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Systematic assessment of blood circulation time of functionalized upconversion nanoparticles in the chick embryo

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ABSTRACT

Nanoparticle-based delivery of drugs and contrast agents holds great promise in cancer research, because of the increased delivery efficiency compared to ‘free’ drugs and dyes. A versatile platform to investigate nanotechnology is the chick embryo chorioallantoic membrane tumour model, due to its availability (easy, cheap) and accessibility (interventions, imaging). In our group, we developed this model using several tumour cell lines (e.g. breast cancer, colon cancer). In addition, we have synthesized in-house silica coated photoluminescent upconversion nanoparticles with several functional groups (COOH, NH$_2$, PEG). In this work we will present the systematic assessment of their in vivo blood circulation times. To this end, we injected chick embryos\textit{ex ovo} with the functionalized UCNPs and obtained a small amount of blood at several time points after injection to create blood smears. The UCNP signal from the blood smears was quantified using a modified inverted microscope imaging set-up. The results of this systematic study are valuable to optimize biochemistry protocols and guide nanomedicine advancement in the versatile chick embryo tumour model.

\textbf{Keywords:} upconversion nanoparticle, chick embryo chorioallantoic membrane, drug delivery, blood circulation time

1. INTRODUCTION

Upconversion photoluminescence is a nonlinear optical process where two or more near infrared excitation photons are converted to higher energy emission photons. Unlike other multiphoton processes (2-photon fluorescence, second harmonic generation), in the upconverting system real intermediate excited states are involved, usually within the f-electrons of lanthanide ions, which enables the process to happen at moderate excitation density ($1 - 10^2$ W/cm$^2$). The synthesis of nanoscale upconverting materials consisting of an inorganic crystalline host matrix doped with lanthanide-ions\textsuperscript{2,3}, increased the interest for biomedical applications\textsuperscript{4}.

Upconversion nanoparticles (UCNPs) have several optical advantages for biomedical imaging, such as background-free imaging capability\textsuperscript{5}, excitation and emission within the tissue optical transparency window\textsuperscript{6} and long emission lifetimes allowing time-gated detection. For effective \textit{in vivo} application, UCNPs should not only exhibit advantageous optical properties but also excellent biochemical properties. The evaluation of UCNP properties in the context of \textit{in vivo} situations is crucial for the translation of UCNPs from the lab to clinical applications.

An identified purpose is the delivery of UCNPs through the leaky tumour vasculature for enhanced detection and/or tumour therapy\textsuperscript{7}. In view of this, a versatile model is the chick embryo chorioallantoic membrane (CAM), an extra-embryonic vascularised membrane responsible for the gas exchange of the developing chick embryo. The development of protocols

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describing *ex ovo* culturing of chick embryos by transferring the embryo into a sterile container, enabled exposure of the CAM and easy access for intervention and imaging. Since the embryos are immune-deficient (up to embryonic development day 14) foreign cancer cells can be introduced without rejection. Grafting tumour cells on the CAM results in their adoption by the CAM and subsequent tumour-induced angiogenesis enables their growth into proliferating, vascularized tumours. The CAM-based tumour development and their microvascular environment can be studied in detail, including the delivery of photoluminescent macromolecules like fluorescent dextran and virus-derived fluorescently labelled nanoparticles to the tumour sites. The presence of nearly all relevant stroma factors e.g. immune cells (at a later stage), extracellular matrix components, blood and lymphatic vessels make the CAM model highly suited for studying tumour-stroma interactions, tumour metastasis, and therapy-induced changes in tumour development, implying the upcoming role of the CAM model in cancer research.

For *in vivo* use, UCNPs should exhibit surface properties that offer a good dispersability in buffers and ensure biocompatible interaction with tissues. Unfortunately, inorganic nanoparticles are naturally colloidally instable, and prone to aggregation due to the vanderWaals attractive forces between them. In biological media UCNPs can bind to proteins, or other molecules, which can influence their photochemical and biochemical properties, and the biological response and distribution *in vivo*. Several strategies in surface chemistry have been developed to repel the UCNPs from each other, for example by adding surface charge to induce electrostatic repulsion or molecular spacers for steric repulsion. Since UCNPs are generally hydrophobic after preparation by the solvothermal decomposition method, UCNPs firstly need to be transferred into the aqueous phase. Silanization and water-dispersible polymer functionalization are commonly used methods. Among them, silica coating with functional groups (-NH$_2$, -COOH, -SH) and PAA, PMAO, PEG wrapping are popular surface modifications for UCNPs *in vitro* and *in vivo* studies. In addition, the functional groups provide opportunity for the binding of molecules (e.g. antibodies) with tumour-specific targeting properties. Biocompatible surface properties prevent or delay the uptake by the immune system thereby increasing the nanoparticle circulation times. Long circulation times enhance the probability that the particles will end up in the tumour and facilitate enhanced tumour visibility or induce therapy. Feedback on the UCNP chick embryo circulation time is thus meaningful for UCNP design. In this proceeding we report our first preliminary *in vivo* results on the blood circulation time of silica coated UCNPs, further functionalized with -NH$_2$, -COOH, and -PEG surface groups in the chick embryo vasculature.

2. METHODS

2.1. Synthesis and characterization of UCNPs

The UCNPs were synthesized and coated in house. Core β-NaYF4:Yb,Er nanoparticles were synthesized following a protocol developed previously. To prepare UCNP@SiO$_2$ a modified water-in-oil microemulsion method was used. Silica coated UCNPs were further conjugated with APTES, APTES plus succinic anhydride and MPEG-silane, to obtain NH$_2$, COOH and PEG surface functional groups, respectively. The resulting particles were mono-disperse, with an average size of ~28±1.5 nm after silica-coating. TEM images, size distribution and emission spectrum of the particles, are shown in Fig. 1.

2.2. Chick embryo *ex ovo* culturing

Fertile eggs were purchases and the embryos were incubated *in ovo* for 3 days while being rotated every 180 min, at 37.5°C and 70% humidity. At embryonic development day (EDD) 3 the eggs were carefully opened and the contents transferred to sterile plastic weighing boats, perforated for oxygen transfer and covered with sterile plastic wrapping. The embryos were returned to the incubator with the same temperature and humidity settings. At EDD 15 the embryos were removed from the incubator and carefully injected with 50 ul 0.5mg/mL UCNP@SiO$_2$-COOH, UCNP@SiO$_2$-NH$_2$ or UCNP@SiO$_2$-PEG respectively, using micrometer-sized glass needles under stereomicroscopic viewing. At several time points after injection a small amount of blood (5 ul) was drawn from the chick embryo, away from the site of injection. A maximum of 6 time points per chick embryo was chosen to reduce physiological impact. To cover a wide range of time points [2 - 720 min] and increase the accuracy of the results given unavoidable biological variations we included a total of 22 chick
embryos in the experiment. The average number of chick embryos per time point per coating was 2.8 with a minimum of 2. The protocol has been evaluated and approved by the Animal Ethics Committee.

2.3. Blood smear preparation

Immediately after drawing the blood samples were prepared as blood smears on microscope objectives, resulting in a monolayer of red blood cells (RBCs) on each slide. The circulating UCNPs present in the blood samples would thus be deposited on the slides as well and could be quantified using upconversion microscopy. No anticoagulants were used in this procedure. We fixed the slides using 100% methanol.

2.4. Upconversion microscopy

The slides were imaged with a wide-field inverted epi-luminescence microscope (Olympus IX70, with objective 40×, NA 1.15) modified to allow external laser illumination at the sample plane (fiber-coupled diode laser at wavelength 980 nm,
Shenzhen LEO Photoelectric Co. Ltd). The excitation laser was directed to the focal plane using a modified Köhler illumination scheme and adjustable iris diaphragm to achieve uniform and controlled excitation power density and spot size at the sample plane. A detailed description of the imaging system is provided in ref 26. During the whole experiment the excitation density was kept at 440±10 W/cm² (720±20 mW on a circular spot of 230 μm in diameter). An EMCCD camera (Andor iXon DU-885) was mounted to the microscope detection port. We used a high-pass absorbance filter as emission filter (cut-off 850 nm, Edmund Optics); a dichroic beam-splitter (cut-off, 511 nm, Semrock) for the reflection of 980 nm toward the sample and passing the visible emitted light to the detection path; and two additional filters (short pass interference filter, cut-off 842 nm, Semrock and band pass filter, KG-5 coloured glass, Thorlabs) as emission filters. The combination of a high-performance interference filter and coloured glass band pass filter was needed to adequately reject the high excitation power scattered by the cells.

2.5 Image acquisition

Each blood smear slide corresponded to a specific time point after injection, UCNP-coating group and chick embryo. A total of 100 slides were collected. We installed an automated X,Y scanner at the sample plane, however, re-focusing after spatial translation was needed so that the process was only semi-automatic. As the slide covers an area of several cm², imaging the whole slide was too time-consuming. As the UCNP-signal decreased with time we recorded more images per slide for the longer time points. A minimum of 20 regions per slide was recorded, corresponding to an area of minimally 0.5 mm². Each UCNP-image was recorded 3 times, and the average image was saved. The exposure time and EMgain were fixed at 0.5s and x100 respectively during the whole experiment. In addition, due to an inhomogeneous distribution of RBCs over the microscope slides we took bright field and UCNP-images of the same area to correct for the number of RBCs (and thus correct for sampled blood volume).

2.6. Image analysis

The fast amount of images (~8000) required an automated analysis. The UCNP-images were 2x2 pixel binned to reduce the read noise. A positive UCNP signal was defined as an SNR of 5 or higher, (the Rose criterion states that an SNR of at
least 5 is needed to be able to distinguish image features at 100% certainty\(^1\). The background noise contained a slight gradient, however, as the UCNP signal was sparsely distributed over the images, it was possible to estimate the local noise level as the mean of a 30x30 pixel area around a central pixel. If the central pixel value (signal) was 5 times higher than the local noise, the pixel was designated UCNP signal. In addition to simply 'adding' the entire UCNP signal per blood smear we also quantified the distribution of UCNP signal by grouping adjacent signal pixels together that belong to one UCNP-node. This way we obtained information on the size distribution of the UCNP nodes in the blood smears. Finally, the bright field images were used to correct for the blood sample volume related to the UCNP signal. The bright field images were histogram equalized to get consistent results, subsequently binarized by thresholding to obtain the total area as a measure for number of RBCs. The process is schematically drawn in Fig. 2.

3. RESULTS

The quantified UCNP-signal in the blood smear images as a function of time after injection is shown in Fig. 3. All data points are averaged over all images and chicken embryos, and corrected for RBC area in the bright field images. As can be seen, the \(\text{--PEG} \) and \(\text{--NH}_2\) coated UCNP@SiO\(_2\) don’t decrease much in the first ±20 minutes, however they rapidly decrease in the following period. We can estimate the blood circulation halftime at \(\tau_{1/2,\text{PEG}} = 25±10\) minutes, and \(\tau_{1/2,\text{NH}_2} = 20±10\) minutes. On the other hand, COOH coated UCNP@SiO\(_2\) already show a reduced signal in the beginning, which does not decrease much with time. No reliable estimate of \(\tau_{1/2,\text{COOH}}\) can be made. A further analysis of the images showed that the size distribution of the detected UCNP nodes in the blood smears is quite different for the \(\text{--COOH}\) group as compared with the \(\text{--PEG} \) and \(\text{--NH}_2\) groups. This is shown in the bar plots in Fig. 4 where the contribution of small, medium and large UCNP nodes (‘clusters’) to the total signal is calculated, for three different coatings and three different time ranges (short, medium and long). Clearly, the \(\text{--COOH}\) group is much more influenced by large UCNP-nodes as compared with the \(\text{NH}_2\) and PEG groups. This early and substantial clustering of the particles in blood is subject of further study, but should be taken into account when interpreting the data points in Fig. 3.

![Fig. 3. Blood circulation behavior of UCNP@SiO\(_2\) functionalized with 3 different surface groups: NH\(_2\), COOH and PEG, in chick embryo circulation. The UCNP-signal as a function of time after injection is quantified from blood smear microscopic images, as described in the text.](http://proceedings.spiedigitallibrary.org/proceedingspdf/96683Y-5.png)
4. CONCLUSION

We have shown our first, preliminary results of the blood circulation time of UCNP@SiO$_2$ coated with different functional surface groups. The results show that a blood circulation half time of around 20 – 25 minutes can be expected for UCNPs in the chick embryo ex ovo model. Up to several hours after injection particles could still be detected in the blood smear samples. Future investigations on the colloidal behavior of the particles are necessary to draw further conclusions on the blood circulation time of COOH-coated particles. Our practical investigation gives important feedback on the behavior of nanoparticles in the blood stream and is an essential step towards nanoparticle-based drug delivery for oncological purposes.

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Introduction

In December 2013, the United Nations declared 2015 as the International Year of Light (IYL), recognizing the immense importance of light-based technologies in our lives, for our futures, and for the development of humankind.

In December 2015, the SPIE Micro+Nano Materials, Devices, and Applications symposium and the new Australian Institute for Nanoscience (AIN) at the University of Sydney’s Camperdown campus offered the opportunity to celebrate the culmination of the IYL and heightened global awareness of the importance of light-based technologies, including nanoscience.

The SPIE symposium is an interdisciplinary forum for collaboration and learning among top researchers in all fields related to nano- and microscale materials and technologies. This 2015 event took place over 4 days, 6-9 December, and included both oral and poster presentations with a focus on nanostructured and biocompatible materials, medical and biological micro/nanodevices, micro/nanofluidics and optofluidics, nanophotonics for biology and medical applications, plasmonics, and solar cell technologies and fabrication.

The University of Sydney is Australia’s first university with an outstanding global reputation for academic and research excellence. Located close to the heart of Australia’s largest and most international city, the Camperdown campus features a mixture of iconic gothic-revival buildings and state-of-the-art teaching, research, and student support facilities. The University of Sydney attracts many of the most talented students in Australia drawn by its range of quality degrees and strong track record of research programs. The University’s academics are leaders in their disciplines nationally and internationally, driving major research initiatives.

Sydney is Australia’s truly international city and one of the world’s most iconic and livable cities in the world, with plenty of open space, famous beaches, glittering harbour, waterways and bushland, great climate and vibrant culture rich of entertainment, cultural activities, and sporting events. Sydney is at the heart of Australia’s economy, and is ranked first in the Asia Pacific in terms of intellectual capital and innovation. Sydney offers a safe and secure environment for individuals and families, with world-class health care, education, transport and telecommunications with a multicultural environment as over a third of Sydney’s population was born overseas.

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