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Zebrafish as an early stage screening tool to study the systemic circulation of nanoparticulate drug delivery systems in vivo

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ABSTRACT

Nanomedicines have gained much attention for the delivery of small molecules or nucleic acids as treatment options for many diseases. However, the transfer from experimental systems to in vivo applications remains a challenge since it is difficult to assess their circulation behavior in the body at an early stage of drug discovery. Thus, innovative and improved concepts are urgently needed to overcome this issue and to close the gap between empiric nanoparticle design, in vitro assessment, and first in vivo experiments using rodent animal models.

This study was focused on the zebrafish as a vertebrate screening model to assess the circulation in blood and extravasation behavior of nanoparticulate drug delivery systems in vivo. To validate this novel approach, monodisperse preparations of fluorescently labeled liposomes with similar size and zeta potential were injected into transgenic zebrafish lines expressing green fluorescent protein in their vasculature. Phosphatidylcholine-based lipids differed by fatty acid chain length and saturation. Circulation behavior and vascular distribution pattern were evaluated qualitatively and semi-quantitatively using image analysis. Liposomes composed of lipids with lower transition temperature ($< 28\ ^{\circ}C$) as well as PEGylated liposomes showed longer circulation times and extravasation. In contrast, liposomes composed of lipids with transition temperatures $> 28\ ^{\circ}C$ bound to venous parts of the vasculature. This circulation patterns in the zebrafish model did correlate with published and experimental pharmacokinetic data from mice and rats.

Our findings indicate that the zebrafish model is a useful vertebrate screening tool for nanoparticulate drug delivery systems to predict their in vivo circulation behavior with respect to systemic circulation time and exposure.

1. Introduction

During the last decades, great expectations were placed on passively and actively targeted nanoparticles for the treatment of severe diseases including solid and metastatic tumors. The visionary concept of the magic bullet, introduced by Paul Ehrlich in 1907 [1], laid the foundation for a new age of cancer treatment. Liposomes, first described by Bangham et al. in 1965 [2], can be considered to be the by far most successfully used nanoparticles in clinical cancer care. Since the first approval of a liposomal anticancer product, namely Doxil/Caelyx in 1995, several lipid-based formulations have reached the market [3,4]. This milestone generated much hope for oncology-based nanoparticulate therapeutics resulting in an increased interest in cancer nanomedicines and a rapid growth of this research field [5].

Importantly, the physico-chemical properties of liposomal formulations have a direct impact on their pharmacokinetics and tissue distribution. Correspondingly, the efficacy and safety of the encapsulated drugs heavily depend on the used nanoparticulate drug delivery system. Recently, Lammers et al. highlighted that shifting the balance between off- and on-target accumulation is the most rational point for clinical use of nanomedicines [6]. Therefore, optimization of pharmacokinetics and biodistribution is of utmost importance for the success of nanomedicines. Unique nanoparticle properties combined with prolonged blood circulation characteristics result in decreased off-target effects and increased probability to reach the target side, e.g. via enhanced permeability and retention effect (EPR) in solid tumors.

The predictability of in vivo performance, however, remains a critical bottleneck in the development of nanoparticles and hampers the translation from in vitro to in vivo applications [3,7]. For example, the pharmacokinetic profile of liposomes can be influenced by multiple parameters

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including lipid composition, amount of cholesterol, PEGylation, or formulation methods as it is described in excellent reviews [8,9,4,10]. These parameters offer virtually endless possibilities for the optimization of nanoparticle formulations. Thus, liposome formulations are often designed empirically and based on physico-chemical properties but rarely optimized after the first in vivo experiments in mice and rats due to high costs and time consumption of in vivo studies. To overcome this issue and to advance the correlation between nanoparticle properties and in vivo performance, different attempts and suggestions have been described [3,7,11,12]. However, there is still an urgent need to incorporate improved pre-clinical test models for the assessment of nano-bio-interactions (e.g. with proteins, membranes, cells) in an early stage of development. This could help to predict the performance in higher animals, systematically pre-select promising nanomedicine formulations and thus facilitate the liposome formulation and development process.

In this study, we focused on the zebrafish as an early and easy accessible vertebrate model to evaluate the circulation behavior of lipid-based nanomedicines in vivo, thus bridging the gap between in vitro cell-based models and in vivo mammalian models. As compared to rodent in vivo models, the zebrafish offers unique advantages including high reproducibility, low husbandry and experimental costs, ethical considerations (3R principle), high level of genetic homology to humans, availability of transgenic lines, and most importantly optical transparency [13,14]. This enables in vivo imaging at spatio-temporal resolution (i.e. down to a cellular level and at various time points) [15]. Consequently, we combined a transgenic zebrafish line expressing green fluorescent protein (GFP) specifically in their vasculature with fluorescently labeled nanoparticles. This offers the possibility to gain advanced insights into the circulation behavior of nanoparticulate drug delivery systems [16]. In order to investigate the predictability of our approach, we have chosen the most established nanoparticulate drug delivery systems, i.e. liposomes. We have used a comprehensive and pioneering work by Semple et al., describing effects of fatty acid chain length, saturation of phosphatidylcholine-based lipids, and cholesterol content on liposome pharmacokinetics in mice [17]. First, we aimed to reproduce the results obtained in mice by Semple et al. in zebrafish embryos, in order to validate the zebrafish model as a sophisticated and early in vivo tool for nanoparticle screening and characterization. Second, we predicted the circulation behavior of a lipid formulation in rats after assessment in our zebrafish model. Finally, we analyzed the effect of cholesterol and PEGylation on the circulation behavior of liposomes in the zebrafish model in vivo.

This comprehensive study can provide the basis for a high-throughput screening platform to assess the circulation behavior of diverse nanoparticulate drug delivery approaches, thus offering a novel concept for nanomedicines development.

2. Material and methods

2.1. Material

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2 dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Corden Pharma Switzerland LLC (Liestal, Switzerland). 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (DAPC 20:4), 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (DAPC 20:0), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was provided by Lipoid (Steinhausen, Switzerland). Cholesterol, branched polyethylenimine Mw 25′000 (PEI), and Sulforhodamine B were purchased from Sigma-Aldrich (Buchs, Switzerland). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Texas red) were purchased from Invitrogen (Basel, Switzerland). SolvableTM and Ultima GoldTM XR were purchased from PerkinElmer (Schwerzenbach, Switzerland).

2.2. Preparation of fluorescent liposomes

Liposomes were prepared as described previously [18]. Lipid and cholesterol stock solutions were prepared in chloroform/methanol (2:1, *v*/v). For the preparation of liposomes either pure phosphatidylcholine-based phospholipids, a combination of DSPC and an indicated mol% of cholesterol or a combination of DSPC/cholesterol and an indicated mol % of DSPE-PEG_{2000} were used [19]. Desired ratios of stock solutions were mixed at a total lipid concentration of 10 mM and dried overnight using nitrogen. Liposomes were fluorescently labeled using 1 mol% of fluorescent dye (as indicated). Dry lipid films were re-hydrated for 10 min at T_m + 10 °C using 0.4 mL of H_2O. For passive encapsulation of a hydrophilic fluorescent dye (i.e. sulforhodamine B), H_2O was replaced by a 1 mM aqueous solution of sulforhodamine B. The resulting multilamellar vesicles were equilibrated for 10 min at T_m + 10 °C and finally extruded 17 times through a polycarbonate membrane with a pore size of 100 nm (Avanti Polar-Lipids). Free hydrophilic dye was removed by size exclusion chromatography using a Sepharose CL-4B column (Sigma-Aldrich) and concentrated using a Millipore Amicon tube (Sigma-Aldrich) with a molecular cut off of 10 kDa (10 min, 4000 rcf).

2.3. Preparation of radioactive liposomes

Tritium labeled doxorubicin ([^{3}H]-doxorubicin) (Campro Scientific, Veenendaal, Netherlands) was encapsulated into DAPC 20:0- and DAPC 20:4-based liposomes using an active loading strategy (i.e. a pH gradient) as follows: Lipid films were re-hydrated using acetate buffer (0.3 M, pH 4.0) and extruded as described before. The external buffer phase was exchanged by PBS (0.01 M, pH 7.8) using gel filtration chromatography (Econo-Pac 10DG, Bio-Rad Laboratories AG, Cressier, Switzerland). [^{3}H]-doxorubicin in EtOH (15 μCi) was placed into a glass vial and EtOH was removed under nitrogen. Subsequently, [^{3}H]-doxorubicin was rehydrated in H_2O (20 μL) and liposome formulations were added. The mixtures were incubated at > T_m for 10 min. Free [^{3}H]-doxorubicin was removed by gel filtration chromatography using PBS (0.001 M, pH 7.4) as a mobile phase. Liposomes were immediately used for pharmacokinetic experiments in vivo in rats.

2.4. Dynamic and electrophoretic light scattering measurements

Dynamic and electrophoretic light scattering measurements for the determination of liposome size and zeta potential were conducted using a Delsa Nano C Particle Analyzer (Beckman Coulter, Nyon, Switzerland) as described previously [20,21]. All measurements were carried out at room temperature (RT) at a lipid concentration of 1 mM in H_2O. For size distribution analysis, the laser was adjusted to 658 nm and scattered light was detected at a 165° angle. Data was converted using CONTIN particle size distribution analysis. For zeta potential analysis, the measurement angle was 15° and data were converted using the Smoluchowski equation (Delsa Nano V3.73/2.30, Beckman Coulter Inc., Brea, CA).

2.5. Cryo transmission electron microscopy (Cryo-TEM)

Size and shape of liposomes were analyzed by Cryo-TEM using a Philips CM200-FEG electron microscope operated at an acceleration voltage of 200 kV as described previously [20]. In brief, liposomes were deposited onto glow-discharged carbon grids (Quantifoil, Jena, Germany), blotted with Whatman 1 filter papers, and vitrified in liquid nitrogen-cooled liquid ethane using a Vitrobot IV plunge-freezing device (FEI Company, Eindhoven, Netherland). Micrographs were recorded with a 4 k × 4 k TemCam-F416 CMOS camera (TVIPS, Gauting, Germany).