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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 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 regained increased attention for cancer diagnosis, because of the new developments in exogenous luminescent materials,6–14 such as rare earth ion doped upconversion 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.15–25 For example, Zhou et al. achieved tri-mode imaging of upconversion luminescence, magnetic resonance and positron emission tomography (PET) in mouse utilizing fluoride-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.
ponding monoclonal antibodies (mAb) of ER-
NaYF4:Yb3+,Er3+ UCNPs
functionalized onto the polyacrylic acid (PAA) stabilized
the hydrophobic NaYF4:Yb3+,Er3+ UCNPs synthesized from an
transplanted in the chick embryo CAM.
form for target labeling of the early stage tumor spheroid
sensitive upconversion luminescence (UCL) imaging nanoplat-
method (as illustrated in Fig. 1), aiming to achieve a highly
sensitive upconversion luminescence (UCL) imaging nanopla-
tform for target labeling of the early stage tumor spheroid
transplanted in the chick embryo CAM.

The surface modification process was carried out to transfer
the hydrophobic NaYF4:Yb3+,Er3+ UCNPs synthesized from an
organic solvent into hydrophilic ones via a simple EDC cross-linking
method (as illustrated in Fig. 1), aiming to achieve a highly
sensitive upconversion luminescence (UCL) imaging nanopla-
tform for target labeling of the early stage tumor spheroid
transplanted in the chick embryo CAM.

The surface modification process was carried out to transfer
the hydrophobic NaYF4:Yb3+,Er3+ UCNPs synthesized from an
organic solvent into hydrophilic ones via a simple two-step
lignad exchange method. As illustrated in Fig. 1, the oleic acid
(OA) capped outside UCNPs were removed by protonation
treatment to obtain ligand free UCNPs,35,36 followed by the
treatment with poly acrylic acid on the ligand free nanoparti-
cles, anchoring the UCNPs with carboxylic groups. Fig. 2A and
B show the TEM images of ligand free and PAA coated NaYF4-
Yb3+,Er3+ UCNPs. Both ligand free and PAA coated nanoparti-
tes have good dispersibility and uniform size distribution
with an average size of around 45 nm. Fig. 2C shows the high
resolution TEM image of an individual nanoparticle, where
the lattice fringes with interplanar spacing are about 0.52 nm,
corresponding to the (100) plane of hexagonal-phase struc-
tured NaYF4. The inset shows the fast Fourier-transform (FFT)
diffractogram, confirming the hexagonal-phase of the UCNPs.
To prove that the PAA molecules were capped on NaYF4:Yb3+,
Er3+ UCNPs, Fourier transform infrared spectroscopy (FTIR)
characterization was performed (Fig. 2D).

The band around 1124 cm−1 is due to the C=O stretching
vibration of the carboxyl groups, and the two strong bands cen-
tered at 1580 cm−1 and 1462 cm−1 are associated with the
asymmetric and symmetric stretching vibration modes of the
carboxylate anions, suggesting the effective COO-RE3+ com-
plexation on the UCNP surface. The band at 1728 cm−1 is
assigned to the C=O stretching vibration of the free carboxyl
groups on the PAA polymer chain.

It is known that the hydrodynamic diameters and surface
charges affect greatly cellular endocytosis and toxicity. Therefore, we have measured the hydrodynamic diameters and
zeta-potential and the results are shown in Fig. S1.† Compared
with the ligand free nanoparticles, an increase in hydrodyna-
mic diameters was observed in PAA coated nanoparticles,
which may indicate the dwelling effect of polymer layers
coated on the surface of UCNPs. A significant change was also
observed in the surface charges, varying from 45.5 mV (ligand
free UCNPs) to −37.9 mV (PAA coated UCNPs), confirming the
existence of carboxyl groups at the surface of UCNPs. The UCL
spectra are similar, indicating that the polymer coating
has a negligible effect on the luminescence properties of the
UCNPs.

Cytotoxicity was investigated on two 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 viabi-
ity, 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
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

Fig. 1 Construction of a UCNPs-mAb nanoplatform.

Fig. 2 (A) TEM image of ligand free NaYF4:Yb3+,Er3+ UCNPs. (B) PAA
coated UCNPs. (C) TEM image of a single nanoparticle, with the corres-
ponding diffractogram (insert). (D) FTIR spectra of the ligand free and PAA coated NaYF4:
Yb3+,Er3+ UCNPs. Both ligand free and PAA coated nanoparti-

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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 MCTS show a condensed structure in 3-D (Fig. S4†), and can mimic more closely the cellular–matrix and cell–cell interactions \textit{in vivo}.\textsuperscript{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 stereo-microscope. 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. 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.
In vitro high concentration (100 μM) experiments revealed that the cellular viability was higher than 90% even at a relatively low concentration of UCNPs. This indicates the potential of UCNPs in the targeted labeling of breast cancer cells. Our work suggests that UCNPs-mAb, in combination with CAM, offer a new possibility in early cancer detection and monitoring.

**Notes and references**
