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COMMUNICATION

Highly luminescent and photostable near-infrared fluorescent polymer dots for long-term tumor cell tracking *in vivo*†

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Near-infrared-emitting polymer dots were prepared by encapsulating the dye NIR775 into the matrix of MEH-PPV dots using a nanoscale precipitation method, and their application for long-term tumor cell tracking *in vivo* is demonstrated for the first time.

In recent years, conjugated polymer dots have attracted increasing attention in the biomedical field because of their attractive chemical and optical features, such as bright fluorescence intensity, excellent photostability, high emission rates, and minimal cytotoxicity.[1-16] Of particular importance is the development of the near-infrared (NIR) emitting polymer dots where autofluorescence background is significantly decreased of *in vivo* fluorescence imaging.[17-20] Recently, Chiu lab has developed deep-red emission polymer dots for *ex vivo* imaging.[21] Rao lab designed self-luminescing NIR polymer dots by integrating bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) in an energy transfer relay for *in vivo* lymph node mapping and tumor targeting.[22] Liu lab developed far-red/near-infrared (FR/NIR)-emissive conjugated polymer dots for *in vivo* tumor imaging.[23,24] Very recently, we reported that the NIR775 dye-doped MEH-PPV polymer dots exhibited long-term colloidal stability and photostability in water at 4 °C for at least nine months, and were successfully applied to imaging vasculature of U87MG tumors in living mice after intravenous injection.[25] To date, only a few studies about the NIR-emitting polymer dots for *in vivo* cell tracking are reported.[24] Herein, we further use the NIR775 dye-doped MEH-PPV polymer dots system as fluorescent nanoprobes for the *in vitro* HeLa cell labeling and *in vivo* long-term HeLa tumor tracking.

Fluorescent cell labeling is as sensitive as radiolabel based imaging techniques, but without any exposure to irradiation. Fluorescent labeling provides an effective means of non-invasively tracking cells repeatedly, thereby providing insight into cell migration to the target site. In general, there are direct and indirect ways of labeling cells with nanoprobes. Direct labeling is comparatively easy, less expensive and a well-established methodology where the cells are labeled with nanoprobes and detected by relevant imaging modalities. Moreover, as each cell contains many nanoprobes that will be passed down to daughter cells, long-term monitoring of the cell's fate is feasible.[26-30] Therefore, we chose direct fluorescent labeling of cells, in which the cells are incubated with the NIR polymer dots in serum free media.

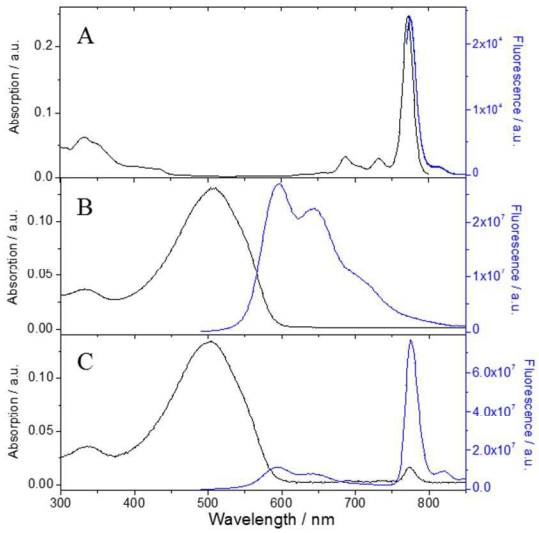

Fig. 1 Absorption and fluorescence spectra of the free NIR775 dyes (A), the free polymer dots (B), and the NIR polymer dots (C).

The NIR polymer dots were prepared according to our previous work by encapsulating the NIR dye, silicon 2,3-naphthalocyanine bis(trihexylsilyloxide) (NIR775), into the matrix of polymer dots, poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV).[15,17] The NIR emission is realized based on a FRET system utilizing NIR775 dye as an acceptor and MEH-PPV polymer as a donor (**Scheme S1**). To minimize the self-quenching effect among encapsulated NIR775 and simultaneously get the highest FRET efficiency, the optimal ration of NIR775 to the MEH-PPV matrix (by weight) was found to be 0.012:1. The synthesized NIR polymer dots were 30–50 nm in diameter (**Fig. S1**). This result is consistent with previous studies.[22]

Fig. 1 shows the absorption and emission spectra of the free NIR775 dyes, undoped MEH-PPV, and NIR775-doped MEH-PPV polymer dots (NIR polymer dots). The free NIR775 dyes dissolved in THF exhibit an absorption peak at 772 nm and strong fluorescence emission at 774 nm. The undoped MEH-PPV polymer dots show a broad absorption band with a maximum at 504 nm and intense emission at 598 nm with a shoulder at 643

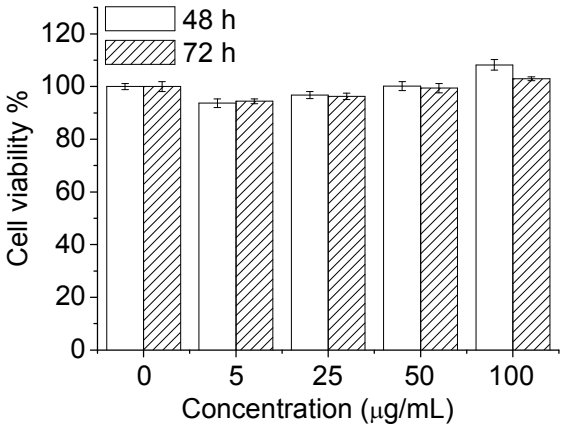

Fig. 2 Viability values (%) of cells estimated by CCK-8 assay versus incubation concentrations of the NIR polymer dots. Data represent mean ± SD (n = 6).

nm. The dominant absorption peaks (around 504 nm) of the NIR polymer dots are due to MEH-PPV, whereas relatively weak absorption (around 772 nm) from the dopant NIR775 can also be observed. With 468 nm excitation, the fluorescence from MEH-PPV is almost completely quenched, and the NIR polymer dots exhibit strong fluorescence peak from the dopant molecule at 776 nm, indicating efficient FRET from the MEH-PPV to NIR775.

The absorbance and fluorescence spectra of NIR775 doped in MEH-PPV dots were similar to the spectra of free NIR775 dyes. However, their excitation spectra are different. The most intense peak of NIR775 doped in MEH-PPV dots is centered at 468 nm, while the excitation peak of the free NIR775 is 764.5 nm (**Fig. S2**). And we compared fluorescence intensity of NIR775 doped in MEH-PPV dots under 468 nm and 764.5 nm excitation, respectively (**Fig. S3**). When excited at 468 nm in aqueous solution, the NIR fluorescence intensity is 10 times stronger than that under excitation at 764.5 nm, confirming efficient FRET occurred from the MEH-PPV matrix to the NIR775 in the synthesized NIR polymer dots.

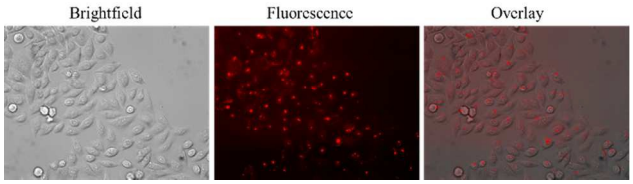

Fig. 3 Fluorescence imaging of HeLa cells incubated with NIR polymer nanoparticles (~5 μg) for 30 h. excitation: 480/30 nm, dichroic beamsplitter: Q570LP, emission: D755/40M; acquisition time: 1 s.

The cytotoxicity of the NIR polymer dots was evaluated by the CCK-8 assay in HeLa cells (**Fig. 2**). The viability of H1299 cells above a 5–100 μg/mL concentration of NIR polymer dots was slightly increased. After 48 h of incubation with polymer dots at a concentration of 100 μg/mL, the cellular viabilities were estimated to be greater than 108%. Even after 72 h of incubation with polymer dots at a concentration of 100 μg/mL, cells maintained greater than 102% cell viability. These results demonstrated the weak toxic effects of NIR polymer dots on cell viability in these conditions.

We chose direct cell labeling techniques for cellular tracking, in which HeLa cells in the culture plate were incubated with 5 μg/mL NIR polymer dots at 37°C overnight, and the cells were washed three times with culture medium before cell imaging. Approximately 100% of HeLa cells were labeled with dots under the fluorescence microscope (**Fig. 3**). And the endocytosed NIR polymer dots within HeLa cells was distributed mainly in the cytoplasm.

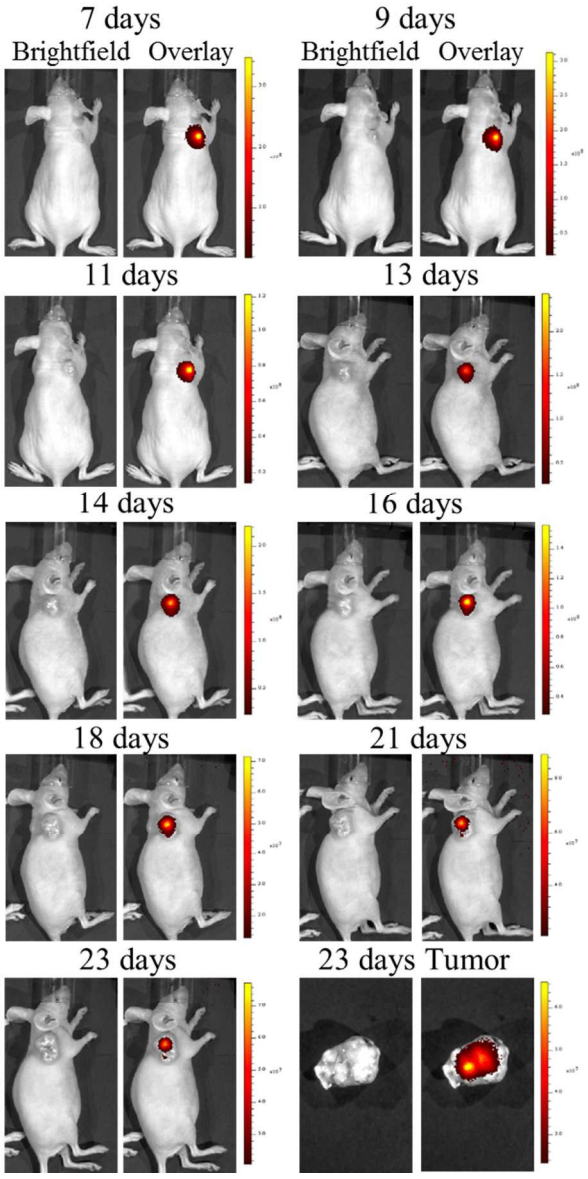

Fig. 4 Time-dependent *in vivo* fluorescence (FL) and brightfield (BF) imaging of the mouse injected with the NIR polymer nanoparticles (~20 μg) incubated HeLa cells and *ex vivo* fluorescence imaging of the tumor excreted from the mouse after 23 days injection. (Excitation filter: 465±15 nm; emission filter: 780±10 nm).

To investigate the long-term labeling capability and toxicity of the NIR polymer dots, 2×10^5 HeLa cells treated with 20 μg of NIR polymer dots were subcutaneously injected into a nude mouse. The mouse was imaged at different time after injection using the IVIS spectrum imaging system (**Fig. 4**). The NIR

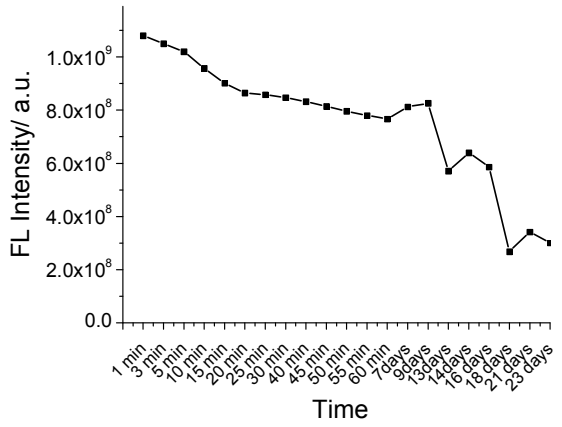

Fig. 5 Region of interest (ROI) analysis of the fluorescence intensity of the NIR polymer nanoparticles (~20 μg) incubated HeLa cells in the mouse over time.

polymer dots remains 75% of its fluorescence upon 7 days injection and still retains 28% of fluorescence after 23 days (**Fig. 5, Fig. S4**), indicating the durable brightness and long-term photostability of the NIR polymer dots. Moreover, *ex vivo* imaging of tumors showed that strong NIR fluorescence signal was detected in almost the whole region of the tumor, suggesting that the NIR polymer dots could be inherited by daughter cells. In addition, after 7 days injection, the size of the grafted tumor reached ~0.2 cm, which is recognized to the naked eyes, and tumor kept growing up to ~1.5 cm after 23 days, indicated that the NIR polymer dots had no obvious effect on the tumor growth. These data indicated that 20 μg could be considered a safe labeling dosage of the NIR polymer dots with no detectable influence on tumor formation ability over 23 days.

We examined the difference in sensitivity by using lower concentration (5 μg, 10 μg) of the NIR polymer dots. Significant fluorescence signals was detected up to 20 days for 10 μg of dots-treated cells and 15 days for 5 μg of dots-treated cells (**Fig. 6**). Furthermore, *ex vivo* imaging of tumors showed that intense fluorescence signal was observed in the tumor formed by 5 μg of dots-treated cells, indicating the high sensitivity of the NIR polymer dots for long-term cell tracking. Tumor tissues from 5 μg of dots-treated cells were excised for sectioning and imaging under a fluorescence microscope. Strong near-infrared fluorescence was observed within the tumor tissue, confirmed the NIR polymer dots in the tumors (**Fig. S5**).

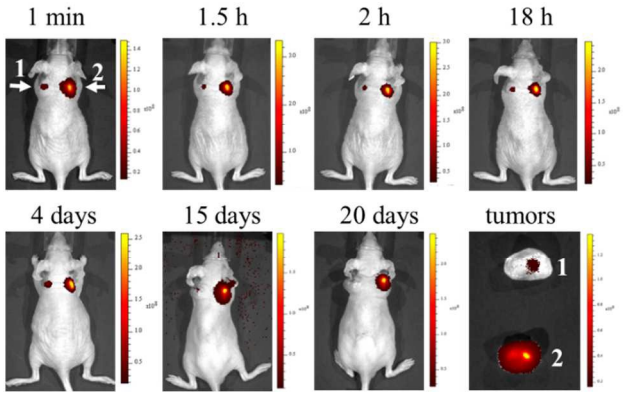

Fig. 6 Time-dependent *in vivo* fluorescence of the mouse injected with different concentration of the NIR polymer nanoparticles (1: ~5 μg, 2: ~10 μg) incubated HeLa cells and *ex vivo* fluorescence imaging of the tumors excreted from the mouse after 20 days injection.

We further test the difference in sensitivity between fluorescence and bioluminescence of NIR polymer dots by conjugated with Luc8 by EDC-mediated coupling reaction according to Rao lab's work.[22] The FRET efficiency of the NIR polymer dots was calculated to be about 0.77, and the overall BRET ratio of the Luc8-conjugated NIR polymer dots, determined by dividing the acceptor emission (551–810nm) by the donor emission (400–550nm), was about 2.5 (corresponding to an efficiency of 0.71). The prepared Luc8-conjugated NIR polymer dots with different concentration (3 μg, 0.6 μg, 0.3 μg, 0.06 μg) were subcutaneously injected into a nude mouse. The mouse was imaged immediately after an intravenous injection of 10 μg of coelenterazine for bioluminescence imaging with 1 minute acquisition time for no emission filter and 3 minutes acquisition time for NIR emission filter (780±10 nm). Following bioluminescence imaging, *in vivo* fluorescence imaging was carried out with 2 second acquisition time (excitation: 465±15 nm; emission: 780±10 nm) (**Fig. 7 and Table S1**). Intense NIR fluorescence signals were clearly visualized in the four injection sites, while bioluminescence imaging showed no obvious signal in the injection Ⅳ, indicating the high luminescence intensity and sensitivity of NIR polymer dots as a probe for fluorescence imaging. Although bioluminescence imaging showed high signal to background ratio with the value was 349.3, 20.2, 11.6, and 6.2 for 3 μg, 0.6 μg, 0.3 μg, and 0.06 μg of Luc8-conjugated dots (without emission filter), respectively. After using the NIR emission filter (780±10 nm), the signal to background ratio was decreased to 12.3, 5.6, 3.8, and 1.8, respectively. Fluorescence imaging using NIR polymer dots as a probe compared to NIR bioluminescence imaging showed a similar signal to background ratio with the value was 3.7, 2.9, 1.5, and 1.0, respectively, and decreased the exposure time by >90-fold, which benefitted the monitoring of quick interactions of cells.

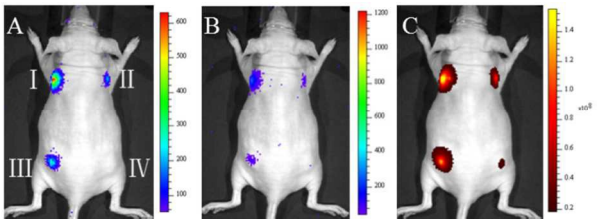

Fig. 7 In vivo bioluminescence (A, B) and fluorescence (C) imaging of the mouse injected with different concentration of the Luc8-conjuagted NIR polymer nanoparticles subcutaneously (Ⅰ: 3 μg, Ⅱ:□0.3 μg, Ⅲ: 0.6 μg, Ⅳ:□0.06 μg). A, open without filter, acquisition time: 1 min; B, with 780±10 nm filter, acquisition time: 3 min; C, Excitation filter: 465±15 nm; emission filter: 780±10 nm, acquisition time: 2 s.

The ability to monitor cell survival, migration and differentiation is essential for the success of cell based therapies. Certain sized nanoparticles allow efficient particle-cell interaction, which permit non-invasive, accurate and real-time cell tracking.

However, it is still big challenge to understanding the fate of cells *in vivo* by fluorescence imaging or magnetic resonance imaging.[26,30-32,] In this study, 2×10^5 HeLa cells incubated with different concentration (5 μg, 10 μg, 20 μg) of NIR polymer dots were subcutaneously injected into a nude mouse. Strong fluorescence signals was detected up to 25 days (**Fig. S6**). The NIR polymer dots still remains 34% (site 1), 23% (site 2), and 33% (site 3) of its fluorescence after 25 days, respectively (**Fig. S7**). At 25 days after injection, the mouse was scarified and surgically opened for imaging. All the three injection sites were detected the NIR fluorescence signals. However, brightfield image of the mouse showed that only two tumors were detected in the site 1 and 2, respectively. These data indicated that the fate of cells *in vivo* is unknown by the direct fluorescent cell labelling method. Although bioluminescence imaging was reported to be able to tracking the fate and function of cell *in vivo*, it is not applicable clinically.[33] On the other hand, *ex vivo* imaging of tumors showed that strong fluorescence signal were observed in the tumors formed by 5 μg and 10 μg of dots-treated cells, highlighting the long-term labeling capability of the polymer dots as a probe for NIR fluorescence imaging.

In conclusion, we prepared the NIR-emitting polymer dots by encapsulating the dye NIR775 into the matrix of MEH-PPV dots using a nanoprecipitation method, and demonstrated the synthesized NIR polymer dots for long-term tumor cell tracking *in vivo* for the first time. These synthesized NIR polymer dots showed no obvious effect on the tumor growth, and exhibited unique capabilities for *in vivo* cell tracking, such as long-term luminescence and photostability, and high sensitivity. This study provides a foundation for the development of the whole-body tumor cell tracking based on the NIR polymer dots as fluorescent nanoprobes.

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Notes and references

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† *Electronic Supplementary Information (ESI) available: [details of the experiment section, TEM, Excitation spectra, Fluorescence spectra, fluorescence images of mice, ROI analysis of the fluorescence intensity]. See DOI: 10.1039/b000000x/*

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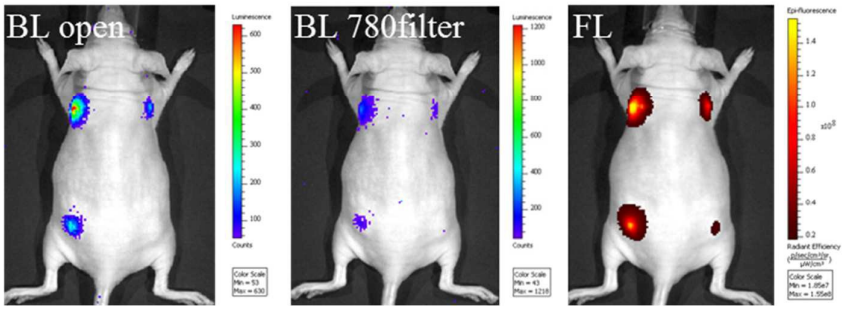

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