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A systematic *in-vivo* toxicity evaluation of nanophosphor particles via zebrafish models

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ABSTRACT

Lanthanide ion-doped nanophosphors are an emerging group of nanomaterials with excellent optical properties, and have been suggested as alternatives to quantum dots. In this letter, we determine the *in-vitro* and *in-vivo* toxicity of β-$NaYF_4$:Ce,Tb nanophosphors using Capan-1 cells and embryonic zebrafish, respectively. In particular, we are the first to report on the *in-vivo* toxicity of β-phase nanophosphors and examine phenotypic developmental abnormalities (growth retardation, heart deformity, and bent tail), apoptotic cell death, and changes in heart function due to the nanophosphors. This study suggests the use of β-$NaYF_4$:Ce,Tb nanophosphors as alternatives for QDs in a wide variety of biomedical imaging applications.

1. Introduction

The emergence of novel nanomaterials is often accompanied by their subsequent employment towards a wide range of applications, in which unique characteristics and functionalities are utilized in an application-specific manner. The small physical scale of nanomaterials that enable their penetration across natural biological barriers (*i.e.* skin, cilia, various membranes), however, must be taken into consideration in determining the level of risk to the general public. In addition, given that the large surface-area-to-volume ratio of nanomaterials plays a direct role in their increased interaction with biological samples, the evaluation of their potential toxicity must precede any prospective biomedical application [1].

Recently, lanthanide ion-doped inorganic nanocrystals (nanophosphors) have garnered increasing interest among researchers as an alternative to quantum dots (QDs) [2–5]. Considering the emerging evidence of quantum dot (QD) cytotoxicity due to the leaching of toxic constituent elements such as Cd or Pb, nanophosphors and their absence of such elements present a viable alternate platform for use across a wide range of biological applications [3]. In addition, unlike QDs, nanophosphors do not exhibit size-dependent optical properties. Specifically, QDs of different sizes have correspondingly distinct emission peaks, thereby underscoring the importance of size uniformity towards generating a homogenous optical signal; nanophosphors, however, are not constrained by size and generate fluorescence via an f–f transition of the Ln^{3+} ion, giving rise to a sharp optical signal [2]. Another advantage of nanophosphors is the absence of photoblinking and photoquenching events [6,7]. Not only do QDs exhibit photoblinking characteristics due to their atomic-like emission, they undergo self-quenching in solution, particularly at high concentrations. Nanophosphors, on the other hand, display a concentration-dependent linear relationship in which their photoluminescent intensity is not diminished via self-quenching events. Such optical advantages of nanophosphors are particularly appealing in the field of bio-imaging, and have recently put them in the spotlight as an alternative to conventional QDs. Yet, despite such interest, more wide-scale biomedical applications have been limited largely due to an absence of a thorough evaluation on their cytotoxicity [8].

Herein, we fabricate green-emitting hexagonal β-$NaYF_4$ nanophosphors [4,9] that use Tb as an activator and Ce as a sensitizer, and examine their biocompatibility to determine their potential as an alternative to QDs. After analyzing their morphological and

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optical properties, we present a thorough evaluation of nanophosphors toxicity at both *in-vitro* and *in-vivo* levels and compare the results with those using CdSe(ZnS) QDs (Scheme 1). While *in-vitro* cytotoxicity experiments are commonly carried out, they are limited to simple tetrazolium-based MTT or MTS assays based on the metabolic activity of a cell [10–12]. Here, we use a combination of a metabolic assay and a DNA quantification assay to provide more perspective on the cytotoxicity of nanophosphors and QDs on cells. Furthermore, while *in-vivo* experiments are utilized to overcome any partial conclusions by providing perspective on a more extensive scale – such as differential organ-dependent toxicity and the sensitivity of a given nanomaterial to the entire organism – rodents are often used. Specifically, while mice and rats are commonly used for *in-vivo* experiments, they are limited by the need to account for a range of variables including those within the same experimental group, their relatively high maintenance cost, and therefore, the tendency to use small sample sizes. From this perspective, zebrafish provide many advantages that include, but are not limited to, the following: 1) fast embryonic development, yielding full organ functionality in a short time frame [13,14], 2) a transparent body, providing excellent light penetration for a variety of optical and fluorescence microscopy experiments [13–15], and 3) an economical and easy maintenance setup [16–19], thereby increasing the possible number of samples in a given experimental group. While such zebrafish-specific advantages confer an ideal model for rapid and large-scale toxicity experiments [20], zebrafish have only been used by others in a limited manner [21–24]. In this work, we report for the first time – to the best of our knowledge – the *in-vivo* toxicity of β-$NaYF_4$:Ce,Tb nanophosphors by conducting a thorough analysis that includes an examination of phenotypic developmental abnormalities, apoptotic cell death, and heart function. In essence, through a comparative analysis of nanophosphor and QD toxicity *in-vitro* and *in-vivo*, we demonstrate the potential to use β-phase nanophosphors as alternatives for QDs for various biomedical applications.

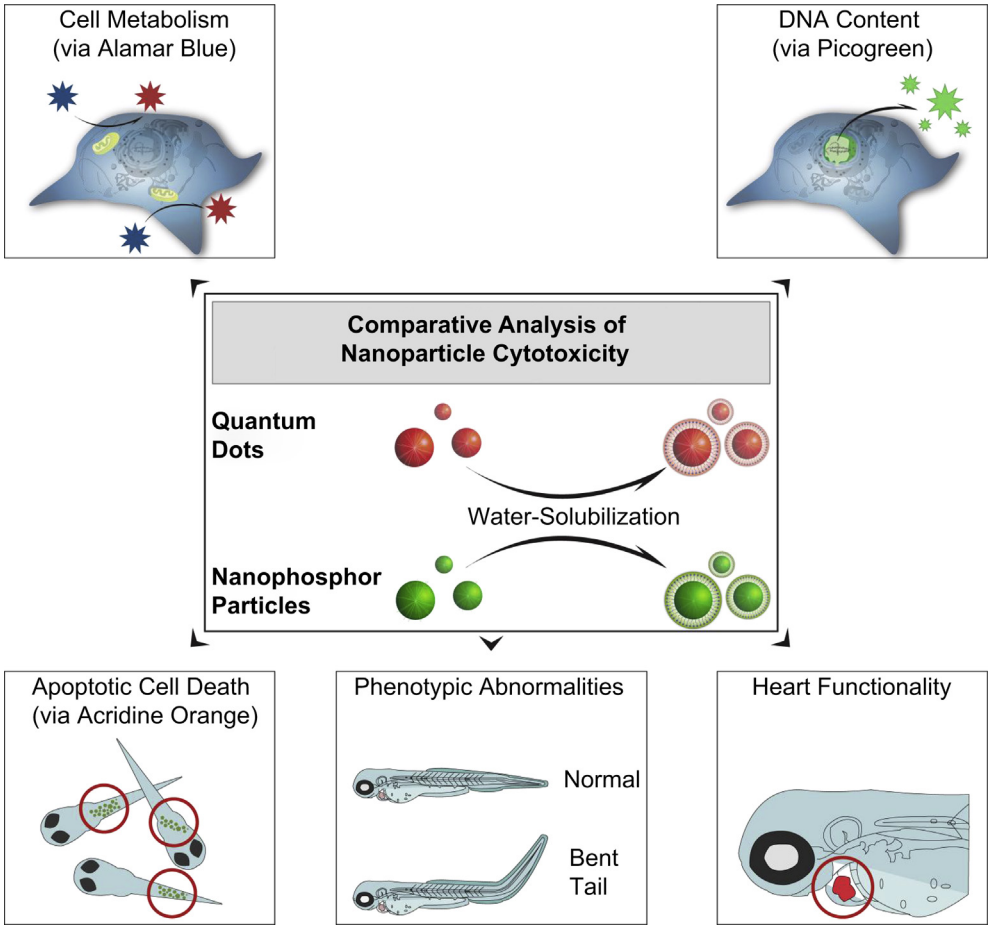
2. Materials and methods

2.1. Raw materials

$YCl_3 \cdot 6H_2O$ (99.99%), $GdCl_3 \cdot 6H_2O$ (99%), $CeCl_3 \cdot 7H_2O$ (99.999%), $TbCl_3 \cdot 6H_2O$ (99.9%), $EuCl_3 \cdot 6H_2O$ (99.99%), NaOH (99.99%), NH_4F (99.99%), oleic acid (OA, technical grade 90%), and 1-octadecene (ODE, technical grade 90%) were purchased from Aldrich and used without further purification. Sodium oleate (>97%) was obtained from TCI.

2.2. Synthesis of nanophosphors

β-$NaYF_4$:$Ce_{0.1}$,$Tb_{0.15}$ (β-$NaYF_4$:Ce,Tb) nanophosphors were synthesized using a previously reported synthesis procedure. One mmoL of $Ln(oleate)_3$ (Ln = Y, Ce, and Tb), 6 mL of OA, and 15 mL of ODE were loaded into a three-neck flask, and heated to 150 °C. Upon formation of a clear solution, the mixture was cooled to 50 °C to which 10 mL of methanol solution containing 2.5 mmoL of NaOH and 4 mmoL of NH_4F was added. The resulting solution was stirred for 40 min to remove the methanol solution, and subsequently heated to 320 °C for 90 min. After washing with ethanol, the synthesized nanophosphors were dispersed in chloroform for further experiments.



Scheme 1. Overview of *in-vitro* and *in-vivo* nanoparticle toxicity evaluation. Nanophosphors and quantum dots, water-solubilized in an equivalent manner, are added to *in-vitro* and *in-vivo* systems. Cell metabolism and DNA content are assessed *in-vitro*, while apoptotic cell death, phenotypic abnormalities, and heart functionality are observed *in-vivo*.