Targeted labeling of early-stage tumor spheroid in chorioallantoic membrane model with upconversion nanoparticles

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Targeted labeling of an early-stage tumor spheroid in a chorioallantoic membrane model with upconversion nanoparticles†

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In vivo detection of cancer at an early-stage, i.e. smaller than 2 mm, is a challenge in biomedicine. In this work target labeling of an early-stage tumor spheroid (~500 μm) is realized for the first time in a chick embryo chorioallantoic membrane (CAM) model with monoclonal antibody functionalized upconversion nanoparticles (UCNPs-mAb).

In clinical oncology the detection of early-stage cancer like carcinoma in situ and tumors smaller than 2 mm is of great importance for improving the cancer cure probability.1–3 Unfortunately, most of the present clinical imaging modalities like ultrasonic imaging, computed tomography (CT), and magnetic resonance imaging (MRI) are not sufficient for detecting the early-stage cancers because of their low resolution and/or poor sensitivity and/or specificity.4,5 Fluorescence imaging has recently gained increased attention for cancer diagnosis, because of the new developments in exogenous luminescent materials,6–14 such as rare earth ion doped up-conversion nanoparticles (UCNPs) that can efficiently convert near infrared (NIR) light to visible and/or shorter wavelength NIR light. In comparison with traditional “down conversion” fluorescent markers that need ultra-violet or visible (UV-Vis) light for excitation, the UCNPs hold many advantages for biomedical imaging, such as minimized background fluorescence, and no photo bleaching.11–14 Furthermore, since UCNPs have a large surface area, bio-functionalized molecules like folic acid, peptides, photosensitizers, doxorubicin (DOX), and si-RNA can be easily conjugated for multifunctional labeling or therapy. Numerous research studies have been reported in this respect on both in vitro and in vivo tests utilizing UCNPs.15–23 For example, Zhou et al. achieved tri-mode imaging of upconversion luminescence, magnetic resonance and positron emission tomography (PET) in mouse utilizing fluorne-18-labeled Gd3+/Yb3+/Er3+ co-doped NaYF4 UCNPs.23 However, these research studies are performed on the mice model in which the imaging is usually executed at a relatively late stage when tumors reach 4–6 mm. In vivo target detection of early stage cancer, i.e. smaller than 2 mm, remains a difficult task in biomedicine.

In this work, target labeling of an early-stage tumor spheroid (~500 μm) was realized for the first time in a chick embryo chorioallantoic membrane (CAM) model with monoclonal antibody functionalized upconversion nanoparticles (UCNPs-mAb). An early-stage tumor spheroid model was built first by transplanting an in vitro cultured 3 dimensional multicellular tumor spheroid (MCTS) of human breast cancer cells MCF-7 onto the chick embryo CAM. The chick embryo CAM is a well-established model which has already been widely used for cancer and angiogenesis research, drug delivery, immunology etc.26–34 Compared with the widely used mice model, the chick embryo CAM has unique advantages in cancer research, including (i) the chick embryo is a naturally immunodeficient system, and various heterogeneous tumor cells can be transplanted into the CAM without any species-specific restrictions, and (ii) since the chick embryo CAM is an extremely thin membrane layer (~200 μm) that usually lies at the top, it is very convenient to observe the motility process of the injected cancer cells or drug molecules under a microscope with little impact on the host. On top of that, the chick embryo model is simple...
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Corresponding monoclonal antibodies (mAb) of ER–NaYF₄:Yb³⁺,Er³⁺ UCNPs functionalized onto the polyacrylic acid (PAA) stabilized form for target labeling of the early stage tumor sensitive upconversion luminescence (UCL) imaging nanoplat-

...organic solvent into hydrophilic ones (without animal manipulation), low cost, easy to maintain, and easily accessible. Since the MCF-7 cell line has a high expression level of estrogen receptor alpha (ER-α), the corre-

...mAb conjugates (0, 5, 10, 20, 50, and 100 μg/mL) to different cell lines, human breast adenocarcinoma MCF-7 and mouse embryo fibroblast 3T3, using different concentrations of UCNPs-mAb conjugates (0, 5, 10, 20, 50, and 100 μg mL⁻¹). After 24 h, no significant change was observed in the cell morphology and proliferation of both cell lines in the presence of the UCNPs-mAb conjugates. The cellular viability was evaluated by the MTT assay of the mitochondrial activities and relevant results are shown in Fig. 2E. Both cell lines demonstrate good viability, even at the maximum concentration of 100 μg mL⁻¹, and the viability is greater than 90%. These results indicate that UCNPs-mAb conjugates have good biocompatibility and could be used for in vivo imaging. Fig. 3 shows the confocal microscopy images of MCF-7 breast adenocarcinoma cells (positive) and 3T3 fibroblast cells (negative) after treatment with UCNPs-mAb (100 μg mL⁻¹) for 8 h. The bright field images show that the cellular morphology is intact, which is...
consistent with the cytotoxicity results of the UCNPs-mAb conjugates. The dark field images show the upconversion luminescence within the MCF-7 cells, whereas little luminescence was observed in the 3T3 cells. The latter is related to the residual non-specific adsorption of the UCNPs on the 3T3 cell membranes. These results indicate that the UCNPs-mAb conjugates can specifically label on the MCF-7 breast cancer cells.

In our study a shell-less cultured chick embryo was developed as the model to research the \textit{in vivo} labeling properties of UCNPs-mAb. A typical shell-less chick embryo is shown in Fig. S3.† The CAM membrane is settled on the top of embryo and yolk, and the blood vessels of CAM can be seen very clearly with naked eyes. In order to assess the \textit{in vivo} targeting behavior of the UCNPs-mAb conjugates on early stage cancer spheroids, MCTSs were cultured \textit{in vitro} and transplanted onto the CAM. Compared with the cancer cells cultured in 2-D, the MCTSs show a condensed structure in 3-D (Fig. S4†), and can mimic more closely the cellular-matrix and cell-cell interactions \textit{in vivo}.42 After 3 days of incubation, the MCTS could be embedded into the CAM membrane (Fig. S5†), and the newly grown blood vessels can be clearly seen surrounding the MCTS. Then UCNPs-mAb were systematically administrated into the chick embryo CAM via venule injection under a stereomicroscope. Owing to the depression of autofluorescence during UCL imaging, the microcirculating behavior of the nanoconjugates in blood vessels was able to be neatly investigated with a modified fluorescence intravital microscope that was equipped with a 980 nm laser. As shown in Fig. S6† on the left is the white image of a typical CAM blood vessel net, and on the right is the corresponding upconversion luminescence image 10 min after the injection of UCNPs-mAb conjugates. We can distinctly see that the nanoparticles fluently flow with the bloodstream and efficiently extravasate from the main blood vessels into the surrounding tissues. Thus the CAM model provides us a simple approach for real-time visualizing the \textit{in situ} interaction of nanoparticles with the vascular networks and also the biotissues, which might be of great value for future nano-bio research studies.

The \textit{in situ} upconversion luminescence imaging of the tumor spheroid was then investigated at different times with an intravital microscope. The UCNPs without any antibody functionalization (non-functionalized UCNPs) were also injected for control, data are shown in Fig. 4A. We see that the non-functionalized UCNPs were present in both the MCTS and the surrounding tissue without specific accumulation within the MCTS, both at 1 h and at 24 h after injection. In contrast, the functionalized UCNPs-mAb were accumulated specifically on the MCTS (Fig. 4B). One hour after injection, the UCNPs-mAb were observed mainly in the surrounding tissue of the MCTS. Twenty-four hours after injection, strong upconversion luminescence was obviously observed in the MCTS, indicating the good targeted delivery of UCNPs-mAb conjugates.

In order to further demonstrate the selective labeling of UCNPs-mAb in tumor cells, the resected MCTS region was histologically examined (Fig. 5). Fig. 5A shows the microscopy image of the H&E stained MCTS embedded into the CAM tissue. Fig. 5B and C are the confocal upconversion luminescence images of CAM and MCTS corresponding to the marked areas in Fig. 5A. As expected, normal CAM regions show very low amount or no luminescence of UCNPs-mAb (Fig. 5B), whereas targeted luminescence of UCNPs-mAb was only observed in the transition zone from the CAM into the MCTS (Fig. 5C). Low fluorescence was detected from the surrounding tissue, resulting in a high contrast between targeted MCF-7 cells and the surrounding tissue. In contrast, from the histological examination of MCTS administered with non-functionalized UCNPs, only very little amount of upconversion luminescence was observed in the MCTS.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Confocal upconversion luminescence images at 100× magnification of UCNPs-mAb incubated with MCF-7 cells (top row) and 3T3 cells (bottom row) for 8 h at 37 °C.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Non-targeted (A) and targeted (B) labeling of MCTS transplanted on the CAM with UCNPs at 1 h (top row) and 24 h (bottom row). From left to right are bright field, dark field (980 nm irradiation) and merged intravital microscopy images at 4× magnification and 2 min exposure time.}
\end{figure}
Conclusions

In conclusion, NaYF$_4$:Yb,Er upconversion nanoparticles have been successfully functionalized and employed for target labeling the cancer at an early stage in the CAM model. PAA coated UCNPs were synthesized by a two-step ligand exchange method, and functionalized with ER-α monoclonal antibodies to obtain UCNPs-mAb conjugates. In vitro research studies reveal that the UCNPs-mAb conjugates have no significant cytotoxicity on mammalian cells, and can specifically label in the MCF-7 breast cancer cells rather than in the normal cells. The cellular viability was higher than 90% even at a relatively high concentration (100 μg mL$^{-1}$) of UCNPs-mAb. The 3-dimensional MCTS (~500 μm) transplanted CAM model has been developed as the early stage tumor model to research the in vivo labeling properties of UCNPs-mAb. Intravital microscopy imaging demonstrated that intravenously injected UCNPs-mAb conjugates have high specificity in labeling the breast cancer. Our work suggests that UCNPs-mAb, in combination with CAM, offer a new possibility in early cancer studies.

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Notes and references