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Zebrafish as a preclinical *in vivo* screening model for nanomedicines

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ABSTRACT

The interactions of nanomedicines with biological environments is heavily influenced by their physicochemical properties. Formulation design and optimization are therefore key steps towards successful nanomedicine development. Unfortunately, detailed assessment of nanomedicine formulations, at a macromolecular level, in rodents is severely limited by the restricted imaging possibilities within these animals. Moreover, rodent *in vivo* studies are time consuming and expensive, limiting the number of formulations that can be practically assessed in any one study. Consequently, screening and optimisation of nanomedicine formulations is most commonly performed in surrogate biological model systems, such as human-derived cell cultures. However, despite the time and cost advantages of classical *in vitro* models, these artificial systems fail to reflect and mimic the complex biological situation a nanomedicine will encounter *in vivo*. This has acutely hampered the selection of potentially successful nanomedicines for subsequent rodent *in vivo* studies. Recently, zebrafish have emerged as a promising *in vivo* model, within nanomedicine development pipelines, by offering opportunities to quickly screen nanomedicines under *in vivo* conditions and in a cost-effective manner so as to bridge the current gap between *in vitro* and rodent studies. In this review, we outline several advantageous features of the zebrafish model, such as biological conservation, imaging modalities, availability of genetic tools and disease models, as well as their various applications in nanomedicine development. Critical experimental parameters are discussed and the most beneficial applications of the zebrafish model, in the context of nanomedicine development, are highlighted.

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1. Introduction

Drug delivery using nanoparticulate carrier systems (*i.e.* nanomedicines) is an effective way to enhance drug concentrations within specific target tissues and minimize side effects in off-target organs [1]. To effectively exploit these unique pharmacokinetic features of nanomedicines, the physicochemical properties of the underlying nanoparticles need to be optimized. Ideally nanomedicines should demonstrate low cytotoxicity, stability in biological environments, controlled blood circulation half-life, cell/tissue specificity and efficacy/functionality under *in vivo* conditions. To this end, nanomedicine formulations are designed and optimized according to almost endlessly tunable parameters. These include chemical composition, size, shape, surface charge or surface modification. Therefore, the preclinical development and evaluation of nanomedicines typically follows the same route taken as for traditional drug development, namely physicochemical characterization, *in vitro* experiments and finally rodent *in vivo* studies [2]. During this development pipeline, the number of investigated nanomedicine candidates decreases with each step, due to increasing experimental costs and complexity. However, as recently outlined by Dai *et al.*, nanomedicine performance is heavily affected by biological features which are mimicked during specific experimental set-ups [3]. In particular, the presence of serum and/or extracellular matrix proteins, heterogeneous cell populations (including cells of the immune system) and dynamic blood flow, particularly varying levels of shear stress, have all been shown to be critical to nanomedicine performance. Despite the vast battery of well characterized and increasingly sophisticated *in vitro* models, there remains a huge gap between cell culture experiments and rodent *in vivo* studies in terms of accurately mimicking the full complexity of a living animal. In light of this, significant efforts have been made in recent years to increase the predictive value of *in vitro* experiments through the development of 3D cell (co-) cultures and/or organ-on-a-chip set-ups [4–7]. However, the reproducible generation and characterization of these sophisticated cell culture models remains extremely challenging [8]. In the case of organs with a complex architecture such as the liver, accurate cell culture models are still missing, among other things due to notoriously difficult cultivation of liver sinusoidal endothelial cells. Furthermore, cell-based systems are often more sensitive in terms of nanomedicine toxicity, as they suffer from poor particle distribution and the inability to compensate stresses *via* homeostatic balances [9].

The absence of early and easy accessible *in vivo* screening tools to assess the effects of various nanomedicine formulation parameters under complex biological conditions has hindered effective nanomedicine formulation design and optimization (*e.g.* accurately tailoring the composition). This has resulted in a high rate of drop-outs during early phases of nanomedicine development [10] and limited understanding of nanomedicines' *in vivo* behaviour [11]. Alternative vertebrate animal models that are available in large numbers, easy to handle, cheap to house and maintain, and applicable to nanomedicine formulation screening approaches, are therefore of great interest to bridge the gap between *in vitro* and rodent *in vivo* studies.

Over time, different *in vivo* model systems such as nematodes (*Caenorhabditis elegans*), frogs (*Xenopus laevis*), chicken embryos, and zebrafish[1] (*Danio rerio*) have been introduced to answer various biological questions related to nanoparticles and nanomedicines [12–14]. While zebrafish are well established as a model system in developmental biology [15–20], increasingly these organisms are being used as *in vivo* models in biomedical research, most prominently as platforms for high throughput screens of small molecule drug candidates either in target- or phenotype-based approaches [21–25]. Critical experimental parameters when using zebrafish for biomedical screening (*i.e.* zebrafish age, sample size, concentrations, wild type versus transgenic lines) have been summarized by Rennkamp et al. [21]. The popularity of these biomedical screens has been further boosted by the development of several partially automated readout technologies (*e.g.* light/dark preference test, open field test, visual motor response test)[26]. Given the current empirical approach to nanomedicine design and optimization, and the almost endless variations in potential nanomedicine composition, shape, size, surface charge and surface modification, nanomedicines could potentially benefit significantly from this emerging *in vivo* model. Indeed, recent studies have demonstrated the potential of the zebrafish as an early and easily accessible *in vivo* tool during nanomedicine development [27,28].

To this end, this review focuses on applications of the emerging zebrafish model (Fig. 1) to facilitate nanomedicine formulation design and optimization prior to rodent studies. Zebrafish characteristics which are of special interest for biomedical research, such as the conservation of key biological features, imaging modalities and the availability of genetic tools and disease models are discussed in detail. Assessment of important nanomedicine characteristics including toxicity, *in vivo* stability and functionality, biodistribution and blood circulation properties, and targeting efficiency within the zebrafish are described and the most suitable experimental set-ups are emphasized. Since comparability and standardization of such experiments are of great importance, critical experimental parameters are also highlighted and discussed.

2. Important zebrafish features for nanomedicine research

Zebrafish larvae have several advantageous properties over adult zebrafish or rodents, which makes them attractive to screen nanomedicines. Firstly, the costs of zebrafish husbandry are low (compared to mice or rats) and larvae are available in large numbers and develop external from the mother. This allows for high-throughput screening set-ups under *in vivo* conditions. Secondly, information networks such as ZFIN (zfin.org), combined with the fact that embryos or frozen sperm can be easily transferred between labs, guarantee fast and easy access to specific transgenic zebrafish lines. Thirdly, the optical transparency of zebrafish larvae, which can be chemically prolonged up to several days (*e.g.* using 1-phenyl-2-thiourea (PTU) to inhibit melanogenesis [29]), enables high resolution (fluorescence) imaging of specific biological events in real time and across entire the living organism. Alternatively to PTU treatment, a transparent zebrafish line (*i.e.* Casper) has been generated which still lacks pigmentation in the adult stage [30]. Finally, numerous molecular and biological tools are available to create new genetically modified zebrafish lines. These include TILLING [31], morpholino oligonucleotides [32], zinc-finger nucleases [33], TALENs [34], and CRISPR/Cas [35], Tol2 transposons combined with bacterial artificial chromosomes [36], and in situ hybridization [37]. Using these tools, many zebrafish lines with particular relevance to nanomedicine development have been generated and are summarized in Table 1. The availability of transparent zebrafish larvae, fluorescent reporter lines and sophisticated imaging techniques, such as confocal or light sheet microscopy, are key factors that enable the investigation of nanomedicine behavior *in vivo* at a macromolecular level.

[1] Developmental stages of zebrafish described as follows: zebrafish = no specific developmental stage, zebrafish embryo = until 48 h post fertilization (hpf), zebrafish larvae = 48 hpf – 3 week post fertilization, adult zebrafish = >3 weeks post fertilization.

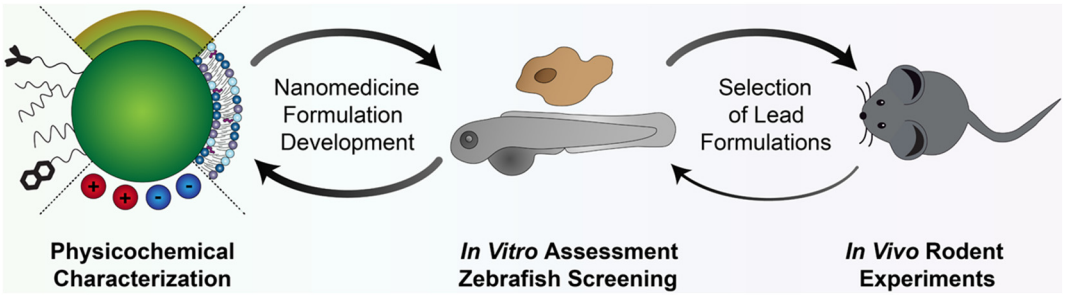

Fig. 1. Schematic representation of nanomedicine formulation design and optimization including the emerging zebrafish model. The complementary application of classical *in vitro* systems and the zebrafish model offers the possibility to screen the effects of varying nanomedicine formulations and physicochemical properties under complex biological conditions. This facilitates the selection of promising lead formulations for subsequent rodent *in vivo* studies.

Proposing the zebrafish as an *in vivo* tool for nanomedicine characterisation raises immediate questions regarding the conservation of relevant biological features and the accuracy of translating findings to mammals. At the genomic level, 76% of human genes (82% of disease-related genes) have orthologues in zebrafish, compared to 80% and 84% in chicken and mice, respectively [47]. Epigenetic markers, which regulate conserved genes between these species, are highly consistent [48]. Zebrafish anatomy and physiology are well described [49–52] and physiological parameters and organ systems of particular interest in terms of nanomedicine toxicity and biodistribution, such as the vascular system [53–55], blood composition [56,57], immune system [58,59] including a lymphatic system [60], blood-brain barrier [61–63] and liver [64,65] have been extensively studied and share many important physiological homologies with their mammalian equivalents [66]. The rapid development of the zebrafish embryo results in a compartmentalized brain and the presence of eyes, ears and internal organs after one day post fertilization (dpf) [29,67,68]. However, maturation of some key organs, particularly the adaptive immune systems, occurs at later developmental stages. When considering a zebrafish study, developmental stage is therefore a critical experimental parameter as discussed in Section 3.

As a key cell type of the mononuclear phagocyte system (MPS), macrophages are among the first cell types that respond to administered nanomedicines [9]. Importantly, early embryonic macrophages have been found to be present and functional at very early stages of zebrafish development (30 h post fertilization, hpf) [69]. In addition, other key cell types defining the mammalian MPS, including monocytes and dendritic cells, are present in adult zebrafish [70]. Macrophage polarization is reported to change from M1 to M2 upon tumor tissue infiltration. This is an important feature of tumor growth and progression [71] and conversion of M1 to M2 macrophages has been demonstrated in larval zebrafish to highlight the diversity and plasticity of zebrafish macrophages as well as similarities to their human counterparts [42]. The rapid development of zebrafish immune cells again highlights the importance of choosing an appropriate zebrafish developmental stage.

Interactions of nanomedicines with cells are heavily dependent on the adsorbed protein corona. In the case of intravenously (*i.v.*) administered nanomedicines, the protein corona comprises blood serum components [72]. In general, zebrafish and human plasma proteomes share striking similarities, in particular regarding the conservation of apolipoproteins and complement factors [73]. Surface opsonization *via* complement factors C3, C4, and C5 is an important initial step towards nanoparticle recognition by macrophages [74] and these proteins are highly conserved in zebrafish, including conservation of respective signalling pathways [75]. Likewise immunoglobulins (Igs), such as IgG and IgM, are known to tag nanoparticles as "foreign" material in the body, again initiating nanomedicine clearance by macrophages [76,77]. As in humans, zebrafish Igs are composed of a light- and heavy-chains, bearing variable V, D, and J segments that are generated through recombination. Junctional diversity and hypermutation further amplify variety of the antibody repertoire. In contrast to humans, where five classes of Igs are known, zebrafish possess only three different Ig isotypes: IgM, IgZ and IgD [78]. Moreover, different cells express varying types of apolipoprotein receptors, which greatly affects the biodistribution of apolipoprotein coated nanoparticles [79–81]. Otis *et al.* characterised the zebrafish as a suitable model for apolipoprotein biology [82], finding a generally conserved physiological role of apolipoproteins despite low genetic sequence similarity. Abundant serum proteins such as albumin, fibrinogen, and transferrin [83] are major components of the characterized protein corona of nanoparticles in mammals [77]. Whereas fibrinogen and transferrin are present in zebrafish [73], a coding gene for the albumin- paralogue, vitamin D binding protein, but not albumin itself, has been found [84].

Regarding the investigation of organ pathology the zebrafish has shown to be a valid tool [85–88]. Zebrafish disease models at varying developmental stages for the cardiovascular system [89–91], liver [92,93], kidney [94,95] and immune system, including the spleen [96–99], are available. Notably, all these organs can significantly influence nanomedicine biodistribution and clearance.

Table 1
Overview of zebrafish lines with particular value for the *in vivo* characterization of nanomedicines. Various promoters and fluorescent reporter proteins can be combined in almost any way. An exemplary selection of existing zebrafish lines is highlighted here together with their specific characteristics and possible applications in nanomedicine formulation development and optimization.

Zebrafish line	Reference	Specific characteristic	Possible application
Casper	[30]	Transparent adults	Long term tumor models, fluorescence imaging of adult zebrafish
Tg(flk1:EGFP)	[38]	Fluorescent vasculature	Blood circulation behavior
Tg(lyve1:EGFP)	[39]	Fluorescent lymphatic system	Lymphatic uptake and distribution
Tg(zmpo:GFP)	[40]	Fluorescent neutrophils	Immune systems interaction
Tg(mpeg1:mCherry)	[41]	Fluorescent macrophages	Immune systems interaction
Tg(tnfa:EGFP-F)	[42]	Fluorescent M1 macrophages	Immune systems interaction
Tg(l-fabp:DBP-EGFP)	[43]	Fluorescent Vitamin D binding protein	Binding to albumin paralogue
TgBAC(cldn5a:EGFP)	[44]	Fluorescent brain endothelial cells	Brain delivery
Stab2 mutant	[28]	No stabilin 2 receptors	Scavenger receptor interactions
LDLR mutant	[45]	Low density lipoprotein receptor deficiency	LDLR dependent biodistribution, hepatocyte or brain targeting
Apoc2 mutant	[46]	Apolipoprotein loss of function	Apoc2 dependent biodistribution

Beside the aforementioned advantageous features of the zebrafish model, there are practical limitations, primarily due to the small size of the experimental system. Firstly, blood sampling from zebrafish larvae and even adult zebrafish is difficult. Secondly, only low amounts of biomolecules, *e.g.* proteins, are available for further analysis due to the small size of zebrafish larvae or respective tumor burden (see Section 4.5). These challenges often require pooling several zebrafish larvae for analysis, which excludes the possibility to observe differences between individual animals, as it is for example possible in rodent *in vivo* studies. However, analysis of high numbers of zebrafish larvae increases the statistical power. In addition, there are technical limitations compared to *in vitro* models. Protocols including the inhibition and fluorescent staining of specific cellular uptake and trafficking mechanisms are mostly designed for *in vitro* set-ups [100,101] and still need to be optimized in order to routinely apply them in zebrafish. Furthermore, generating stable transgenic zebrafish lines expressing fluorescent proteins or specific targeting receptors requires several months, which can be an experimental constraint, especially when compared to simply transfecting cells *ex vivo*. Overall, zebrafish-based test systems have to be validated carefully by comparing them to established protocols. This will ultimately increase acceptance in the scientific community and facilitate the use of the zebrafish model during preclinical screening of nanomedicine formulations.

3. Critical parameters of zebrafish experiments

The success and reproducibility of nanomedicine zebrafish studies is affected by several experimental parameters which are discussed in the following section (Fig. 2). Developmental stage and experimental timing are the first parameters that must be carefully defined when planning a zebrafish study. The stages of zebrafish embryonic development and the presence of major vertebrate organ systems are well described and easy to predict [67,102]. As already indicated, zebrafish development including the gradual loss of transparency, organ maturation and the development of the immune system occurs over relatively short time frames (hours to days) and may influence experimental outcomes. Depending on the study objective, different stages of development are recommended as start point. For example, generation of genetically modified zebrafish lines using mutagens, such as *N*-Ethylnitrosourea (ENU), are performed on adult zebrafish whereas genetic constructs/systems, such as capped mRNA, expression plasmids or CRISPR-Cas, are preferentially injected at the single-cell stage [103]. Exploiting the optical transparency of the zebrafish larvae, fluorescently labeled nanomedicine injections are often coupled with fluorescence-based imaging. Importantly, the injection time points of different nanomedicine formulations, required controls and replicates should be planed carefully to ensure consistent intervals between injection and imaging.

Various administration routes of nanomedicines into the zebrafish have been used. These include, for example, oral administration, simple addition to the zebrafish media and both *i.v.* and intraperitoneal (*i.p.*) injections. Choosing a suitable administration route is partly dependent on the required dosing accuracy and zebrafish developmental stage. Oral administration by gavage has been performed in adult zebrafish [104,105], as well as in larvae [106], but will not be further discussed given the very few reports of orally administered nanomedicines. Injecting nanomedicines into blood circulation is often performed *via* the easily accessible duct of Cuvier, a comparatively large blood vessel of the embryonic zebrafish that continuously remodels and reduces in size until 120 hpf [107]. Alternatively, local CNS (*i.e.* brain ventricle) [108], retro-orbital [109] or *i.p.* injections [110] have been described. These injections are generally performed at later developmental stages. Finally, direct injection into the blood island/caudal hematopoietic tissue (CHT) is often used in infection models within the embryonic fish.

Using a microinjector-system, precise injections of samples can be achieved. By adjusting air pressure and volume, injection volumes can be calibrated by injecting samples into mineral oil followed by drop size measurements with a scale included in the microscope ocular, the injection base [111] or using microscopy calibration slides. For injections *via* the duct of Cuvier, special care must be taken to avoid injection into the yolk sac. Material injected into the yolk will not enter circulation, leaving an unknown sample volume in circulation. The blood volume of a zebrafish larvae at 2 dpf is around 60 nl, therefore *i.v.* injected sample volumes should not exceed low nanoliter ranges (*i.e.* up to 3 nl) [112].

Experimental temperature is another critical factor. Incubation temperature, during early zebrafish developmental stages, affects the rate of development and the innate immune response of zebrafish larvae [113]. In general, it is known that zebrafish cope with stress (*i.e.* chemical exposure, pain) by choosing regions of higher water temperatures. Temperature dependent physiological processes (*e.g.* immune response) can in turn affect experimental results [114–116], meaning experimental temperature must be carefully selected and standardized, especially when assessing processes involving the zebrafish immune system (*e.g.* nanomedicine clearance by macrophages). Furthermore, physicochemical nanoparticle properties can also vary dependent on body temperature thereby influencing nanomedicine pharmacokinetics [27]. Of particular note here are lipids (*e.g.* DMPC) with phase transition temperatures between 28°C (zebrafish) and 37°C (mammalian) for which small variations in experimental temperature can become critical.

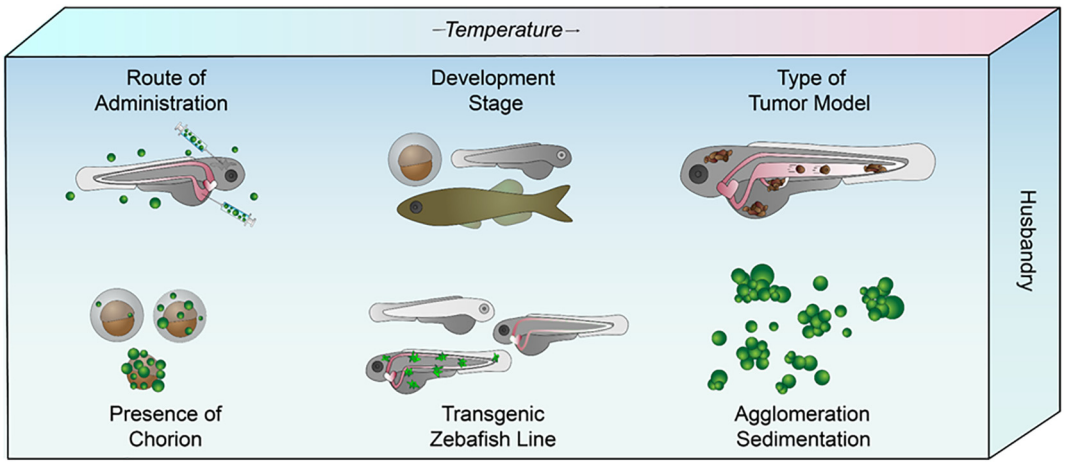

Fig. 2. Critical experimental parameters affecting the results of nanomedicine zebrafish studies. The appropriate selection of zebrafish developmental stage and the most suitable zebrafish line is crucial. In addition, the route of administration of nanomedicines, such as incubation in media or intravenous injection, strongly influences experimental results. Zebrafish husbandry and maintenance dictates many factors, such as water conductivity, pH, feeding, and fish density. They should be considered and controlled to guarantee consistent experimental conditions.

Notably, elevated temperatures over longer periods of time can affect physiological processes of the zebrafish, mediated by the presence of heat shock proteins [117,118]. Therefore, performing experiments under varying temperature conditions should be considered carefully, strictly controlled and exact conditions should be reported.

4. Applications of the zebrafish in nanomedicine development

Given the many advantages over rodent counterparts, zebrafish larvae are increasingly used as model systems during nanomedicine formulation development and optimization. Nanomedicine toxicity, biodistribution and systemic circulation, stability, functionality and targeting efficiency have all been successfully assessed within the complex biological, *in vivo* environment of a living zebrafish larvae (Fig. 3). Table 2 summarizes the experimental details of various nanomedicine studies using the zebrafish model. Since nanomedicines are most frequently developed as potential cancer therapies, the generation, compatibility (with well characterized mouse models [119–122]) and use of zebrafish cancer models will be discussed in Section 4.5 of this review.

4.1. Toxicity assessment of nanoparticulate drug delivery systems

Toxicological assessment of nanomedicines was one of the first applications to combine nanomedicines and zebrafish and is covered in several reviews [123–125]. In general, nanotoxicity studies involve exposing zebrafish embryos to nanoformulations *via* addition to the zebrafish media. This approach is, at the very least, questionable with respect to dosing accuracy, actual exposure and the stability of nanoparticles in zebrafish media. In testing the overall toxicity of nanomedicines, properties of the encapsulated drug will also affect experimental outcomes. Based on varying logP values, drugs permeate differently into zebrafish skin, a factor that will significantly affect the ability to control and standardize dosage and exposure [23]. Henn et al. showed that results of toxicological assays or drug screenings (*i.e.* chemical exposure), were differentially affected by the chorion surrounding the zebrafish embryo [126]. In a related study, Paatero et al. tested toxic effects of different nanoparticles following incubation with normal and dechorionated embryos, as well as injection of the same samples into 4 hpf embryos [127]. This study revealed differing abilities of nanoparticles to penetrate biological barriers, which significantly influenced toxicological profiles. To overcome this variable, the chorion can be removed by either enzyme supported- or mechanical dechorionation [126]. To standardize exposure of single zebrafish embryos in toxicological screens, the presence or absence of the chorion, assay volume, nanoparticle concentration and number of zebrafish/well has to be clearly stated. Going one step further, Pan et al. quantified nanoparticle uptake in individual zebrafish embryos through inductively coupled plasma mass spectrometry of digested zebrafish embryos/larvae obtained *via* an aqua regia-based microwave digestion protocol [128]. Although rigorous, this approach is time consuming which hampers its use for the screening of large nanomedicine libraries. Taken everything together, direct injection of nanomedicines into zebrafish larvae allows for a precise control of zebrafish exposure and should therefore be the method of choice. As an example, Vibe et al. assessed the toxicity of free and nanoparticle formulated drug upon injection into zebrafish larvae [129]. Thus, uncertainties regarding the actual drug exposure were excluded enabling the observation and analysis focused solely on nanoformulation effects. In contrast, simple addition of formulations to the fish water should be avoided since the amount of test substance taken up by the animal cannot be controlled. An exception are long-term exposure studies in the field of eco-nanotoxicology.

Nanoparticle aggregation and sedimentation are heavily affected by the suspension media. In contrast to cell culture media containing bicarbonate/CO_2 and buffering agents, sample addition to un- or only slightly buffered zebrafish media can induce pH changes (possibly resulting in false positive results) and should therefore be carefully monitored [128]. If nanoparticle toxicity is tested *via* addition to the zebrafish media, colloidal stability should also be assessed in that same media (*i.e.* E3 medium [130]) to ensure uniform exposure. Along these lines, Kiene *et al.* first determined the highest non-toxic nanoparticle concentration *in vitro* before proceeding to test their nanoparticles in a zebrafish embryo toxicology assay (mortality, morphology, hatching rate) with accompanying size and polydispersity measurements [131].

Toxicological readouts, such as survival or malformations, can be assessed in a controlled and relatively fast manner [132]. However, given the degree and severity of malformations is often subjective. In an attempt to improve comparability and reproducibility of these experimental outcomes semi-quantitative scoring systems have been

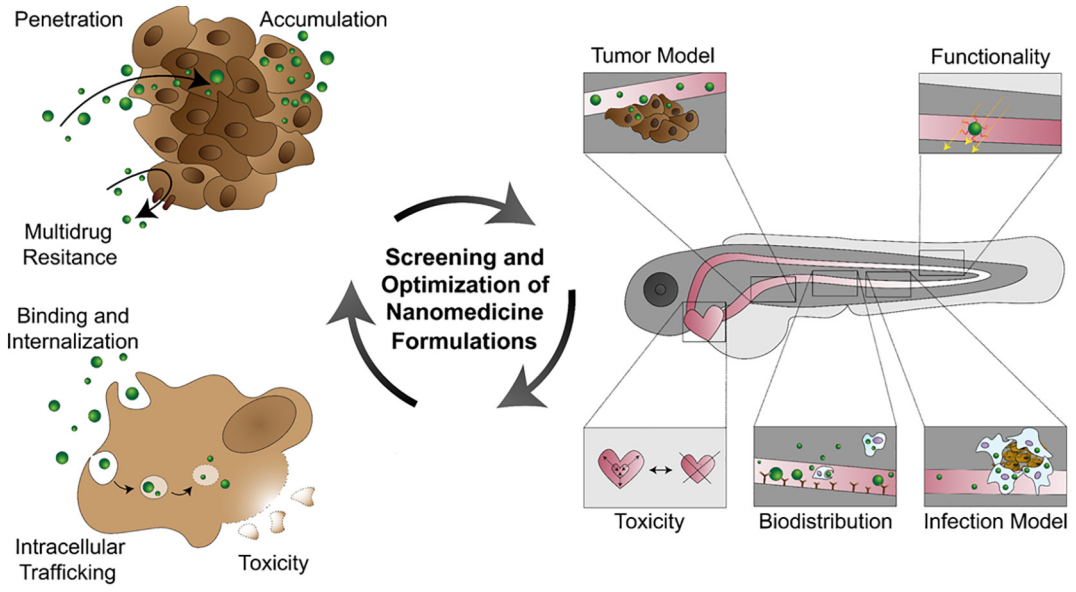

Fig. 3. Complementary application of *in vitro* and zebrafish model experimental set-ups for nanomedicine formulation design and optimization. The complementary application of classical 2-D (bottom left) and sophisticated 3-D (top left) *in vitro* systems and the zebrafish model (middle right) offers the possibility to assess nanomedicine interactions with biological environments under complex biological conditions. The availability of an optimized preclinical, *in vivo* screening platform increases the chance to identify potentially successful nanomedicine formulations prior to translation into rodent *in vivo* studies.

Table 2
Summary of nanomedicine studies using zebrafish to investigate toxicity, biodistribution, functionality and tumor treatment.

Application	Experimental readouts	Administration (nanomedicine/cancer cells)	Zebrafish line	Developmental stage	Reference
Nanotoxicology	Mortality, Blood flow, Tissue penetration	Incubation*/Injection	*Tg(kdrl:EGFP s843)*	6–24 hpf	[127]
	Morphology, Hatching, Mortality, Induction of Heat-Shock Protein	Incubation*	Wild-type, *Tg(hsp70:GFP)*	0 hpf	[128]
	Mortality, Swim bladder inflation, Yolk discoloration, Pericardial edemas	Injection	*Tg(nacre$^{-/-}$;roy$^{-/-}$)*	72 hpf	[129]
	Mortality, Morphology, Hatching	Incubation*	Wild-type (AB)	4 hpf	[131]
	Mortality	Injection	N.A.	1-cell stage	[132]
	Development, Morphology, Mortality	Incubation*	Wild-type (AB)	2 hpf	[133]
	Morphology, Mortality	Incubation*	Wild-type (AB)	0–2 hpf	[134]
	Development, Morphology, Apoptotic cell death, Heart functionality	Incubation*	Wild-type (AB), *Tg(cmlc2:EGFP)*	6–30 hpf	[136]
	Gill injury, Biochemical liver and kidney toxicity, Mortality	Incubation*	Wild-type	Adult	[138]
	Mortality, Morphology, Heart rate, Apoptotic cell death, Tissue distribution	Incubation*	N.A.	0 hpf	[139]
	Locomotion, Hatching, Morphology, Mortality, Body weight, Thyroid hormone levels	Incubation*	Wild-type (AB)	2 hpf	[140]
	Mortality, Morphology, Locomotion, Neuronal cell volume	Incubation*	Wild-type (AB), *Tg(lsl1:EGFP)*	48–96 hpf	[141]
Biodistribution	NP extravasation and blood circulation behavior	Injection *via* duct of Cuvier	*Tg(kdrl:GFP)*	48 hpf	[27]
	Scavenging receptor, macrophage and neutrophil mediated nanoparticle clearance	Injection *via* duct of Cuvier	*Tg(kdrl:GFP)^{s843}, Tg(kdrl:RFP-CAAX)^{s916}, Tg(mpeg:GFP)^{gl22}, Tg(mpeg:RFP-CAAX)^{ump2}, TG(flt1^{enh}:RFP)^{hu5333}, Tg(flt4^{BAC}:YFP)^{hu7135}, Tg(mpx:GFP)^{uwm1}*, CRISPR/Cas9 mutants	52–56 hpf	[28]
	Macrophage clearance	Injection *via* duct of Cuvier	*Tg(kdrl:EGFP), Tg(mpeg1:Gal4;UAS:Kaede)*	48 hpf	(Sieber et al., manuscript in preparation)
	NP localization in blood vessels	Injection *via* heart	*Tg(kdrl:GFP), Tg(gata1:DsRed)*	52 hpf	[149]
	Endothelial cell association, Tissue penetration	Injection *via* duct of Cuvier	*Tg(kdrl:GFP)*	52 hpf	[150]
	Endocytosis and stability	Injection *via* duct of Cuvier	*Tg(kdrl:GFP)*	52–56 hpf	[151]
	Transport across biological barriers	Incubation*	Wild-type	Embryo, Larvae, Adult	[153]
	Presence in whole body, brain, eye, and intestine, Intestinal absorption	Incubation*, Oral administration (gavage), Injection into intestine	Wild-type	24–168 hpf, Adult	[154]
	Ability to cross the BBB	Injection *via* heart	Wild-type	144 hpf	[155]
	Presence in brain, blood stream, and spinal cord	Injection *via* caudal vein, spinal cord, and brain	*Tg(-3mnxl:TagBFP), Tg(isl1:GFP), Tg(GFAP:EGFP), Tg(fli1a:EGFP)*	96–144 hpf	[156]
	Uptake into infected and uninfected macrophages	Injection *via* caudal vein	Casper, *Tg(mpeg1:mcherry), Tg(lyz:DsRed2), Tg(fli1:EGFP)*	72–120 hpf	[163]
	Accumulation in tuberculosis granulomas	Injection *via* posterior caudal vein	*Tg(fli1a:EGFP)*, Wild-type	48-52hpf	[164]
Functionality	Photothermal triggered drug release	Injection *via* brain ventricle	N.A.	120 hpf	[170]
	Photothermal induction of ROS generation	Injection of cancer cell/NP mixture *via* duct of Cuvier	Casper	30 hpf	[171]
	Reduction of xenograft cell viability upon singlet oxygen generation	Injection *via* cardinal vein	N.A.	48 hpf	[172]
	Laser induced formation of plasmonic nanobubbles	N.A.	N.A.	Blastula stage	[173]
	Enzyme activity, Stability	Injection *via* duct of Cuvier	Wild-type (ABC/TU)	48 hpf	[174]
	Enzyme activity, Stability	Injection *via* duct of Cuvier	Wild-type (ABC/TU), *Tg(mpeg1:Gal4;UAS:Kaede)*	48 hpf	[175]
	Transfection efficiency	Injection into embryo interlayer	N.A.	1-cell stage	[180]
	mRNA delivery	Injection into yolk, *via* hindbrain ventricle, caudal vein, trunk, and pericardial cavity	Wild-type (AB)	1-cell stage, 24–48 hpf	[181]
	Gene silencing	Injection	*Tg(fli:EGFP)*	Sphere stage	[182]

(continued on next page)

Table 2 (*continued*)

Application	Experimental readouts	Administration (nanomedicine/cancer cells)	Zebrafish line	Developmental stage	Reference
Tumor Treatment	Tumor proliferation analysis	Cancer cell injection *via* the perivitelline space, NP *via* yolk	N.A	48–144 hpf	[229]
	Cancer cell targeting	Cancer cell injection *via* duct of Cuvier, NP *via* the caudal vein	*Tg(fli1:EGFP)*	48–53 hpf	[230]
	Tumor growth inhibition	Cancer cell injection *via* the perivitelline space, NP incubation*	*Tg(FLK-1:EGFP), Tg(FLK-1:mCherry)*	48 hpf	[231]
	Tumor growth inhibition, Tumor spreading	Injection of cancer cell/NP mixture into yolk	N.A.	96 hpf	[232]
	Tumor vascularization	Injection of cancer cell/NP mixture *via* perivitelline space	*Tg(fli1a:EGFP)*, Wild-type	48 hpf	[233]
	Tumor vascularization	Injection of cancer cell/NP mixture into yolk	Wild-type (AB)	48 hpf	[234]
	Metastasis	Pretreated cancer cell injection into yolk	Tg(kdr*l*:mCherry)	48 hpf	[235]
	NP tumor accumulation, Tumor growth, NP circulation, NP macrophage uptake	Cancer cell injection *via* duct of Cuvier, NP injection *via* caudal vein	Wild-type (AB), *Tg(fli1:EGFP)y1, Tg(mpeg1:mCherry)*^{UMSF001}	48–96 hpf	[236]
	Angiogenesis, Tumor growth, Metastasis	Cancer cell injection *via* perivitelline space, NP incubation*	*Tg(FLK-1:EGFP)*	48 hpf	[238]
	Tumor cell migration/invasion	Cancer cell *via* hindbrain ventricle, NP incubation*	5D tropical strain	48–96 hpf	[239]
	PCR analysis of VEGF RNA as a marker of cancer cell growth	Injection *via* common cardinal vein	*Tg(fli1:GFP)*	120 hpf	[240]
	PCR analysis of cancer cell specific RNA	Cancer cell injection into yolk,	Tübingen Wild-type	48–144 hpf	[241]
	Evaluation of optimal targeting ligand density	Cancer cell and NP injection *via* duct of Cuvier	*Tg(kdrl:EGFP), Tg(mpeg1:Gal4;UAS:Kaede)*	48 hpf	(Witzigmann et al., manuscript in preparation)

For each study, experimental readouts are included. Administration routes of nanomedicines and/or cancer cells (for the generation of zebrafish tumor models) are indicated as well as transgenic zebrafish lines used and zebrafish development stage during the course of experiments. References are sorted according to their application and listed in order of their appearance in the manuscript. It has to be noted that simple incubation of zebrafish with nanomedicines (*) is not recommended as exposure and dose cannot be controlled. NP = nanoparticle, hpf = hours post fertilization, N.A. = information not available.

proposed by different experts [133,134]. Accompanying these scoring systems are newly developed nanotoxicity readouts focusing on behavioral aspects of zebrafish larvae and aimed at greater automation and sample throughput. These include automated procedures for studying locomotion parameters such as swimming speed and depth [135].

Interestingly, nanomedicine toxicity can been visualized at a cellular level within zebrafish embryos/larvae [136]. For example, acridine orange staining of apoptotic cells and tissues is possible [137]. Furthermore, toxicological endpoints such as disruption of gill [138], skin [139], and the endocrine system [140], as well as complex toxicity mechanisms (*e.g.* immunotoxicity, genotoxicity, neurotoxicity, or reproductive toxicity) have all been reported [123]. Nasrallah et al. performed a locomotion assay in *Tg(Isl1:EGFP)* zebrafish larvae, stably expressing green fluorescent protein in motor neurons, following nanoparticle incubation [141]. Finally, a highly sensitive analysis of nanoparticle toxicity was recently proposed by Pan et al. [128], that takes advantage of the endogenous expression of heat shock proteins in response to toxic compounds [142]. Using a newly generated transgenic zebrafish line, expressing green fluorescent protein (GFP) under the control of a heat shock protein promoter, the authors were able to show nanoparticle toxicity induced GFP expression with 20-fold greater sensitivity as compared to a classical toxicology analysis in wild type zebrafish [128].

4.2. Biodistribution and systemic circulation of nanomedicines

Most *i.v.* administered nanomedicines aim to alter the biodistribution of their drug payload, prolong blood circulation lifetimes of drugs and/or promote passive accumulation of drugs in fenestrated solid tumors. Organ biodistribution, circulation half-life ($t_{1/2}$) and area under the curve (AUC) are therefore important pharmacokinetic parameters of nanomedicines that have been traditionally assessed in mice, rats or dogs [143,144]. These dynamic parameters can be assessed in zebrafish larvae, with the accompanying advantage of being able to extensively optimize nanomedicine formulations under realistic and complex *in vivo* conditions early in the development process and prior to first trials in higher animals.

Biodistribution is heavily affected by nanoparticles' propensity to be recognized and cleared by cells of the reticuloendothelial system (RES or MPS) [145–147]. By screening nanoparticles in zebrafish larvae, both the systemic circulation and the clearance of nanoparticles, primarily by macrophages and scavenger receptor expressing cells, can easily be assessed [27,28]. Correlating data ascertained in zebrafish embryos to higher order mammals. We recently demonstrated that zebrafish larvae are an accurate *in vivo* model to predict pharmacokinetic properties in rodents [27]. In this study, different lipid-based nanoparticle formulations demonstrated different binding affinities to venous tissues of the zebrafish larvae. Based on the analyzes of acquired confocal images, zebrafish extravasation and circulation factors were defined to enable semi-quantitative descriptions of nanoparticles' blood circulation. With this approach, investigational nanomedicine formulations can be compared to established and well-defined formulations (*e.g.* long circulating PEGylated liposomes). Here it is important to note that predictions of absolute pharmacokinetic parameters (allometric scaling from zebrafish to mice), such as $t_{1/2}$ or AUC, are not yet possible but are the subject of current investigations. To further elucidate the underlying biological mechanisms of nanoparticle clearance,

we uncovered the presence of the stabilin-2 scavenger receptor, expressed exclusively in the caudal vein (CV) and caudal hematopoietic tissue (CHT) of the zebrafish larvae [28]. Highly expressed on mammalian liver sinusoidal endothelial cells (LSECs) and other clearance organs [148], stabilin-2 is primarily responsible for the removal of macromolecular and colloidal waste from blood circulation. By injecting different nanoparticles in zebrafish larvae, we showed a preference of this receptor for all anionic nanoparticles, a finding which was verified in mice. Nanoparticle uptake by macrophages represents another important clearance mechanism of nanomedicines. Transgenic zebrafish expressing fluorescent proteins in all macrophages [41] are therefore a valuable tool to assess this important pharmacokinetic parameter. Our group has recently investigated macrophage clearance of liposomes of various sizes and surface modifications (*i.e.* PEGylation) in zebrafish embryos. Importantly, a good correlation between macrophage uptake in zebrafish larvae and liposome accumulation in the spleen of rats, a main reservoir of mammalian macrophages, was found (Sieber et al., manuscript in preparation).

To assess the ability to accurately mimic dynamic flow, Garcia et al. assessed various parameters including blood flow velocity, shear stress and flow disturbances in zebrafish larvae. It was found that depending on the vasculature architecture (*i.e.* branch points, curvature), and concomitant changes in velocity and shear stress, nanoparticle accumulation was strikingly altered. This study further highlights the value of the zebrafish larvae model when it comes to nanoparticle studies under dynamic conditions [149], a parameter which cannot be mimicked *in vitro* in such a detailed manner. Jiang et al. investigated the impact of surface charge on nanoparticle interactions with endothelial cells, under flow, in zebrafish larvae [150]. In this case, vessel diameter dependent changes of blood flow velocity as well as nanoparticle surface charge was found to influence nanoparticle binding to endothelial cells and penetration into surrounding tissue. Askes et al. investigated cellular uptake and trafficking of nanoparticles in cell culture and zebrafish larvae at various timepoints [151]. Here, nanoparticle endocytosis and lysosomal accumulation, including long-term stability in these cellular compartments, was demonstrated both *in vitro* and *in vivo*.

Biological barriers, such as the blood brain barrier (BBB), prevent nanomedicines reaching their site of action. The BBB is of particular interest, as effective drug delivery to the brain remains a major challenge in terms of drug delivery. Active targeting of nanomedicines to receptors (over-)expressed at the BBB is one popular approach taken to try and breach this biological barrier [152]. Li et al. successfully demonstrated this approach in larval zebrafish envisioned based on already described zebrafish features such as a functional BBB and the expression of tight junctions, transferrin receptors, and efflux transporters [153–155]. Furthermore, transgenic zebrafish lines expressing fluorescent proteins in neuronal cells or astrocytes have been used to examine brain accumulation of nanoparticles upon injection at different sites within zebrafish embryos [156]. However, it is important to note that current information on BBB development in the zebrafish is scarce and this topic remains controversial. Accurate descriptions of the functional development of the BBB in zebrafish larvae, determined using established protocols [157,158] and with appropriate control experiments, will be of great value to the zebrafish community. In the context of nanomedicines, BBB permeability is relevant and can be assessed using fluorescent dyes with different molecular weights [159].

4.3. Nanomedicines targeting macrophage resident pathogens

Nanomedicines are a promising therapeutic option to treat infectious diseases. Ideally, this should involve the preferential biodistribution of nanomedicines, and subsequent targeted drug delivery, to niches formed by bacteria. Interestingly, zebrafish larvae have emerged as more accurate model system for many infectious diseases. Mice often fail to replicate important features of human pathology which is particularly striking in the case of tuberculosis (TB) where compact granulomas, characteristic of human tuberculosis infections, are present in zebrafish embryo TB models [160,161] but absent from the most widely used mouse models [162]. Taking advantage of the high persistency of TB in macrophages, Fenaroli et al. were able to show rifampicin loaded nanoparticles, administered to zebrafish larvae and targeted to macrophages, resulted in increased survival rates and decreased bacterial load, whereas free rifampicin showed only a reduced efficacy [163]. Complementary to the transgenic macrophage fluorescent line, the authors also exploited established transgenic zebrafish lines expressing fluorescent proteins in neutrophils or in vascular endothelial cells, to precisely describe both the infection status and nanomedicine treatment process. In a follow up study, the authors investigated the possibility of macrophage independent nanomedicine-based delivery strategies to tuberculosis granulomas *via* an enhanced permeability and retention (EPR)-like process. Here, the versatility in experimental set-ups of zebrafish larvae enabled injection of *Mycobacterium marinum* into the neural tube of zebrafish larvae, resulting in the formation of tissue granuloma (*i.e.* model organism to study tuberculosis infection). Subsequent injection of PEGylated nanoparticles resulted in reduced clearance by macrophages and accumulation of nanoparticles within granulomas, supporting the authors' hypothesis [164].

4.4. In vivo evaluation of advanced functional nanomedicines

Nanomaterials are used in a broad range of applications including stimuli responsive systems, enzyme/protein delivery, and gene therapeutics [165–169]. Successfully developing such complex and sophisticated systems is heavily dependent on their stability and functionality under *in vivo* conditions. These parameters can often be ideally assessed in zebrafish.

4.4.1. Stimuli responsive nanomedicines

Nanoparticle drug delivery systems can be designed to release their cargo upon a specific trigger (*e.g.* pH changes, redox changes, photoirradiation). Yan et al. injected photo-responsive, curcumin loaded nanoparticles in zebrafish larvae [170] to demonstrate triggered drug release upon light irradiation or temperature increase (37 °C). Here, the inherent fluorescence of curcumin could be used as a reporter within the living embryo. In addition, curcumin released in the zebrafish larvae heart was able to improve heart function (*i.e.* heartbeat rate and cardio muscular contractility). Photoirradiation, in combination with nanoparticles, has also been used to generate reactive oxygen species (ROS) to kill tumor cells within zebrafish larvae [171,172]. Nanomedicines are also ideal candidates for combined diagnostic and therapeutic function, so-called theranostics. In zebrafish larvae, theranostic plasmonic nanobubbles, generated around gold nanoparticles, have been successfully used to both identify and kill xenografted cancer cells upon focused laser illumination and without harming surrounding tissue [173]. As a more general comment here, the small and transparent zebrafish larvae are, of course, an ideal proof-of-concept *in vivo* system to validate and optimize photo-responsive systems. However, the micrometer thick tissue of a transparent zebrafish larvae does not resemble clinically relevant tissue and, in taking these technologies forward into the clinic, significant challenges to efficiently deliver light into deep and opaque tissue must be additionally met.

4.4.2. Enzyme and protein delivery

For nanoparticle-mediated enzyme delivery, enzyme activity upon immobilization is a key question to be assessed. An important parameter for enzymatic activity is access to substrates and co-factors. This process can be affected by many factors *in vivo*, including blood flow, shear stress, cell interactions (*e.g.* immune cells) and protein adsorption. Using classical enzymatic activity assays, it is simply not possible to mimic all these complex and intertwined determinants.

Transparent zebrafish larvae are an ideal platform for initial *in vivo* assessment of enzyme delivery systems since many enzymes can process colorimetric or fluorescent substrates (a requirement for image-based analysis). Recently, enzyme loaded nanoparticles and free enzyme were injected into the blood circulation of zebrafish larvae followed by an enzyme activity assay [174]. Both enzyme preparations were shown to be active and neither elicited acute toxicity (*i.e.* seizures, heart failure, signs of denaturation). Interestingly, immobilized enzymes remained associated with particles after injection (distinct localized areas) in contrast to the very diffuse distribution of free enzyme throughout the zebrafish larvae. Qualitative analysis of staining patterns enabled assessment of both the stability and functionality of enzyme loaded particles *in vivo*. In this example, zebrafish larvae had to be euthanized prior to the enzyme activity assay, however this limitation can be overcome by choosing a suitable enzyme reaction, preferably one producing a fluorescent product, as demonstrated by Einfalt et al. [175]. In this study, polymer-based artificial organelles, containing protein gates and a model enzyme, were developed. After demonstrating functionality *in vitro*, zebrafish larvae were then used to demonstrate activity *in vivo*. In this case, fluorescently labeled artificial enzyme-containing organelles were injected into the blood circulation of zebrafish larvae. After successful uptake of artificial organelles by macrophages in the caudal region of the zebrafish larvae, the enzyme substrate was injected. Importantly, the formation of a fluorescent product demonstrated the intracellular functionality of the designed system in a complex *in vivo* environment.

4.4.3. Gene therapy

The past decades have seen significant progress in the development of non-viral vectors for gene therapy [169,176]. Typically, an excess of positively charged or ionizable lipids or polymers is required to efficiently complex polyanionic nucleic acids within discrete nanoparticle formulations. Since cationic nanoparticles are often highly cytotoxicity *in vitro* [177], much effort has been made to optimize formulation design, *e.g.* screening polymer/lipid to nucleic acid ratios, use of ionizable cationic lipids (with optimized pK_a values) and/or including helper lipids, to minimize cytotoxicity and other detrimental features (*e.g.* aggregation in serum) associated with cationic particles. These efforts have recently resulted in the clinical translation of the first non-viral RNAi therapeutic [178]. Many challenges however remain, perhaps most significant being the ability to efficiently target genes to cells beyond the liver. Once again, the ability to quickly screen many and varied gene delivery systems within realistic *in vivo* situations would hugely benefit the field [179]. Encouragingly, zebrafish embryos/larvae have already been used to show successful nanoparticle-mediated gene transfection [171,180,181] and gene silencing [182], highlighting the suitability of this model organism for the design and optimization of gene delivery systems.

4.5. Nanomedicines for cancer therapy

In the past 30+ years, very few nanomedicines have been granted market approval for the targeted treatment of solid tumors, although several are currently in clinical trials [183]. Successful translation of nanomedicines, from the preclinical setting to the clinic, therefore remains a persistent and major hurdle. The development of new *in vivo* platforms to quickly screen, analyse, and optimize large numbers of cancer nanomedicines has the potential to facilitate the selection of lead formulations and to accelerate their preclinical development. However, *in vivo* cancer models must be designed and applied with utmost care to avoid misleading conclusions as to the nanomedicines' efficacy, particularly given cancer is a pathophysiologically heterogeneous disease (*e.g.* varying tumor size, presence and extent of the extracellular matrix, specific cell types, location). In the following sections, existing zebrafish cancer models are described including comparisons to their rodent counterparts and their applications during nanomedicine development are discussed.

4.5.1. Zebrafish cancer models

Zebrafish can be used to study the pathophysiology of various cancers including melanoma, rhabdomyosarcoma and hepatoma [184–186]. Most tumor models generated in zebrafish are histologically comparable to human tumors and possess important hallmarks of cancer, such as genomic instability, invasiveness, transplantability, existence of cancer stem cells and conservation of tumor suppressor genes and oncogenes [185,187–189]. Furthermore, the availability of various genetically modified zebrafish lines such as Casper [30] (transparent at adult stage), *Tg(kdrl:eGFP)* [38] (fluorescent embryonic and early larvae vasculature), *Tg(mpx:eGFP)* [190] (fluorescent neutrophils), *Tg(mpeg1:eGFP)* [41] (fluorescent macrophages) and *Tg(cd41:eGFP)* [191] (fluorescent thrombocytes) facilitates detailed investigation of important cancer features such as angiogenesis, neutrophil-mediated metastasis, and immune responses, at a cellular level and in living organisms [192–194]. For example, Nicoli et al. injected cancer cells, loaded with fluorescent dye, into zebrafish larvae expressing GFP in their vascular endothelial cells, to dynamically assess tumor angiogenesis *in vivo* [195,196]. In another study, Feng *et al.* investigated host inflammatory response, upon cancer cell transplantation, making use of various transgenic fluorescent zebrafish lines [197].

Zebrafish cancer models can be created through embryo exposure to carcinogens, forward genetic screens, reverse genetic knockouts, transgene expression or xenotransplantation of mammalian cancer cells [185]. Carcinogenic chemicals, such as *N*-nitrosodimethylamine [198], 7,12-dimethylbenz(*a*)anthracene [199] or *N*-ethyl-*N*-nitrosourea [200], can be directly dissolved or dispersed into the zebrafish media, making this approach straightforward. Since many oncogenes and tumor suppressor genes are also important for tumor development, new cancer-related genes can be identified through forward genetic screens, including the selection of phenotypes with proliferation defects [201,202]. The creation of reverse genetic knockout enables creation of human-like cancer mutations through specific knockout of a gene known to be linked to cancer development, for example the tumor suppressor, *TP53* [203]. Here it is important to note that these methods are affected by an evolutionary gene duplication event in zebrafish [204], resulting in the presence of redundant genes that can compensate for engineered genetic mutations. Therefore, induction of tumors by transgenic expression of mammalian oncogenes, within single cell zebrafish embryos, is a frequently used approach. This also allows for the combination of human oncogenes and fluorescent markers to be co-expressed in specific tissues [205]. However, transgene expression is time consuming and laborious. As an alternative, zebrafish cancer models can be generated by xenografting human cancer cell lines or patient-derived tumor cells within zebrafish embryos [206,207]. The success and reproducibility of this approach is however strongly affected by different experimental parameters, such as developmental stage, site of injection and experimental temperature, and care must therefore be taken to ensure these parameters are strictly controlled. Most studies involving the generation of zebrafish xenografts report cancer cell injection at 2 dpf, as highlighted in different reviews [192,208–210]. At this time point, gastrulation is complete, the main anatomical organization of the zebrafish body is established, larvae are fully transparent and have not yet developed an adaptive immune system. As a result, injection of human cancer cells into zebrafish embryos does not require immunosuppression. Furthermore, zebrafish, at this developmental stage, feed exclusively from their yolk, minimizing fish-to-fish variations caused by differential diet.

The site of xenograft injection is critical in determining resultant tumor access to blood and/or vascularization. The yolk is commonly preferred as xenograft injection site, given its large size, the ability to carry large tumor burdens and the naturally nutrient rich environment that promotes tumor proliferation. The main limitation of xenograft

injections within the yolk is the subsequent restricted access to blood which limits tumor vascularization (*i.e.* blood vessels do not sprout to xenograft). Alternatively, cancer cells can be injected into the perivitelline space, resulting in spontaneous tumor vascularization *via* the ingrowing subintestinal vessels [196]. This approach is often used to test novel angiogenesis inhibiting compounds [211].

Direct cancer cell injections into blood circulation, *via* the duct of Cuvier [212] or the cardinal vein [213], have also been demonstrated. This approach gives less control over the ultimate site of tumor formation, however most *i.v.* administered cancer cells will tend to accumulate in the caudal part of the zebrafish larvae vasculature, due to the narrowed blood vessels and reduced blood flow velocity of this tissue, which guarantees blood access of the established xenograft.

In general, injecting mammalian cancer cells into zebrafish larvae presumes an accurate control of experimental temperature given mammalian cells require an optimal temperature of 37 °C whereas the optimal temperature for zebrafish husbandry is 28 °C. Fortunately, zebrafish larvae, from 2 dpf onwards, are able to survive and develop at temperatures up to 35 °C [214] for several days, while adult zebrafish can withstand water temperatures up to 38 °C [215]. As mentioned previously, in carrying out zebrafish experiments at elevated temperatures, the potential activation of heat shock protein pathways should also be carefully considered.

4.5.2. Zebrafish and rodents as complementary model organisms in cancer research

As stated previously, most tumor models generated in zebrafish are histologically comparable to human tumors and possess many important hallmarks of cancer. For both mice and zebrafish, genetic cancer models and xenotransplants are available. Each has its own advantages and disadvantages. Genetically induced tumors (*i.e.* gene knockout or knock-in) in zebrafish, for instance, suffer from later onset and lower incidence rates (close to 30%) compared to orthologue mouse models [185]. This can be somewhat compensated by working with larger numbers of zebrafish, given the fecundity and comparably cheap husbandry costs of zebrafish. In addition, most cancer models can be effectively exploited in zebrafish larvae as observed larval phenotypes are strongly predictive of adult phenotypes. Nevertheless, some zebrafish organs are anatomically less complex (*e.g.* kidney, pancreas) than their mammalian counterparts or indeed absent all together (*e.g.* mammary and prostate glands and lungs) [189]. This clearly hampers, or prevents, the generation of representative tumor models within these organs. In addition, tumor classification and characterization of zebrafish cancer models is often difficult given the lack of zebrafish-specific antibodies required for tissue staining, flow cytometry or western blots. Regarding the conservation of oncogenic pathways, for example high conservation of *BRAF* and *NRAS* oncogenes and absence of *BRCA1* and *INK4α/ARF* tumor suppressors, the inability to accurately characterize tumor pathology can be critical [47,216]. Upon cancer cell xenotransplantation, successful tumor formation often requires the addition of supplementary cytokines, such as growth factors. The use of solubilized tissue basement membrane matrices (*e.g.* Matrigel® or Cultrex®), containing transforming growth factor and fibroblast growth factor, has been shown to support tumor growth in both rodents and zebrafish xenograft models [196,217]. Addition of tissue-specific growth factors to the zebrafish media is possible but has yet to be established as standard protocol. Regarding xenograft rejection, the adaptive immune system of zebrafish is not fully functional until 1 month post fertilization, making common immunosuppression protocols used in rodents, redundant in zebrafish embryos [218]. For xenotransplantation in adult zebrafish, immunosuppression through irradiation or dexamethasone treatment is effective [30,219].

In general, xenograft cancer models in zebrafish have several advantageous features over their rodent counterparts. Due to the small size of zebrafish embryos, zebrafish xenografts require a relatively small number of transplanted tumor cells (max. 2000 cells/zebrafish compared to up to 1 million cells/mouse) [192,220]. This is particularly relevant in cases where human primary cells are to be xenografted, given these cells are difficult to obtain in large numbers. As such, the potential use of zebrafish cancer models towards personalized cancer therapy has not gone unnoticed [221]. Indeed, given the relatively short amount of time needed to generate patient-derived xenografts in zebrafish larvae, it is possible to assess the effectiveness of various patient treatment options under more realistic and predictive biological conditions in zebrafish larvae [222].

Assessment of tumor growth and tumor cell migration in zebrafish is generally done before and after treatment. By transplanting fluorescently labeled cancer cells, these two parameters can be determined dynamically in living organisms at cellular resolution and can even be quantified [214,223]. In contrast, imaging of transplanted cancer cells in rodents generally relies on luminescence measurements, which suffer from limited resolution [189]. High resolution live imaging of tumors is possible in rodents by intravital microscopy [224]. However, this procedure requires invasive surgical procedures.

4.5.3. Screening of cancer nanomedicines

Various xenograft cancer models used in rodents can be successfully translated to zebrafish, including models for metastasis [225]. At present, xenografting remains the method of choice for zebrafish-based drug discovery and development of cancer nanomedicines, as emphasized in specific reviews [209,226]. Key advantages of this approach include the ability to transplant hundreds of larvae a day, the ability of zebrafish to support relatively large tumor burdens, the observed rapid onset of cancer, transparent larvae (for live fluorescence imaging) and the use of human cancer cell lines, which are stained or genetically modified to express fluorescent markers [185,209]. In addition, Stoletov et al. were able to observe characteristic fenestrations in the tumor vasculature following transplantation of cancer cells overexpressing vascular endothelial growth factor into zebrafish embryos [219,227]. This may prove a valuable model for the characterization of nanomedicines designed to passively accumulate within solid tumors *via* the EPR effect. However, the generation of such an 'EPR zebrafish model' will require thorough characterization given the developing vasculature of zebrafish larvae is intrinsically leaky, as evidenced by the increased observed extravasation of long circulating nanoparticles into the tissues of healthy larvae [27]. In addition, the size of the xenografted tumor is a potentially critical parameter. On the one hand, tumors in zebrafish larvae are inherently limited in size, raising questions over the conservation of key characteristics of large tumors, such as functional tumor microenvironment or the presence of a hypoxic/necrotic core. On the other hand, as observed in rodent tumor models, the use of adult zebrafish to generate larger tumors could result in the overestimation of nanomedicine performance due to exaggerated tumor growth rates and disproportionally large tumors [228]. Moreover, by using adult zebrafish, the many advantages of zebrafish larvae, such as optical transparency and availability, are lost.

If carefully implemented, zebrafish larvae xenograft models can be a valuable tool to optimize specific aspects of nanomedicine performance under *in vivo* conditions. Gao et al. successfully used zebrafish larvae to generate tumor models using multidrug resistant cancer cells, since comparable models in mice often lose drug resistance over long experimental time periods [229]. Making use of the optical transparency of zebrafish larvae, injected cells can be pre-treated (with membrane dyes or genetically modified) to obtain fluorescent cancer cells or cells expressing specific proteins/receptors. Going one step further, Yang et al. injected transfected cancer cells, expressing a coiled coil forming peptide, into zebrafish larvae [230]. Through subsequent injection of nanoparticles decorated with a complementary coiled coil peptide, selective cancer cell delivery of fluorescent nanoparticle cargos, *via* membrane fusion, was demonstrated.

Several ways of applying nanomedicine formulations to zebrafish xenografts have been demonstrated, including addition to the zebrafish

media [231], direct co-injection of a nanomedicine/cancer cell mixture [232–234] or injection of pre-treated cancer cells [235]. While issues surrounding the addition of nanomedicines to zebrafish media have already been discussed in this review, as potential cancer therapies, neither co-injection nor pre-treatment of cancer cells with nanomedicines accurately reflect any realistic course of treatment. Moreover, these administration routes do not exploit a key advantage of using zebrafish larvae, namely the ability to assess blood circulation behavior, a key parameter of all clinically approved cancer nanomedicines. Ideally, zebrafish larvae xenografts should be established and characterized before nanomedicine injection into blood circulation, as described by Evensen et al. [236]. To the best of our knowledge, this latter study is the most extensive application of zebrafish xenografts with regards to nanoparticle characterization. In a separate study, Zhou et al. exploited an established zebrafish model of cancer metastasis [225,237] to investigate the role of TGF-β during cancer metastasis and demonstrated cytokine function in both zebrafish and human cancer cells. Based on these findings and extensive characterization of this cancer model, Zhou et al. were subsequently able to develop a nanomedicine formulation for the co-delivery of two drugs to cancer cells [235]. This study highlights the importance of using well-established zebrafish cancer models during nanomedicine development.

Various readouts to assess cancer nanomedicine efficacy in zebrafish cancer models are available. Many studies report antiangiogenic properties of nanomedicines [233,234,238] following reported protocols [196]. In addition, evaluation of tumor growth or metastasis have been reported, to various degrees of detail [229,231,232,238]. However, assessing such experimental readouts in a reproducible, robust, and representative way remains a major issue. To address this, Wehmas et al. developed imaging software protocols to reproducibly measure glioblastoma cell migration and invasion in zebrafish larvae following nanomedicine treatment [239]. Other groups have reported polymerase chain reaction (PCR) procedures to assess RNA levels of specific tumor markers, such as vascular endothelial growth factor (VEGF) [240,241], as a quantitative assessment of nanomedicine treatment efficacy.

5. Discussion and conclusion

The potential value of the zebrafish model for nanomedicine development has been demonstrated by many studies. In particular, zebrafish larvae are uniquely placed to bridge the gap between *in vitro* models and rodent *in vivo* studies given their availability in large numbers, their optical transparency, the availability of numerous transgenic fluorescent lines and the relatively low costs of husbandry and experimental set-ups. These features facilitate the rapid and cost-effective assessment of nanomedicines, under *in vivo* conditions and down to the macromolecular level. Nanomedicine assessment in whole living organisms is particularly important as nanomedicine-bio interactions are determined by a combination of biological processes, anatomical features and molecular mechanisms. Up to date, this dynamism and complexity simply cannot be accurately mimicked *in vitro* [242].

Zebrafish models can be used for formulation screening using reported experimental set-ups and conventional imaging technologies. Screening of up to 20 nanomedicine formulations per day, researcher, and microscope is feasible. Nevertheless, to reach high-throughput screening capabilities, fully automated injection, imaging, and analysis protocols will need to be developed. Towards this goal, significant progress has been made to fully automate zebrafish injections [243-247] using organ-targeted microinjection systems [248]. However, these systems remain highly customized and are not widely available. Furthermore, automated injection into zebrafish blood circulation, given the precision required, remains a major unmet challenge. Nevertheless, given the zebrafish model for biomedical applications is rapidly gaining interest, further technical developments to improve screening speed and accuracy are anticipated.

Zebrafish are not yet considered a standard model for nanomedicine research. Further studies are necessary to fully characterize the model, evaluate potential applications, and assess its predictive value for studies in higher animals. Prediction of therapeutic efficacy or exact pharmacokinetics requires precise characterisation of physiological features. As highlighted above, the protein corona has a strong effect on nanoparticle behavior in biological environments [249,250]. Several publications have demonstrated the source of serum proteins (*i.e.* sheep, rat, human, rabbit) differentially influences the extent to which nanoparticles aggregate as well as their targeting efficiencies [249,251]. Nevertheless, the importance of assessing nanoparticle formulations *in vivo*, with respect to the adsorbed protein corona, have been highlighted by Hadjidemetriou et al. Here, striking differences between protein corona compositions formed *in vitro* (plasma incubation) and those formed *in vivo* (rodent injection and recovery) were observed resulting in markedly different profiles of both nanoparticle receptor binding and cellular uptake [252].

Further research is therefore needed to investigate the protein composition of zebrafish plasma, which has not yet been fully characterized [73]. Similarly, there are still several fundamental biological unknowns of the zebrafish that need to be fully characterized, including presence or absence of Kupffer cells and blood-brain barrier integrity. In terms of experimental parameters, both the route of nanomedicine administration and the developmental stage of the zebrafish are critical. Nanoparticles should always be administered to zebrafish larvae *via* the same anticipated route used in higher order animals and ultimately patients. For example, incubation of nanoparticles in zebrafish media does not reveal toxicological information of the same formulation administered into blood circulation. With respect to development stage, it is critical to appreciate that zebrafish development is a very rapid process, especially during early developmental stages (up to larvae). The presence and maturation of different cells and organs during early development can change in the timeframe of hours and must be considered carefully, particularly with respect to timing of nanomedicine administration and imaging over different experimental days.

Despite these open questions and challenges, zebrafish models are highly predictive when it comes to the characterization of pharmacokinetic properties of nanoformulations. For example, disparities between *in vitro* and in vivo results have been demonstrated when determining the optimal ligand density of receptor targeted nanomedicines. *In vitro*, higher ligand densities often result in an increased cellular uptake. However, this often does not translate to rodent *in vivo* studies, where intermediate ligand densities are often optimal so as to balance circulation, targeting, and clearance profiles [253]. Witzigmann et al. used a zebrafish larvae xenograft model to optimize nanoparticle targeting ligand density. As expected, optimal ligand densities *in vitro* did not correlate with in zebrafish larvae studies. However, the latter was precisely predictive of subsequent rodent *in vivo* experiments (Witzigmann et al., manuscript in preparation).

The vast majority of nanomedicine research in zebrafish is performed in zebrafish larvae (see footnote[1]). This is primarily due to their easy availability and favorable imaging properties (dimension and transparency). These advantages diminish at later developmental stages. Zebrafish larvae are therefore ideal tools to assess nano-bio interactions occurring over short timeframes. This includes cell specific binding, clearance by cells of the innate immune system and functionality of advanced nanosystems (*i.e.* enzyme/gene delivery). Zebrafish models might not be well suited to assess long-term effects of nanomedicines (*e.g.* evaluation of chronic toxicity of nanomedicines following repeated administration).

In conclusion, formulation design and optimization of nanomedicines in complex biological environments are key steps prior to first rodent *in vivo* experiments. Due to the inherent complexity and diversity of physicochemical parameters of nanomedicines, selection of appropriate biological model systems during preclinical development of nanomedicines is crucial (Fig. 4). Several factors influence this

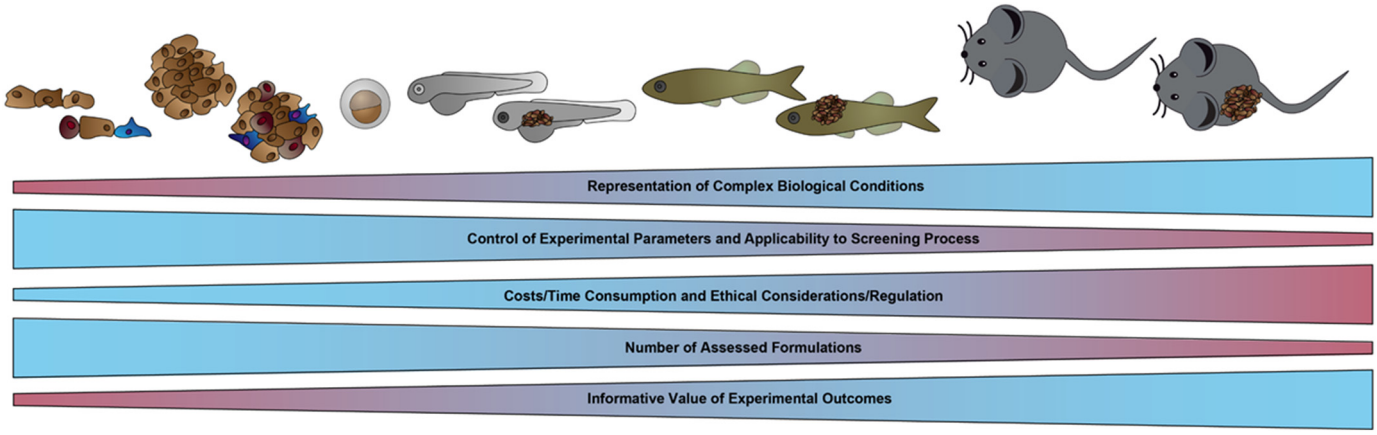

Fig. 4. Comparison of different preclinical models for nanomedicine development. Factors affecting the appropriate selection of a model system during nanomedicine formulation design and optimization are represented. Depending on the experimental complexity and rationale, each model system has its specific advantages and disadvantages. An optimized screening strategy relies on a smart combination of several available model systems and is key to success during clinical translation.

selection, including the extent to which complex biological conditions are mimicked, user control over experimental parameters, ability to screen appropriate numbers of samples, time, and costs. To this end, every available (*e.g.* 2D/3D cell culture) or emerging model system (zebrafish, organ-on-a-chip) has its own advantages and disadvantages. Ultimately, both *in vitro* and *in vivo* model systems (*i.e.* zebrafish) should be used to complement, enrich, and inform the design and optimization of nanomedicines prior to first injections in rodents. An approach, which aligns with reduce, replace and refine (3Rs) legislative guidelines concerning the ethical use of animals in research. With this arsenal of techniques, we may finally see the long awaited advance of many new nanomedicines from bench to bedside.

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