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Layered nanoprobe for long-lasting fluorescent cell label**

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Abstract

A long-lasting particle-based fluorescent label is designed for extended cell imaging studies. This onion-like nanoprobe is intrigued by the layer-by-layer fabrication technology. The nanoprobe is assembled with multiple layers of optically quenched polyelectrolytes whose fluorescent signal can be released later by intracellular proteolysis. Upon incubation with cells, the assembled nanoprobe is taken up efficiently. The tight packing and layered assembly of the quenched polyelectrolytes slow subsequent intracellular degradation, and then result in a prolonged intracellular fluorescence signal for up to 3 weeks with no noticeable toxicity.

Keywords

layer-by-layer; protease; cell tracking; fluorescence; slow release

1. Introduction

Layer-by-layer fabrication technology has been used to develop biomaterials for various biological applications.^[1-5] One major advantage of this fabrication technology is materials with different properties could be layered sequentially resulting in a multi-functional assembly. Recent advances in cell therapies, such as T-cell, islet cell, myocyte, and stem cell therapies, have opened new possibilities in various clinical applications.^[6-9] To further improve their therapeutic value, information about the location, functionality, and fate of injected cells is vital.^[10-12] Monitoring cell trafficking inside the body is important, and conventional techniques, such as histology, do provide clear evidence but require biopsies and thus, cannot provide real-time information.

Imaging is the best and only non-invasive way to track injected cells *in vivo*. To follow injected cells, they must be labeled with appropriate imaging reporters that offer bright signals for extended periods of time with no cytotoxicity. Ineffective labeling approaches would reduce the sensitivity and limit the duration of studies. Several transgenic reporters, such as green fluorescent protein, luciferase, and thymidine kinase, can satisfy these prerequisites; however, introducing a foreign gene into therapeutic cells is a concern in clinical applications.^[13-15] Chemical reporters, including organic fluorochromes and inorganic nanocrystals, have been used,^[16,17] but most chemical reporters provide only transient signals, especially in dividing cells. Recently, we found that multilayered small interfering RNA (siRNA)-coated gold nanoparticles (AuNPs) show extended gene silencing effects in various tumor cells.^[5] Based on this observation, we designed a long-lasting imaging nanoprobe for live cell labels.

2. Results and Discussion

A labeling nanoprobe was prepared with multiple layers of polyelectrolytes, sequentially assembled on an inert AuNP, using alternating charged polymers (**Figure 1a**). AuNPs were selected as the core of multilayered fluorescent nanoprobe for their uniform size, shape-dependent optical and electronic features, as well as feasibility for surface modification.^[18,19] The negatively charged polyelectrolyte used was polyacrylic acid (PAA), and the positively charged layer was biodegradable poly-L-lysine (PLL) decorated with fluorescein isothiocyanate (FITC).^[20,21] Because the positively charged lysine residues in PLL can be degraded by many intracellular proteases, such as cathepsins B and L, PLL was selected as the backbone to provide a slow-release capability.^[22-24] When multiple FITC residues were loaded onto one PLL backbone, self-quenching occurred due to the close proximity of neighboring fluorochromes. Importantly though, this quenched signal could be recovered after proteolytic degradation of the PLL backbone. The optimal loading ratio (FITC/PLL = 10/1) that provided the maximum quenching/dequenching effect and protease accessibility was determined from a series of PLL conjugates with differing amounts of FITC. A greater than 34-fold change in fluorescence signal was observed upon treatment with a model protease, trypsin (**Figure S1a**). Higher loading of FITC provided no further benefit. For comparison, FITC was also loaded on a non-degradable poly-D-lysine (PDL) at the same ratio (FITC/PDL = 10/1) and treated in the same way. As expected, the fluorescence signal was not increased (**Figure S1b**), suggesting that proteolysis is required to release the quenched fluorescence signal.

To assemble the multilayered fluorescent AuNPs, the negatively charged AuNPs (40 nm) in water were dropped into the positively charged PLL-FITC solution (average $M_w = 50$ kDa) for the first layer of coating. The reaction mixture was incubated for 30 min, and then the coated particles were spun down by centrifugation. After three washes with sterilized water, the PLL-FITC-coated AuNPs (1L AuNPs) were added to the negatively charged PAA ($M_w = 15$ kDa) solution. By repeating these procedures (**Figure 1a**), multilayered AuNPs – up to five layers of PLL-FITC and four layers of PAA (5L AuNPs) – were successfully fabricated by electrostatic interactions. The successful alternating layers of charged polyelectrolytes were evidenced by the zigzag pattern of the surface zeta potentials (**Figure 1b**). The PLL coating brought the surface charge from negative to positive, whereas the PAA coating switched it from positive to negative. The hydrodynamic diameter of the prepared AuNPs was measured by dynamic light scattering (DLS) after coating with each layer. The size of initial bare AuNPs was 40 nm and the prepared particle size increased steadily with the number of layers (1L: 63 nm; 3L: 112 nm; 5L: 144 nm) (**Figure 1c**).

AuNPs prepared with different number of layers were then subjected to protease activation. As shown in **Figure 1d**, particles were stable in phosphate-buffered saline (PBS), but when treated with protease, the fluorescence signal change was layer-dependent. The optimal formulation was 5L AuNPs (five layers of PLL and four layers of PAA), the fluorescence of which changed by 195-fold. The fluorescence signal change of 7L AuNPs, however, was similar to that of 5L AuNPs despite the two additional layers of PLL-FITC added to the particles. Under the same conditions, when protease-resistant PDL-FITC was used in the assembly, no fluorescence change was seen with the PDL-FITC coated AuNPs (5D AuNPs, five layers of PDL and four layers of PAA), as observed with the free PDL-FITC peptide (**Figure S1b**), again suggesting that protease degradation is required to release the packed fluorochromes. Comparing the protease-induced fluorescence changes between free PLL-FITC and assembled particle, a much larger change was seen with the assembled particles. This was presumably caused by the layer-by-layer packing, because fluorescence quenching could occur not only within each PLL molecule but also between layers, and the plasmonic resonance of Au core^[25-26]. Additionally, the kinetics of the fluorescence release of the

nanoprobe was much slower than that of free PLL-FITC molecules, suggesting the protease required more time to digest the tightly packed layers.

The ability of the multilayered fluorescent nanoprobe to label live cells was investigated by comparing the nanoprobe with commercially available cell labeling reagents, CM-DiI and CMTMR, using fluorescence microscopy. HeLa cells were incubated with cell trackers and various multilayered fluorescent nanoprobe (Figure 2). When incubated with CMTMR, the fluorescence signal was strong at short time points but decreased quickly and lasted only for 2 days (Figure 2a). When incubated with 3L AuNPs, the intracellular fluorescence signal was sustained for approximately 3 days (Figure 2b). However, a prolonged fluorescence signal was found with 5L AuNPs. A high fluorescence signal was maintained for more than 14 days with negligible background (Figure 2c). CM-DiI, PLL-FITC without AuNPs, and 1L AuNPs were also tested with HeLa cells; however, their signal strengths were much weaker than that of the 5L AuNPs (Figure S2). As expected, there was no signal when non-degradable 5D AuNPs were used (Figure 2d). To investigate the sub-localization of multilayered nanoprobe, HeLa cells were stained with Hoechst and LysoTracker after treatment with 5L AuNPs (Figure 3). Merged fluorescence signal of FITC and LysoTracker clearly showed the lysosomal delivery of multilayered fluorescent nanoprobe. Two hours after incubation, the merged image of FITC and LysoTracker signals in some cells showed significant overlapping, indicating the particles were located and activated in lysosomes. While in some other cells, FITC fluorescent signal was distributed evenly in whole cells. It suggested that somehow the nanoprobe could flee the lysosomes. At day 3, high FITC signal was found in nucleus. Further supported the hypothesis that the particles were able to escape from lysosomes and continued to release fluorescent signals.

Similar signal patterns were seen with MDA-MB231 cells using either commercially available cell trackers or our multilayered fluorescent nanoprobe (Figure S3). However, in MDA-MB231 cells, the fluorescence signal could only be followed for 5 days, because of the fast growing nature of MDA-MB 231 cells. Among the probes tested, only 5L AuNPs could maintain a strong fluorescence signal to day 5 in MDA-MB231 cells. The cytotoxicity of the prepared multilayered fluorescent nanoprobe was evaluated in HeLa (Figure 2f) and MDA-MB231 (Figure S3g) cells. No significant toxicity was detected in any nanoprobe-treated cells.

To gain a better understanding of signal retention after cell division, a suspension of the Jurkat T cell line was tested with 5L AuNPs (Figure 4a) or the commercially available cell tracking agents, CM-DiI and CMTMR (Figure 4b,c). After incubation with various labels, aliquots of cells were collected and analyzed by flow cytometry (Figure S4). The initial fluorescence intensity of each label was controlled to a similar level for a fair comparison. Similar retention patterns were seen with the CM-DiI and CMTMR cell trackers. The intensity in CM-DiI- and CMTMR-treated cells had declined to 8 % and 3 % of the original intensity, respectively, after 4 days of incubation. At day 7, the labels were largely gone, and the signal had declined to a background level. As the doubling time of the Jurkat T cell line is approximately 48 h, this result suggested the label lasted for about three divisions. In contrast, the 5L AuNPs offered a much longer signal duration. At day 4, the signal intensity was still 24 %. At days 7 and 14, the signal had declined to 12 % and 7 %, respectively. Even after 21 days, the 4 % remaining signal was still detectable by FACS. These results suggest that the slow release mechanism prolonged the duration of action of the cell labels and that the nanoparticles were suitable for labeling dividing cells.

The potential toxicity of the nanoprobe in Jurkat cells was further assessed by monitoring a T-cell-specific surface marker. Specifically, after treatment with 5L AuNPs, cells were stained with an anti-CD3-phycoerythrin (PE) antibody and SYTOX Blue to confirm cell

phenotypic integrity and viability, respectively. No significant difference between 5L AuNPs-treated and non-treated cells was seen up to 7 days (**Figure 5**). Near identical FACS scatter plots of the 5L AuNPs-treated and non-treated cells also supported that 5L AuNPs cause no cell damage 21 days after treatment (**Figure S5**), indicating the safety of this long-lasting nanoprobe.

In summary, the tightly packed polyelectrolytes showed slow proteolytic degradation, so that the attached fluorochromes were not released at once, and this slow-release process resulted in a persistent supply of fluorochromes, maintaining the intracellular fluorescence signal at a high level for a prolonged period. Potentially the release kinetics could be controlled by varying polyelectrolytes with different properties, such as length, ionic strength and bond stability. A systemic evaluation of the negatively charged polyelectrolytes is currently under study for a better-controlled probe. Because each particle acts as a reservoir, the developed nanoprobe also worked well with dividing cells. During division, the nanoprobe could be distributed into daughter cells, and then refilled the daughter cells with newly released fluorochromes (Figure 5d). When FITC reporter was replaced by an near-infrared reporter, the developed long-lasting labeling strategy could have enormous potential in *in vivo* tracking of cells, because the intracellular fluorescence signal lasts for weeks, much longer than current labeling approaches.

3. Experimental Section

Chemicals and materials

Poly-L-lysine ($M_w = 30,000 \sim 70,000$), poly-D-lysine ($M_w = 30,000 \sim 70,000$), poly acrylic acid (35 wt. % solution, average $M_w = 15,000$), fluorescein 5(6)-isothiocyanate (FITC), and the monoclonal anti-CD3-PE antibody were obtained from Sigma-Aldrich (St. Louis, MO). Bare AuNPs (40 nm) were purchased from BB International (Cardiff, UK). SYTOX Blue, CM-DiI, CMTMR, Hoechst 33342 and LysoTracker were from Invitrogen (Carlsbad, CA). Amicon Ultracel membranes (10 kDa) were from Millipore (Billerica, MA), and CellTiter aqueous one solution was from Promega (Madison, WI).

Preparation of PLL-FITC and PDL-FITC

Different amounts of FITC (0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 mg) in dimethylformamide (DMF) solution (500 μ L) were reacted with 1 mg PLL or PDL in NaHCO_3 (1 mM, 500 μ L) with constant shaking in the dark at room temperature for 2 h. The reaction products were separated using molecular weight cut-off membrane filters (10 kDa, Millipore). The resulting PLL-FITC and PDL-FITC was collected and washed several times with sterilized water until the color was clear.

Proteolytic activation of PLL-FITC

To measure the dequenching effect, the prepared PLL-FITC (PLL 0.2 nmole in 5 μ L) and PDL-FITC (PDL 0.2 nmole in 5 μ L) in PBS were incubated with 10 μ L trypsin-EDTA (0.25%, Sigma-Aldrich) in a 96-well culture plate at 37 °C, and the increase in the FITC fluorescence signal was assessed using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) at 480 nm excitation and 530 nm emission for 3 h.

Preparation of multilayered fluorescent AuNPs

AuNP solution (40 nm, 3.15×10^9 particles in 700 μ L) was added drop-wise into a PLL-FITC solution (PLL, 1.8 nmole in 500 μ L). After incubating for 30 min in the dark with gentle shaking, the solution was centrifuged (30 min at 16,100 g , using a microcentrifuge; Eppendorf, Hauppauge, NY). The supernatant was removed, and the gel-like deep red pellet was suspended in pure water and centrifuged again (30 min at 16,100 g). After a further

wash, PLL-FITC-coated AuNPs were resuspended in pure water (500 μL). Next, the PLL-FITC-coated AuNPs were added to a PAA solution (23.3 mM, 500 μL). The reaction solution was incubated in the dark for 30 min with gentle shaking, followed by three washes. The deposition procedures were repeated to a total of nine layers of polyelectrolyte (five layers of PLL-FITC and four layers of PAA). Zeta potentials of prepared AuNPs in water were measured using a ZetaPALS (Brookhaven Instruments Corp., Holtsville, NY) and sizes were measured using a Zetasizer Nano-ZS (Malvern, Worcestershire, UK) according to the manufacturer's protocol.

Protease-assisted fluorescence release from multilayered AuNPs

The protease-induced fluorescence change of the multilayered fluorescent AuNPs was determined by incubating various formulated particles (7.88×10^8 particles) in a 96-well culture plate with or without trypsin (25 μL) in PBS at 37 °C and the increase in the FITC fluorescence signal was analyzed using a Spectramax M2 plate reader (Molecular Devices) at 480 nm excitation and 530 nm emission for 8 h.

Cell lines

HeLa cells and MDA-MB231 human breast cancer cells were cultured in DMEM medium (Mediatech Inc., Manassas, VA), and Jurkat cells were cultured in RPMI 1640 medium (Thermo Scientific, Rockford, IL). All culture media were supplemented with 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, and 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich) in a humidified atmosphere with 5% CO₂ at 37 °C.

Live cell imaging using fluorescence microscopy

Fluorescence images of live cells were acquired using a fluorescence microscopy system (Olympus, Tokyo, Japan). Briefly, HeLa and MDA-MB231 cells were collected by trypsinization, counted, and plated in a 96-well black clear-bottom culture plate (Corning Life Sciences, Pittston, PA) at a density of 1×10^3 cells per well. After 1 day, AuNPs (2.52×10^8 particles) were added and incubated for 12 h. Cells were then washed three times with PBS and cultured in phenol red-free medium and imaged with a fluorescence microscopy at the time points indicated. In a separate set of experiment, cells were treated with CellTrackers, CM-DiI (2 μM), or CMTMR (7.5 μM , Invitrogen). One day after cell seeding, HeLa and MDA-MB231 cells were incubated with CM-DiI for 5 min at 37 °C and then further incubated at 4 °C for an additional 15 min according to manufacturer's protocol. After removing the medium and then for an additional 15 min. at 4 °C. For CMTMR study, cells were incubated with CMTMR for 30 min at 37 °C and the medium was then replaced with fresh medium and incubated for a further 30 min. After labeling with CM-DiI or CMTMR, cells were washed three times with PBS and cultured in phenol red-free medium and imaged under a fluorescence microscopy as indicated.

For the sub-localization of multilayered nanoprobe experiment, HeLa cells were collected by trypsinization, counted, and plated in a 96-well black clear-bottom culture plate (Corning Life) at a density of 1×10^3 cells per well. After 1 day, AuNPs (2.52×10^8 particles) were added and incubated for 12 h. Cells were then washed three times with PBS and cultured in phenol red-free medium. At each time point (2 hr, 1 day and 3 day), cells were stained with LysoTracker (1 μM , Invitrogen) for 1 h and Hoechst 33342 (1 μM , Invitrogen) for 5 min at 37 °C according to manufacturer's protocol. After three washes with PBS, cells were imaged under a fluorescence microscopy.

Cytotoxicity assessment of the multilayered fluorescent AuNPs

A cell proliferation assay was performed to assess the cytotoxicity of the various treatments. Briefly, HeLa and MDA-MB231 cells were collected by trypsinization, counted, and plated in a 96-well culture plate at a density of 5×10^3 cells per well. One day after seeding, AuNPs (2.52×10^8 particles) were added and the cells were cultured for a further 24 h. At day 2, 20 μ L CellTiter solution (Promega) was added to each well and incubated for an additional 3 h, and then the absorbance of the solution was measured at 490 nm using a Spectramax M2 plate reader (Molecular Devices).

Fluorescence retention in Jurkat cells

Jurkat cells (1.0×10^6 cells) were seeded in a 12-well culture plate (BD Falcon, San Jose, CA) together with 5L AuNPs (1.26×10^9 particles) in 400 μ L RPMI 1640 medium and cultured for 24 h in the dark at 37 °C in a 5% CO₂ atmosphere. Cells (4×10^5) were then transferred to a FACS tube after three washes with PBS, and the FITC fluorescence signal inside the cells was measured using a BD LSR II flow cytometry (BD Biosciences, San Jose, CA). Remaining cells were cultured in 6-well culture plates (BD Falcon) in 4 mL RPMI 1640 medium. The procedure was repeated at different time points, up to 21 days. In a separate set of experiments, Jurkat cells (1.0×10^6) were incubated with CM-DiI (1 μ M) or CMTMR (2 μ M) following the manufacturer's instructions. On the designated day, the fluorescence signal of CM-DiI or CMTMR was measured using a PE filter set by a BD LSR II flow cytometry.

Assessment of CD3 expression profiles and cytotoxicity after treatment with 5L AuNPs

One day after incubating Jurkat cells (1.0×10^6) with 5L AuNPs (1.26×10^9 particles) in a 12-well culture plate in 400 μ L RPMI medium, a fraction of the cells (4×10^5) was moved to a FACS tube, followed by three PBS washes. Anti-CD3 antibody (3 μ L, Sigma-Aldrich) and SYTOX Blue (2 μ L, Invitrogen) in 1 mL PBS were added according to the manufacturer's instructions. PE fluorescence for CD3 expression and DAPI for cytotoxicity in Jurkat cells were measured for up to 7 days using a BD LSR II flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Preparation and characterization of multilayered fluorescent AuNPs. (a) Preparation process for multilayered fluorescent AuNPs using PLL-FITC and PAA as the charged polyelectrolytes. (b) Zeta potential after each coating of polyelectrolytes. (c) Diameter of bare AuNPs and polyelectrolyte-coated AuNPs. (d) Protease-assisted FITC release from various AuNPs. Multilayered fluorescent AuNPs (1L: FITC 4.5 nmole, 3L: FITC 6.5 nmole, 5L: FITC 8.5 nmole, 7L: FITC 10.5 nmole, 5D: FITC 11 nmole) were incubated with or without trypsin in PBS and the fluorescence intensity was examined to 8 h.

Figure 2.

Live cell imaging and cytotoxicity in HeLa cells. After incubation for 30 min with cell tracker, CMTMR (a) or incubation for 12 h with various multilayered fluorescent AuNPs, such as 3L (FITC 2.1 nmole) (b), 5L (FITC 2.7 nmole) (c), and 5D (FITC 3.6 nmole) (d), fluorescence signal were assessed using fluorescence microscopy (magnification : 20 X). Cell viability (f) was evaluated after incubation for 24 h with various multilayered fluorescent AuNPs using CellTiter solution.

Figure 3.

Subcellular localization of the multilayered nanoprobes. HeLa cells were incubated without (a) or with (b) 5L AuNPs (FITC 2.7 nmole) for 12 h and washed three times with PBS. At each time point (2 h, 1 day and 3 day), cells were stained with LysoTracker (1 μ M) for 1 h, Hoechst (1 μ M) for 5 min and then fluorescence signal was investigated with fluorescence microscopy (magnification : 40 X) using different filter sets (Hoechst : blue / 5L AuNPs : green / LysoTracker : red).

Figure 4. Fluorescence retention through generations in Jurkat cells. After treatment with 5L AuNPs (FITC 13.6 nmole), CM-DiI (1 μ M), or CMTMR (2 μ M), fluorescence intensity was evaluated by flow cytometry (a-c) for 21 days.

Figure 5.

Cell phenotype and viability after treatment with 5L AuNPs in Jurkat cells. CD3 expression (PE) and cell viability (DAPI) without staining (a) and double staining without treatment (b) or treatment with 5L AuNPs (c) were assessed for 7 days using flow cytometry. (d) Illustration of cellular uptake and signal release from multilayered fluorescent AuNPs through generations.