrations of NPOH (25, 35, 50 and 125 μg/L) for 48 hpi induced behavioral alterations and LC50 was estimated at 0.0334 ppm. At higher concentrations (> 50 μg/L), 100% of mortality was achieved. According to Bilberg et al. (2011), increasing concentrations of nanoformulations induce higher mortality levels. As no mortality was observed in the group treated with 125 μg/L of surfactants, it was confirmed that the lethal effects were induced by the nanoemulsified POH.

Different nanomaterials are being widely used for applications in industrial and consumer products, such as metalworking nanofluids. However, little is known about their physicochemical attributes on toxicity and the effects they might produce in the environment and human health. This is clearly evidenced in the studies carried out by Niyaghi et al. (2014), where the authors referred to the great ability of zinc oxide nanoparticles (ZnO NPs) to improve lubrication and thermal conductivity, as well as to the promising possibility of using them as metalworking lubricant and coolant additives due to their low cost compared to other NPs. In that work, Niyaghi et al. (2014) designed microemulsions to stabilize these NPs and studied their toxicity in zebrafish embryos. They found that the addition of 20 nm spherical ZnO NPs caused a significantly higher mortality than the formulation without NPs. The LC50 was estimated at 45 ppm and 90 ppm, for microemulsions with ZnO NPs and microemulsions without ZnO NPs, respectively. In addition, a higher average number of malformations in surviving embryos was observed with the addition of ZnO NPs to the metalworking nanofluids (Niyaghi et al. 2014). The work also revealed that ZnO metalworking nanofluids had significantly higher toxicity than the prepared microemulsions. This demonstrates the need for precautionary development of metalworking nanofluids.

Based on all the abovementioned, we can conclude that the smaller the material, the greater the toxic effects in zebrafish, as the particle size is directly related to morphological alterations due to enhanced absorption through the gills (Souza et al. 2016). Moreover, many nanoparticles are toxic by themselves, but in many cases this toxicity could be reduced if the nanoparticle is combined in an emulsion. So, the mere fact of combining them could be considered a good option when designing low toxicity systems. In addition, as reported by Niyaghi et al. (2014), these NPs might be more stable inside the emulsion.

Liposomes

Liposomes are composed of derived lipids and phospholipids that mimic the properties of biological membranes. They have been widely used as carriers for delivery systems, since they offer great advantages such as controlled size during their production, composition with high drug/lipid ratios, and the ability to be in circulation for long periods. In addition, liposomes can be loaded with a desired molecule in a selective manner since hydrophilic and amphiphilic molecules can be entrapped in their core whereas hydrophobic molecules can be partitioned into the lipid bilayer. Liposomes have been used to transport small molecules (Fernandez Ruocco et al. 2013; Keller 2001) and macromolecules such as proteins (Hirota and Duzgunes 2011) and DNA (Chiaramoni et al. 2007,

2008, 2010; Hirota and Duzgunes 2011). Liposomes can also be further engineered with functional moieties to improve their performances in terms of circulation longevity, target-specific delivery, enhanced intracellular penetration, contrast enhancement for image-guided therapy, and stimuli-sensitivity.

Some authors have studied the efficiency of a desired drug when it is encapsulated in a liposome carrier system. For example, Yan et al. (2016) reported a study with liposomes of the antioxidant resveratrol-modified stearate, which forms nanoparticles capable of releasing drugs. These authors encapsulated an antiapoptotic drug and studied mortality in zebrafish embryos. They found that the toxicity of the drug was significantly higher in embryos incubated with the encapsulated drug than in those incubated with the free drug. Another example is that of a study carried out by Yuan et al. (2013), where an anti-angiogenic drug was incorporated in PEG-liposomes. In zebrafish, development defects were observed both with the encapsulated drug and with the drug in its free form. The authors demonstrated that liposomes do not disturb the anti-angiogenic role of the drug. Then, the authors used this liposome system for an i.v. therapy in mice and observed no side effects. In the same line, Ruyra et al. (2013) introduced an immunostimulant cocktail in liposomes and studied toxicity in zebrafish embryos and larvae by evaluating the hatching rate, mortality and malformations. They found that embryos were able to hatch and develop normally and observed no morphological defects. In addition, they worked with liposomes with different formulations that ended in different surface charge, and observed different survival of larvae, highlighting the importance of an appropriate design of the carrier. They studied the in vivo toxicity of cationic liposomes in different formulations, and observed that toxicity was affected by the dose and the concentration of encapsulated lipopolysaccharide in the lipidic bilayer of liposomes. When comparing the survival between embryos and larvae, Ruyra et al. (2013) found that the mortality in larvae was higher. Although not discussed by the authors, the difference may be due to the presence of the chorion in the embryo, which acts as a protective barrier.

Polymeric liposomes Lipopolymers are a particular kind of liposomes that can be obtained by a photopolymerization process. Since photopolymerizable lipids combine the plasticity of lipids with the robustness of polymers, they have received much attention in several biological areas (Alonso-Romanowski et al. 2003; Chiaramoni et al. 2007, 2008, 2010; Fernandez Ruocco et al. 2013; Gasparri et al. 2011). Lipopolymers have many potential applications in medicine because they present great stability, given by the photoactivable bonds in photopolymerizable lipids (Yavlovich et al. 2010). Polymerizable lipids contain a diacetylene moiety along their acyl chain. These lipids can be polymerized by UV irradiation to form chains of covalently linked lipids in the bilayer. Closely packed and properly ordered, diacetylene lipids undergo polymerization via 1,4-addition to form alternating ene-iyne polymer chains upon irradiation with 254 nm light (Gou et al. 2008).

Since lipopolymers are more stable than regular liposomes, they are very useful as drug delivery systems. This is mainly because the systems allow substance release in particular tissues, avoiding losses in other undesired cellular types. In this way, the toxicity is reduced while the therapeutic action is increased (Qin et al. 2011).

A wide variety of polymerizable lipids have been used for biological applications, such as 1,2-bis (10,12-tricosadinoyl)-sn-glycero-3-phosphocholine (DC8,9PC), one of the most studied. (Temprana et al. 2010) demonstrated that the polymerization efficiency of DC8,9PC and 1,2-dimirystoil-sn-glycero-3-phosphocholine (DMPC) in a [1:1] molar ratio is higher than that obtained when the molar ratio is [1:0.5] or [1:0.25]. In these mixtures, only DC8,9PC is polymerizable, so in irradiated bilayers there is a coexistence of large unpolymerized regions with polymerized domains, as shown in Fig. 4.

Fernandez Ruocco (2011) studied the effect of lipopolymers in zebrafish, and found that the complex formed between lipopolymers and L-arginine (0.025:0.0025 mg/mL) induced higher spontaneous movements respect to untreated animals. The authors proposed that the combination between lipopolymers and L-arginine results in a metabolic activation

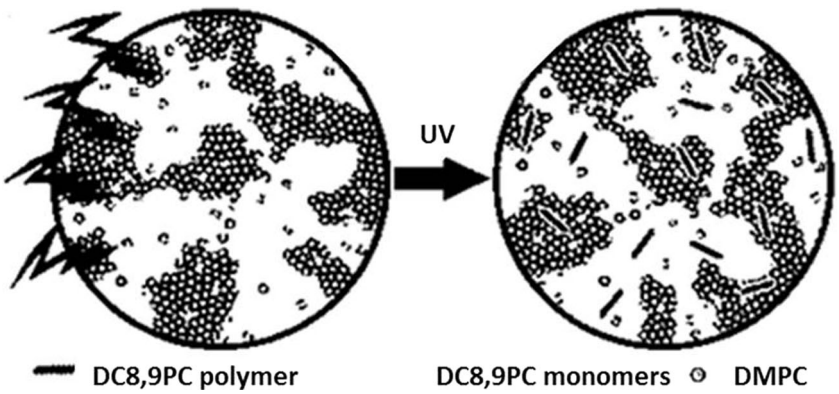

Fig. 4 Proposed model for lipopolymers formed with 1,2-bis (10,12-tricosadinoyl)-sn-glycero-3-phosphocholine (DC8,9PC) and 1,2-dimirystoil-sn-glycero-3-phosphocholine (DMPC) in a 1:1 M ratio(Temprana et al. 2010)

that is not induced by any of the two components independently (Fernandez Ruocco 2011).

Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are basically obtained from nanoemulsions of melted lipids in an aqueous phase in the presence of surfactants, formed at relatively high temperatures, under mechanical dispersion and high pressure homogenization, which, when allowed to cool at room temperature, return to solid state in a nanodisperse population (Siekmann and Westesen 1992). Their main usefulness is to be used as carriers for controlled release. The control of parameters during production could lead to different particle-size populations, thus obtaining the so-called solid lipid microparticles (SLMs) when their diameter exceeds the nanoscale. Nanostructure lipid carriers (NLCs) are considered a second generation of SLNs (Müller et al. 2002) in which the lipid is replaced by a mixture of it with a second lipid that is liquid at room temperature (e.g., an oil) that causes imperfections within the lipid crystal lattice of the particle, allowing a slower release and higher stability of the loaded actives.

In general, the lipids used are triglycerides (e.g., myristylmyristate), partial glycerides (e.g., glyceryl monostearate), fatty acids (e.g,. palmitic acid), steroids (e.g., cholesterol) and waxes (e.g., cetyl palmitate).

Yostawonkul et al. (2017) carried out in vivo toxicological studies in chorionated zebrafish embryos as a model to test a novel potential mucoadhesive carrier for hydrophobic drug molecules for cancer treatment (Table 5) (Yostawonkul et al. 2017). The carrier, an oleoyl-quaternized-chitosan coated NLC (CS-NLC), was studied in comparison to uncoated NLC. The authors observed that embryonic survival rates after 72 h of exposure to both carriers were dose-dependent. However, CS-NLC was more lethal and teratogenic than uncoated NLC. CS-NLC also produced malformations that were not observed in embryos treated with NLC. The authors stated that the mode of action that caused these effects should be studied. While the increase in the hydrodynamic diameter of NLC after surface coating was around 16%, the change of surface charge drastically changed from negative (uncoated NLC) to positive (CS-NLC). This difference could be responsible for the higher toxicity and teratogenicity, as cationic nanoparticles are generally more toxic than anionic ones (Frohlich 2012). Thus, CS-NLC might destabilize the cell membrane, cause cellular lysis and finally induce embryo death. The findings on the high toxicity of the carrier led the authors to conclude that its dosage should be carefully optimized.

Frederiksen et al. (2003) studied whether the encapsulation of gamma-cyhalothrin—a highly toxic insecticidE—into SLMs reduces its aquatic toxicity while retaining its desired activity (Table 5). For this purpose, these authors used adult zebrafish to test the toxicity of SLMs prepared under different homogenization conditions to obtain formulations that differed in particle size, with negative Zeta potential. The authors found that toxicity on zebrafish was independent of the particle diameter and 10-fold lower than the toxicity determined for the traditional emulsifiable concentrate formulation of the active.

The size of materials plays a critical role in how the organism responds to them. Small sizes lead to an exponential increase in surface area relative to volume, which makes the nanomaterial surface more reactive to its surrounding environment. At the scale of micrometers, it is expected that changes in size do not involve determinant changes in surface characteristics, whereas, at the nanoscale, a small change in size implies determinant surface alterations. This could explain the absence of size effect of SLMs in comparison to its possible contribution in NLCs.

The surface charge plays a predominant role in toxicity. Another key factor involved in the toxicity of SLN-derived systems is the composition of the solid lipid matrix. In the works mentioned above, the toxicity of SLN-derived systems was not assessed in terms of changes along time: the composition of the SLN-derived system can be modulated to obtain from burst releases to long-term stability of the actives into the lipid core of the particles. In terms of ecotoxicity, it would be interesting to perform studies considering longer previous storage periods.

On the other hand, the effects between embryos and adult fishes exposed to both particles cannot be compared linearly. Although there are studies demonstrating that it is possible to replace adult zebrafish with embryos and larvae, because there is a correlation in the effects observed, it is necessary to analyze the same system to compare results.

Other nanoparticles

The majority of the NPs studied for their toxicity in zebrafish are inorganic (Wehmas et al. 2015; Zhao et al. 2013). Little is known about biodegradable NPs, except for chitosan NPs (Wang et al. 2016; Yuan et al. 2016). Chitosan NPs are selected as vehicles for drug and gene delivery in brain target systems. Their main features are biodegradability and biocompatibility (Hu et al. 2011). Studies on these NPs have shown induced toxicity in zebrafish embryos by decreasing their hatching rate in a dose-dependent manner and malformations in the surviving embryos. This toxicity seems to be due to physiological stress. Wang et al. (2016) found that the toxicity of chitosan NPs as regards the hatching rate and the induction of malformations was dose-dependent. Some studies suggest that the main cause of the toxicity of NPs relies on their exposure time and concentration in the zebrafish

Table 5 Studies on the toxicity of solid lipid nanoparticles in zebrafish embryos

Nanoparticle	Hydrodynamic rato (nm)	Stage	Assays	Critical biophysical parameters	Reference
AgNP	21.5	Embryos/larvae (0–120 hpf)	Behavior Hatching rate Malformation	Spherical shape, negative charged nanoparticles. Unstable through time in experimental conditions. Dose-dependent hatching inhibition and locomotor toxicity	Ašmonaitė et al. 2016
Chitosan nanoparticle	200 and 340	Embryos/larvae (0–96 hpf)	Cellular apoptosis Hatching rate HSP70 protein Malformation Mortality ROS production	Ionic cross-linked chitosan, negative charged nanoparticles. Round shape with almost homogeneous structure. Dose- and time-dependent toxicity. The malformations depend on the NP size	Hu et al. 2011
Chitosan nanoparticle	85	Embryos/larvae (0–120 hpf)	Hatching rate Malformation Mortality	Ionic cross-linked chitosan, negative charged nanoparticles with sodium tripolyphosphate. Round shape almost homogeneous structure. Dose-dependent toxicity.	Wang et al. 2016
Chitosan nanoparticle (CS-NP) / Tween-modified chitosan nanoparticle (TmCS-NP)	247/251	Embryos/larvae (0–120 hpf)	Behavior Cellular apoptosis Development of primary and secondary motor neurons Free swimming activity Hatching rate Histopathology of skeletal muscle Light–dark swimming activity Malformation Mortality ROS production Spontaneous movements Tactile sensitivity test	Ionic cross-linked nanoparticles. Round shape almosy homogeneous structure. Dose-dependent toxicity, with increased oxidative stress effect higher for the TmCS-NP	Yuan et al. 2016
CoFe2O4NP	40	Embryos/larvae (0–96 hpf)	Antioxidant defense system (96 hpf) Cardiotoxicity CAT activity Cellular apoptosis Developmental toxicity GPx content Hatching rate Malformation MDA content ROS production SOD activity	Negative charge NP. CoFe2O4 NPs showed rapid aggregation/agglomeration and sedimentation in E3 medium over time, reducing the exposure concentration. Dose-dependent toxicity	Ahmad et al. 2015

Table 5 (continued)

Nanoparticle	Hydrodynamic rato (nm)	Stage	Assays	Critical biophysical parameters	Reference
Fluorescent silica nanoparticle	60 and 200	Embryos/larvae (0–96 hpf)	Embryo development Hatching rate Morphological alteration Mortality	Nanoparticles prone to aggregation, concentration-dependent. In media, both sizes of NP (200 and 60 nm) settled at the bottom of the well. Due to aggregation they could not enter the corion. Therefore, they are considered as non-toxic.	Fent et al. 2010
MONPs: ZnONP; TiO2NP; CeO2NP; SnO2NP	Variable depending on protocol preparation and media	Embryos/larvae (0–96 hpf)	Results on embryos varied according to preparation method and type of nanoparticle. Only ZnONP came out as toxic.	Biophysical characterization revealed differences among the same NP prepared in different media. As a general rule, every NP suspension presented impurities as free ions and aggregation in E3 media. Originally all NP were in the 2.8–11.6 nm range in diameter, but then showed an increase in size of ~150–1000×. All of them presented a spherical shape. The MONP showed littletoxicity due to NP agglomeration. As a consequence bioavailability was reduced. The ZnO NP was the most toxic towards embryos.	Wehmas et al. 2015
TiO2NP	33,4 (aggregate to 149,4 nm)	Embryos/larvae (0–96 hpf)	Hatching rate Malformation Mortality Locomotor activity ROS production Histopathology	Spherical shape NP. Prone to aggregation; from their original size of 33.4 ± 1.9 nm, in water they aggregated to 149.4 ± 1.3 nm. Dose-dependent toxicity. Accumulation of the NP observed in the brain.	Hu et al. 2017
MgONP	20 (666.2 in E3 media)	Embryos/larvae (0-144hpf) Adults	Cellular apoptosis (AO) Hatching rate Malformation Mortality ROS production	The NPs have polyhedral shape and present differente Z potential values according to the size; larger size, positive Z potential values. Prone to aggregation in media, sedimentation was observed 48 h after incubation. Dose-dependent toxicity.	Ghobadian et al. 2015
ZnONP	50–100	Embryos/larvae (0-144hpf) Adults	Antioxidant defense system (144 hpf) CAT activity DNA damage (Olive tail movement) GPx content Hatching rate Malformation MDA content ROS production SOD activity Mortality	Rod-shape powder, prone to aggregation in suspension.Dose-dependent toxicity.	Zhao et al. 2013
TiO2NP	20 (aggregate up to 1 μm)	Adults (0–120 hpf)	Hatching rate Morphological alteration Mortality Swimming behavior	NP showed aggregation and sedimentation through the assay with increasing concentration. No toxic effect was observed.	Chen et al. 2011

embryos: it might be more related to an immune response due to the presence of the foreign material in the body rather than to the nanoparticle per se (Wang et al. 2016). Regarding chitosan nanoparticles with Tween 80, a dose-dependent increase in toxicity was also observed (decreased hatching rate, increased mortality and incidences of malformation) (Yuan et al. 2016). In this last study, neurobehavioral alterations were also observed in zebrafish embryos exposed to NPs, chitosan and its counterpart with the detergent, suggesting that these NPs could affect embryonic development and larval neuro behavior (Yuan et al. 2016). Results of this study are described in Table 6.

Studies on the toxicity of biodegradable NPs should be encouraged because they are assumed to be innocuous, but as aforementioned, some toxicity level could be noticed and should be addressed. If this point is addressed, then explanations as to why the toxicity is caused could be found and solved much more easily.

Other nanoparticles popular in nanotechnology are metal oxide NPs, whose semiconducting properties serve their purpose in commercial sunscreen to block ultraviolet radiation (Wehmas et al. 2015). For instance, zinc oxide NPs are used in the fields of optoelectronics, cosmetics, catalysts, ceramics, and pigments, etc (Zhao et al. 2013). Magnesium oxide NPs are very stable under harsh process conditions and are considered non-toxic for the human health. These two properties have allowed using these NPsin vaccines and bactericidal compounds in aqueous environments (Ghobadian et al. 2015). Studies on inorganic NP have shown that, even at sublethal concentrations, these NPs can cause alterations in the hatching rate, malformations, survival and production of reactive oxygen species (Ahmad et al. 2015; Ašmonaitė et al. 2016; Fang et al. 2015; Ghobadian et al. 2015; Hu et al. 2017; Wehmas et al. 2015; Zhao et al. 2013). Fent et al. (2010) managed to achieve a fluorescent silica nanoparticle non-toxic for zebrafish embryos. Nonetheless, this type of nanoparticle is not toxic only because it aggregates in such a way that the resulting size does not allow chorion penetration. Due to this, toxicity of the fluorescent silica nanoparticle is hard to test (Fent et al. 2010). Despite the many studies found on several metal oxide NPs, results vary from work to work. The causes for the toxicity of these NPs in zebrafish embryos have been attributed to several factors, including size, composition, aggregation, ions in solution, etc., depending on the authors. Results are described in Table 6.

As a whole, the toxicity of nanoparticles should be tested in wide-screening concentration assays. The zebrafish represents an ideal in vivo model to this aim. Despite the different conclusions reached in the different works described above, it is imperative to reach a consensus as to how to test this toxicity and inform the results. Although the reasons as to why a nanoparticle might be toxic are important, it is more urgent to know whether, considering the way it is manufactured and the way it will be used, such a nanoparticle is an environmental or health risk.

Conclusions

Nanotechnology has become one of the most used technologies world-wide, and has conquered a wide variety of products such as medicines, pharmaceuticals, agricultural products, cosmetics, cleaning materials, electronic components, food additives, sports equipment and surface coatings (Hu et al. 2011; Patil and Kim 2017). However, little is known about the impact of nanoparticles in the environment and human health. The zebrafish is an adequate model to make a quick and cheap screening of novel nanoparticles, which allows the analysis of their possible toxic implications. The most common experiments to test toxicity are the hatching rate, malformations, cellular apoptosis, production of reactive oxygen species and mortality. In this review, information about several micro- and nanoparticles, including carbon nanotubes, dendrimers, emulsions, liposomes, metal nanoparticles, and solid lipid nanoparticles, has been shown, with special focus on the relationship between their biophysical properties and toxicity in zebrafish. Although the results found by the different authors are controversial, it is clear that variations in structure and composition of the same nanoparticle may cause differential toxicity. For this reason, it is critical to standardize

Table 6 Studies on the toxicity of nanoparticles in zebrafish embryos

Particle type	Stage	Assays	References
Nanostructure lipid carriers	Embryos/larvae (3–72 hpf)	Coagulation of fertilized eggs Malformations Pericardial edema Yolk sac edema	Yostawonkul et al. 2017
Solid lipid microparticles	Adults	Mortality	Frederiksen et al. 2003
Author	**Nanoparticle**	**Size**	**Zeta Potential (mV)**
Yostawonkul et al. 2017	NLC	126.1–147.1 nm	−48.1; +44.9
Frederiksen et al. 2003	SLM	0.73–100 μm	−33.6

protocols for the preparation of nanoparticles. Finally, not all studies can be compared since each has used different assays, reducing the possibility of a complete understanding of the toxicity of the different nanoparticles. It is thus imperative to normalize a test battery to evaluate their toxicity in zebrafish.

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Compliance with ethical standards

Conflicts of interest C.S. Martinez declares that she has no conflicts of interest. D.E. Igartúa declares that he has no conflicts of interest. M.N. Calienni declares that she has no conflicts of interest. D.A. Feas declares that she has no conflicts of interest. M. Siri declares that she has no conflicts of interest. J. Montanari declares that he has no conflicts of interest. N.S. Chiaramoni declares that she has no conflicts of interest. S.delV. Alonso declares that she has no conflicts of interest. M.J. Prieto declares that she has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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