Nonprotecting Group Synthesis of a Phospholipase C Activatable Probe with an Azo-Free Quencher

Benjamin K. Liebov, Alejandro D. Arroyo, Natalia I. Rubtsova, Sofya A. Osharovich, E. James Delikatny, and Anatoly V. Popov

Department of Radiology, University of Pennsylvania, 3620 Hamilton Walk, Philadelphia, Pennsylvania 19104, United States

ABSTRACT: The near-infrared fluorescent activatable smart probe Pyro-phosphatidylethanolamine (PtdEtn)-QSY was synthesized and observed to selectively fluoresce in the presence of phosphatidylcholine-specific phospholipase C (PC-PLC). PC-PLC is an important biological target as it is known to be upregulated in a variety of cancers, including triple negative breast cancer. Pyro-PtdEtn-QSY features a QSY21 quenching moiety instead of the Black Hole Quencher-3 (BHQ-3) used previously because the latter contains an azo bond, which could lead to biological instability.

INTRODUCTION

Advances in the field of molecular imaging represent an ongoing effort to improve the physician’s ability to detect, monitor, and treat a variety of diseases, including cancer. Linder et al. describes these advances in terms of the development of three classes of molecular imaging probes: nonspecific agents, which fluoresce permanently, a specific or targeted probe with a fluorophore, and an activatable smart probe with a fluorophore that will only fluoresce upon enzymatic cleavage or activation via another biological mechanism. In this paper, we report the synthesis of an activatable smart probe designed to detect enzyme activity in lipid metabolism with known cancerous associations.

Lipids play an important role in biological systems as they are known to participate in the regulation of cell signaling. Because many cancers exhibit variations in their metabolic and signaling profiles, enzymes in lipid metabolism have become exciting targets for molecular imaging probes. Phosphocholine (PC) is a lipid metabolite that has been observed in increased levels in prostate,4,6,9,13 brain,4,6,9,12 and breast4,9,13–17 cancer cells and solid tumors. Elevated levels of PC have also been associated with the degree of malignancy,18–20 and decreased levels of PC are observed in response to chemotherapeutic treatment.21 PC has become an important and distinctive cancer biomarker. To detect PC by an activatable smart probe it is necessary to understand the surrounding biological processes. PC can be produced by multiple metabolic pathways, including the anabolic phosphorylation of choline by choline kinase or as the product of the catabolism of phosphatidylcholine by phospholipase C (PLC), along with diacylglycerol.22–28 These metabolic processes are considered a potential factor in the irregular choline metabolism observed in a variety of cancers.29–31 Elevated activity of phosphatidylcholine-specific PLC (PC-PLC) has been observed in a variety of cancer types, including the triple negative breast cancer line, MDA-MB-231.32 On the basis of these observations, our research efforts have centered on the development of molecular imaging probes that will selectively fluoresce in the presence of PC-PLC.

Previously, we reported the synthesis and selectivity of an activatable smart probe that selectively fluoresces in the presence of PC-PLC.22–24 This specific activation is achieved by the precise construction of the probe based on a core structure of the phospholipid, phosphatidylethanolamine (PtdEtn). If left unmodified, PtdEtn can be enzymatically cleaved by a variety of enzymes. However, if a bulky moiety is placed at the sn-2 position of the glycerol backbone without a spacer, only cleavage by PC-PLC is observed.22 Our lab exploited this selective cleavage to design a next-generation activatable probe selective for PC-PLC. The final goal would be to design a probe that can more easily delineate cancerous tissue from healthy tissue.

Along with the selectivity achieved by the PtdEtn backbone, to build the activatable probe, a precise fluorophore/quencher pair must be implemented. The former has already been explored in our previous research with the fluorophore, pyropheophorbide a (Pyro, λ_{ex} = 410 and 665 nm; λ_{em} = 670–725 nm). Pyro achieves two goals: first, it is bulky enough to prevent cleavage of the phospholipid by any enzyme other than PC-PLC (as described above) and second, it fluoresces in the near-infrared (NIR) window. NIR fluorophores have been of interest recently because the NIR window is known for low

ABSTRACT: The near-infrared fluorescent activatable smart probe Pyro-phosphatidylethanolamine (PtdEtn)-QSY was synthesized and observed to selectively fluoresce in the presence of phosphatidylcholine-specific phospholipase C (PC-PLC). PC-PLC is an important biological target as it is known to be upregulated in a variety of cancers, including triple negative breast cancer. Pyro-PtdEtn-QSY features a QSY21 quenching moiety instead of the Black Hole Quencher-3 (BHQ-3) used previously because the latter contains an azo bond, which could lead to biological instability.

INTRODUCTION

Advances in the field of molecular imaging represent an ongoing effort to improve the physician’s ability to detect, monitor, and treat a variety of diseases, including cancer. Linder et al. describes these advances in terms of the development of three classes of molecular imaging probes: nonspecific agents, which fluoresce permanently, a specific or targeted probe with a fluorophore, and an activatable smart probe with a fluorophore that will only fluoresce upon enzymatic cleavage or activation via another biological mechanism. In this paper, we report the synthesis of an activatable smart probe designed to detect enzyme activity in lipid metabolism with known cancerous associations.

Lipids play an important role in biological systems as they are known to participate in the regulation of cell signaling. Because many cancers exhibit variations in their metabolic and signaling profiles, enzymes in lipid metabolism have become exciting targets for molecular imaging probes. Phosphocholine (PC) is a lipid metabolite that has been observed in increased levels in prostate, brain, and breast cancer cells and solid tumors. Elevated levels of PC have also been associated with the degree of malignancy, and decreased levels of PC are observed in response to chemotherapeutic treatment. PC has become an important and distinctive cancer biomarker. To detect PC by an activatable smart probe it is necessary to understand the surrounding biological processes. PC can be produced by multiple metabolic pathways, including the anabolic phosphorylation of choline by choline kinase or as the product of the catabolism of phosphatidylcholine by phospholipase C (PLC), along with diacylglycerol. These metabolic processes are considered a potential factor in the irregular choline metabolism observed in a variety of cancers. Elevated activity of phosphatidylcholine-specific PLC (PC-PLC) has been observed in a variety of cancer types, including the triple negative breast cancer line, MDA-MB-231. On the basis of these observations, our research efforts have centered on the development of molecular imaging probes that will selectively fluoresce in the presence of PC-PLC.

Previously, we reported the synthesis and selectivity of an activatable smart probe that selectively fluoresces in the presence of PC-PLC. This specific activation is achieved by the precise construction of the probe based on a core structure of the phospholipid, phosphatidylethanolamine (PtdEtn). If left unmodified, PtdEtn can be enzymatically cleaved by a variety of enzymes. However, if a bulky moiety is placed at the sn-2 position of the glycerol backbone without a spacer, only cleavage by PC-PLC is observed. Our lab exploited this selective cleavage to design a next-generation activatable probe selective for PC-PLC. The final goal would be to design a probe that can more easily delineate cancerous tissue from healthy tissue.

Along with the selectivity achieved by the PtdEtn backbone, to build the activatable probe, a precise fluorophore/quencher pair must be implemented. The former has already been explored in our previous research with the fluorophore, pyropheophorbide a (Pyro, λ_{ex} = 410 and 665 nm; λ_{em} = 670–725 nm). Pyro achieves two goals: first, it is bulky enough to prevent cleavage of the phospholipid by any enzyme other than PC-PLC (as described above) and second, it fluoresces in the near-infrared (NIR) window. NIR fluorophores have been of interest recently because the NIR window is known for low
autofluorescence, minimal absorption by water and hemoglobin, and low tissue scattering. Notable examples are indocyanine green or IRDye 800CW, both of which fluoresce in the NIR window and can be observed by detectors with tissue depth penetration of up to 1 cm. The relative ease of detection of a NIR fluorophore like Pyro makes it an ideal candidate. An additional feature of Pyro is that it is a known photosensitizing agent, therefore our probe could be used not only for detection of cancer but also for treatment through the production of singlet oxygen when stimulated at the proper wavelength.

Despite the utility of Pyro as a NIR fluorophore, to create an activatable smart probe, the fluorescence must be quenched when not in the presence of the activating enzyme, PC-PLC. This is achieved by coupling a quenching moiety to the smart probe. Previously, our laboratory used the NIR Black Hole Quencher-3 (BHQ-3, absorbance range 620−730 nm) because it precisely absorbed the fluorescence emitted by Pyro through Förster resonance energy transfer. The completed probe Pyro-PtdEtn-BHQ was successfully isolated by our lab (Scheme 1) and tested against various sources of PC-PLC. In previously published papers, we reported the biological testing of Pyro-PtdEtn-BHQ against a series of phospholipases, including PC-PLC. Testing on cancer cells was carried out with DU145 human prostate cancer cell line, which has increased PC-PLC levels, and in vivo studies were performed on mouse models bearing DU145 tumors. Despite the success of Pyro-PtdEtn-BHQ, BHQ-3 has been shown to experience stability problems in vivo due to the presence of an azo bond in the structure. Moreover, the shelf-life of Pyro-PtdEtn-BHQ chloroform solution is less than 2 months, which makes it inconvenient for practical use as it requires freshly synthesized probes. The synthesis is complicated, and the overall yield of Pyro-PtdEtn-BHQ is as low as 15%.

Because of the quenching range afforded by BHQ-3, it has been used in a variety of fluorescent probes. Unfortunately, as was just mentioned, one of the drawbacks noted for BHQ-3 is the instability in the azo bond present in its structure. A 2015 article published by Uddin et al. exploits the ease of cleavage of the azo bond of BHQ-3 as a probe activation mechanism to monitor hypoxia in mouse models. Although this example uses the azo bond for advantageous purposes, the
bulk of the literature on BHQ-3 describes a molecule with excellent quenching potential but with the potential for structural instability. An alternative quenching moiety with similar quenching capabilities but without an unstable azo bond could lead to a new generation of more stable activatable probes.

In this paper, we present the development of a new activatable smart probe that incorporates an alternative, more stable quenching moiety: QSY21, which has a similar quenching range (580–720 nm) to BHQ-3 (620–730 nm). Both quench in the NIR range, but their difference lies in their structures as QSY21 does not contain an azo bond (see structure in Scheme 2) and is therefore considered to be more stable. Moreover, a variety of activatable smart probes have already been synthesized using QSY21 to detect biological processes.

This paper presents the synthesis and initial biological evaluation of the novel PC-PLC selective probe, Pyro-PtdEtN-QSY.

**RESULTS AND DISCUSSION**

When the initial Pyro-PtdEtN-BHQ activatable probe was developed, it required the use of a BOC-protected phosphatidylethanolamine (LysoPtdEtN-BOC, see Scheme 1). By protecting the primary amine of the phospholipid we were able to prevent the coupling of the Pyro moiety at that position and saw exclusive substitution at the desired sn-2 position, which is required to achieve selective cleavage via PC-PLC.

Although the use of the LysoPtdEtN-BOC was feasible, it would have been more economical to use the unprotected phospholipid LysoPtdEtN. Unfortunately, when BHQ-3-SE was reacted with unprotected LysoPtdEtN, the desired product, LysoPtdEtN-BHQ, could not be isolated.

However, when unprotected conditions were tested using the azo-free quenching moiety, QSY21 (as succinimidyl ester, QSY21-SE), the desired product, LysoPtdEtN-QSY, was isolated in decent yield as a blue oil (Scheme 2). A detected side product was the product of hydrolysis of QSY21-SE; this did not inhibit the production or isolation of LysoPtdEtN-QSY. Not using a BOC protecting group proved to be not only financially advantageous but it also reduced the number of synthetic steps as we no longer had to remove the protecting group. An additional disadvantage of BOC-deprotecting step was partial chemical cleavage of ester bonds at the glycerol backbone under the strong acidic conditions that dramatically decreased the yield of Pyro-PtdEtN (20%, Scheme 1). That is why this alteration most likely is responsible for the higher yield and cleaner product obtained.

Once LysoPtdEtN-QSY was isolated, Pyro is coupled to the sn-2 position of the phospholipid using a carbodiimide, N-(3-dimethylaminopropyl)-N’-ethyl carbodiimide hydrochloride (EDC·HCl). An excess of Pyro and EDC·HCl is used to drive the reaction toward the desired product, Pyro-PtdEtN-QSY (Scheme 2). Initially, Pyro appears green but once incorporated into the probe, its fluorescence is quenched by QSY21 and the fully assembled probe, Pyro-PtdEtN-QSY, appears aquamarine. This stark color change between Pyro and Pyro-PtdEtN-QSY simplifies the product isolation using column chromatography. The overall yield of Pyro-PtdEtN-QSY was as high as 61%, more than 4 times higher than for Pyro-PtdEtN-BHQ.

The shelf-life of Pyro-PtdEtN-QSY chloroform solution by now is more than 8 months, which makes it an ideal probe for practical use.

With Pyro-PtdEtN-QSY in hand, we set out to test its selective activation by PC-PLC. To confirm its specificity, the probe was exposed to PC-PLC as well as several other phospholipases, including sphingomyelinase (SMase), sPLA2 (IB porcine), and phospholipase D (PLD). In this (and next) experiment, the probe was incorporated into lipid nanoparticles (LNP) in 140 mM NaCl–10 mM HEPES, pH 7.4 (control). When Pyro-PtdEtN-QSY was exposed to PC-PLC, an increase of fluorescence of Pyro-PtdEtN-QSY (Figure 1).

![Figure 1. Selective cleavage of Pyro-PtdEtN-QSY by PC-PLC in a buffered solution compared to several different phospholipases: PC-PLC, SMase, sPLA2 (IB porcine), PLD, and buffer 140 mM NaCl–10 mM HEPES, pH 7.4 (control). No positive control was used.](image-url)
results of this experiment are evident from Figure 2. Fluorescence is observed almost immediately only when

Pyro-PtdEtn-QSY is exposed to PC-PLC. The fluorescence continues to increase with time, whereas the buffered control, PLD, and sPLA2 (IB porcine) experiments remain quenched even after 6 h. This experiment confirms the conclusions of previous enzyme tests, demonstrating that Pyro-PtdEtn-QSY is successfully quenched until it is exposed to PC-PLC and fluorescence is restored. Moreover, this study is more accurate in confirming the potential translational use of the probe as a detector for PC-PLC in in vitro and in vivo models.

■ CONCLUSIONS

The NIR fluorescent probe Pyro-PtdEtn-QSY shows remarkable selectivity for PC-PLC. The synthesis of the probe does not require the use of protecting group chemistry essentially skipping a step that had been previously required for a PC-PLC selective probe. Additionally, probe stability is increased using a quenching moiety that does not have an azo bond. This structural change could lead to increased stability when exposed to biological conditions. Therefore, Pyro-PtdEtn-QSY is an excellent candidate for in vitro and in vivo studies of cancer cell types with increased PC-PLC levels, such as MDA-MB-231, triple negative breast cancer.

■ EXPERIMENTAL PART

Materials and General Methods. Dry solvents were purchased from Fisher Scientific. Pyropheophorbide a (Pyro) was purchased from Frontier Scientific, Newark, DE. 1-Palmitoyl-2-hydroxy-sn-glycero-phosphoethanolamine (16:0 Lyso-phosphatidylethanolamine, LysoPtdEtn) and 1,2-diesteroyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] ammonium salt (DSPE-PEG2000) in chloroform, 5 mg/mL, were purchased from Avanti Polar Lipids, Inc., Alabaster, AL. QSY21 succinimidyl ester (QSY-SE) was purchased from Thermo Fisher Scientific, Grand Island, NY. Other reagents/reactants were purchased from Sigma-Aldrich and used without further purification. Silica diol (Premium RF, 70A, 40–75 μm, product # - 62570-01) and silica diol thin-layer chromatography plates (w/ UV254, glass backed, 200 μm, 10 × 20 cm 25/pk, product # - 2914136) were purchased from Sorbtek Chromatography, Norcross, GA. All chemical reactions with QSY21 and/or Pyro were carried out in the dark under dry Ar. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were recorded on a Bruker Ultraflex III MALDI TOF/TOF (Bruker Daltonics, Billerica, MA) using positive-mode ionization with a terthiophene (Ter; 2,2′:5′,2″-terthiophene) matrix. Absorbance and fluorescence data, as well as time-dependent release of fluorescence, were measured using a SpectraMax M5 fluorescent plate reader (Molecular Devices, San Jose, CA) and an IVIS Spectrum in vivo imaging system (PerkinElmer, Hopkinton, MA).

Phospholipases. Phospholipases were obtained from Sigma-Aldrich (St. Louis, MO), dissolved in 140 mM NaCl–10 mM HEPES buffer, pH = 7.4, and stored in aliquots at ~80 °C. Mammalian phospholipase isoforms were used whenever available: sPLA2 (type IB, porcine pancreas), otherwise bacterial isoforms were employed: PC-PLC (Bacillus cereus), SMase (Streptomyces chromofuscus), and PC-PLD (Streptomyces chromofuscus).

Experimental Procedures. Synthesis of LysoPtdEtn-QSY.

LysoPtdEtn (0.050 g, 0.110 mmol) and QSY-SE (0.020 g, 0.0245 mmol) were added to a 100 mL round-bottom flask with stir bar, followed by dry acetonitrile (~50 mL), diisopropylethylamine (40 μL, 0.230 mmol), and 4- (dimethylamino)pyridine (DMAP, 0.0087 g, 0.0712 mmol). The reaction flask was purged with argon and covered in aluminum foil. The blue reaction solution was stirred for 2 days. Reaction progress was tested via thin-layer chromatography (TLC) on functionalized silica diol (Sorbtech cat # 2933187) using a methanol/chloroform solution (5:95). The product was observed at Rf = 0.47. The reaction solution was concentrated in vacuo until ~3 mL remained. The reaction solution was loaded onto a silica diol (Sorbtech cat # 62570-01) column. A gradient of methanol/chloroform eluent was used (0:100 to 20:80) to elute several blue fractions. The blue fractions were tested via TLC, and on the basis of these results, the proper fractions were concentrated to a dark blue oil. The oil was left under vacuum overnight to reveal the product (0.0239 g, 0.0214 mmol, 87% yield). The product, LysoPtdEtn-QSY, was confirmed by MALDI mass spectrometry and UV–vis spectrometry (Supporting Information, SI).

Synthesis of Pyro-PtdEtn-QSY. LysoPtdEtn-QSY (0.0190 g, 0.0170 mmol) was put into a 20 dram vial charged with a stir bar, followed by Pyro (0.0118 g, 0.0542 mmol), N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC-HCl, 0.1848 g, 0.9640 mmol), DMAP (0.0225 g, 0.1841 mmol), and dry dichloromethane (~4 mL). The reaction vial was purged with argon, covered in foil, and
Preparation of Pyro-PtdEtn-QSY-Loaded Lipid Nanoparticles. DSPE-PEG2000 (1 mL of 5 mg/mL solution in CHCl₃, 1.78 μL chloroform. An aquamarine band was collected between minimal chloroform and placed on a silica diol preparative TLC plate. The plate was eluted with 10:90 methanol/chloroform. An aquamarine band was loaded onto a silica diol (Sorbtech cat 6871 R 62570-01) column (~0.5 cm). Methanol/chloroform (1:99) was mixed in a 15 mL glass ask. (Pyro-PtdEtn-QSY concentration in the initial chloroform solution was determined using the Beer–Lambert law by measuring the optical density at 410 nm and employing an extinction coefficient of 110 000 M/cm.) The solvent was evaporated under nitrogen flow. The obtained lipid film was dried under high vacuum overnight. The flask was then flushed with Ar and held in the water bath at 80 °C for 3 min, then rehydrated with 1 mL of hot (80 °C) degassed buffer solution under Ar flow. Buffer (140 mM NaCl–10 mM HEPES) was used at pH 7.4. The mixture was vortexed until an optical clear dispersion was obtained and allowed to cool down. Upon reaching room temperature, the solution was filtered through a 0.22 μm MILLEX-GV filter.

Enzyme-Mediated Probe Cleavage. Fluorescence experiments: 90 μL of phospholipase solution in buffer (140 mM NaCl–10 mM HEPES, pH = 7.4) was placed into a black well flat clear bottom 96-well plate (10 units per well). Pyro-PtdEtn-QSY lipid dispersion (10 μL) in buffer (140 mM NaCl–10 mM HEPES, pH = 7.4) was added to the phospholipase to a final concentration of 1 mM. Fluorescence was monitored over time at 37 °C on a Molecular Devices SpectraMax MS plate reader under excitation 409 nm and emission 656 nm.

Enzyme-Mediated Probe Cleavage: IVIS Spectrum Experiments. The IVIS Spectrum (PerkinElmer, Hopkinton, MA) is an advanced preclinical optical imaging system ideal for fluorescence imaging in vivo and in vitro. Black well flat clear bottom 96-well plates were prepared as above with solutions of 1 mM Pyro-PtdEtn-QSY and 10 units of phospholipases in 140 mM NaCl–10 mM HEPES buffer, pH = 7.4. Fluorescence was monitored over time at 37 °C with excitation between 415 and 445 nm (430 nm filter) and emission at 650–670 nm (660 nm filter).

Spectroscopic data for the characterization of PtdEtn-QSY and Pyro-PtdEtn-QSY.
phosphorylation in human breast cancer cells; NMR application of phosphocholine and adenosine triphosphate (ATP) are markers of phospholipase C by Enzyme-Activated Near-Infrared Probes. Bioconjugate Chem. 2011, 22, 4849–4861.

Aberrant Choline Phospholipid Metabolism in Breast Cancer. Mol. Cancer Ther. 2015, 14, 899–908.

(26) Chiorazzo, M. G.; Bloch, N. B.; Popov, A. V.; Delikatny, E. J. Synthesis and Evaluation of Cytoxic Phospholipase A2 Activatable Fluorophores for Cancer Imaging. Bioconjugate Chem. 2015, 26, 2360–2370.

(27) Aula, S. P.; Popov, A. V.; Delikatny, E. J. Choline kinase alpha-Partnering the ChoK-Hold on tumor metabolism. Prog. Lipid Res. 2016, 63, 28–40.

(28) Aula, S. P.; Popov, A. V.; Delikatny, E. J. Direct Inhibition of Choline Kinase by a Near-Infrared Fluorescent Carbocyanine. Mol. Cancer Ther. 2014, 13, 2149–2158.

(29) Podo, F.; Ferretti, A.; Kajiwara, A.; Zhang, P.; Ramoni, C.; Barletta, B.; Pini, C.; Baccarini, S.; Pulciani, S. Detection of phosphatidylcholine-specific phospholipase C in Friend leukemia cells before and after erythroid differentiation. Anticancer Res. 1993, 13, 2309–2317.

(30) Xingzhou, W.; Lu, H.; Zhou, L.; Huang, Y.; Chen, H. Changes of phosphatidylcholine-specific phospholipase C in hepatocarcinogenesis and in the proliferation and differentiation of rat liver cancer cells. Cell Biol. Int. 1997, 21, 375–381.

(31) Abalsamo, L.; Spadaro, F.; Bozzuto, G.; Paris, L.; Cecchetti, S.; Lugini, L.; Iorio, E.; Molinari, A.; Ramoni, C.; Podo, F. Inhibition of phosphatidylcholine-specific phospholipase C results in loss of mesenchymal traits in metastatic breast cancer cells. Breast Cancer Res. 2012, 14, R50.

(32) Tromberg, B. J.; Zhang, Z.; Leproux, A.; O’Sullivan, T. D.; Cerussi, A. E.; Carpenter, P. M.; Mehta, R. S.; Roblyer, D.; Yang, W.; Paulsen, K. D.; Pogue, B. W.; Jiang, S.; Kaufman, P. A.; Yodh, A. G.; Chung, S. H.; Schnall, M.; Snyder, B. S.; Hylton, N.; Boas, D. A.; Carpy, S. A.; Isakoff, S. J.; Mankoff, D. Predicting Responses to Neoadjuvant Chemotherapy in Breast Cancer: ACRIN 6691 Trial of Diffuse Optical Spectroscopic Imaging. Cancer Res. 2016, 76, 5933–5944.

(33) Teraphongphom, N.; Rosenthal, E. L.; Kong, C. S.; Warram, J. M. Specimen mapping in head and neck cancer using fluorescence imaging. Laryngoscope Invest. Otolaryngol. 2017, 2, 447–452.

(34) O’Sullivan, T. D.; Cerussi, A. E.; Cuccia, D. J.; Tromberg, B. J. Diffuse optical imaging using spatially and temporally modulated light. J. Biomed. Opt. 2012, 17, No. 071311.

(35) Durduran, T.; Choe, R.; Baker, W. B.; Yodh, A. G. Diffuse Optics for Tissue Monitoring and Tomography. Rep. Prog. Phys. 2010, 73, No. 076701.

(36) Liu, H.; Zhao, M.; Wang, J.; Peng, M.; Wu, Z.; Zhao, L.; Yin, Z.; Hong, Z. Photodynamic therapy of tumors with pyropheophorbide-a-loaded polyethylene glycol-poly(lactic-co-glycolic acid) nanoparticles. Int. J. Nanomed. 2011, 6, 4905–4918.

(37) Cheng, J.; Tan, G.; Li, W.; Li, J.; Wang, Z.; Jin, Y. Preparation, characterization and in vitro photodynamic therapy of a pyropheophorbide-a-conjugated Fe3O4 multifunctional magnetofluorescence photosensitizer. RSC Adv. 2016, 6, 37610–37620.

(38) Zhou, A.; Wei, Y.; Wu, B.; Chen, Q.; Xing, D. Pyropheophorbide A and C (RGDyK) Comodified Chitosan-Wrapped Upconversion Nanoparticle for Targeted Near-Infrared Photodynamic Therapy. Mol. Pharmaceutics 2012, 9, 1580–1589.

(39) TM, G. L. BBQ-650 (Blackberry Quencher 650). http://www.genelink.com/news/site/products/mod_detail.asp?modid=240.

(40) Technologies, L. B. BQH-3 Carboxylic Acid FAQs. https://www.biosearchtech.com/products/synthesis-reagents/carboxylic-acid-succinimidyl-esters/bhq3-carboxylic-acid?tab=page_8916.

(41) Guo, Z.; Park, S.; Yoon, J.; Shin, I. Recent progress in the development of near-infrared fluorescent probes for bioimaging applications. Chem. Soc. Rev. 2014, 43, 16–29.

(42) Simard, B.; Tomanek, B.; van Veggel, F. C. J. M.; Abulrub, A. Optimal dye-quinque partners for the design of an “activatable” nanoprobe for optical imaging. Photonem. Photobiol. Sci. 2013, 12, 1824–1829.

(43) Uddin, M. I.; Evans, S. M.; Craft, J. B.; Marnett, L. J.; Uddin, M. J.; Jayagopal, A. Applications of Azo-Based Probes for Imaging Retinal Hypoxia. ACS Med. Chem. Lett. 2015, 6, 445–449.

(44) Bollerstadt, R.; Gowda, A.; McNichols, R. Fluorescence Resonance Energy Transfer-Based Near-Infrared Fluorescence Sensor for Glucose Monitoring. Diabetes Technol. Ther. 2004, 6, 191–200.

(45) de la Zerda, A.; Bodapati, S.; Teed, R.; May, S. Y.; Tabakman, S. M.; Liu, Z.; Khuri-Yakov, B. T.; Chen, X.; Dai, H.; Gambhir, S. S. Family of Enhanced Photoacoustic Imaging Agents for High-Sensitivity and Multiplexing Studies in Living Mice. ACS Nano 2012, 6, 4694–4701.

(46) Jolivel, V.; Arthaud, S.; Botia, B.; Portal, C.; Derset, B.; Clave, G.; Leprince, J.; Romieu, A.; Renard, P.-Y.; Touzani, O.; Liger, H.; Noack, P.; Massonneau, M.; Fournier, A.; Vaudry, H.; Vaudry, D. Biochemical Characterization of a Caspase-3 Far-Red Fluorescent Probe for Non-invasive Optical Imaging of Neuronal Apoptosis. J. Mol. Neurosci. 2014, 54, 451–462.
(48) Kokko, T.; Kokko, L.; Loevgren, T.; Soukka, T. Homogeneous Noncompetitive Immunoassay for 17β-Estradiol Based on Fluorescence Resonance Energy Transfer. *Anal. Chem.* 2007, 79, 5935−5940.

(49) Le Reste, L.; Hohlbein, J.; Gryte, K.; Kapanidis, A. N. Characterization of Dark Quencher Chromophores as Nonfluorescent Acceptors for Single-Molecule FRET. *Biophys. J.* 2012, 102, 2658−2668.

(50) Li, X.; Mu, J.; Liu, F.; Tan, E. W. P.; Khezri, B.; Webster, R. D.; Yeow, E. K. L.; Xing, B. Human Transport Protein Carrier for Controlled Photoactivation of Antitumor Prodrug and Real-Time Intracellular Tumor Imaging. *Bioconjugate Chem.* 2015, 26, 955−961.

(51) Madiyar, F. R.; Bhana, S.; Swisher, L. Z.; Culbertson, C. T.; Huang, X.; Li, J. Integration of a nanostructured dielectrophoretic device and a surface-enhanced Raman probe for highly sensitive rapid bacteria detection. *Nanoscale* 2015, 7, 3726−3736.

(52) Ofori, L. O.; Withana, N. P.; Prestwood, T. R.; Verdoes, M.; Brady, J. J.; Winslow, M. M.; Sorger, J.; Bogoy, M. Design of Protease Activated Optical Contrast Agents That Exploit a Latent Lysosomal Effect for Use in Fluorescence-Guided Surgery. *ACS Chem. Biol.* 2015, 10, 1977−1988.

(53) Ogawa, M.; Kosaka, N.; Choyke, P. L.; Kobayashi, H. Tumor-Specific Detection of an Optically Targeted Antibody Combined with a Quencher-Conjugated Neutravidin “Quencher-Chaser”: A Dual “Quench and Chase” Strategy to Improve Target to Nontarget Ratios for Molecular Imaging of Cancer. *Bioconjugate Chem.* 2009, 20, 147−154.

(54) Sun, X.; Zhang, A.; Baker, B.; Sun, L.; Howard, A.; Buswell, J.; Maurel, D.; Masharina, A.; Johnson, K.; Noren, C. J.; Xu, M.-Q.; Correa, I. R. Development of SNAP-Tag Fluorogenic Probes for Wash-Free Fluorescence Imaging. *ChemBioChem* 2011, 12, 2217−2226.

(55) Verdoes, M.; Oresic Bender, K.; Segal, E.; van der Linden, W. A.; Syed, S.; Withana, N. P.; Sanman, L. E.; Bogoy, M. Improved Quenched Fluorescent Probe for Imaging of Cysteine Cathepsin Activity. *J. Am. Chem. Soc.* 2013, 135, 14726−14730.

(56) Xing, B.; Khanamiryan, A.; Rao, J. Cell-permeable near-infrared fluorogenic substrates for imaging β-lactamase activity. *J. Am. Chem. Soc.* 2005, 127, 4158−4159.

(57) Zhang, S.; Metelev, V.; Tabatabaei, D.; Zamecnik, P.; Bogdanov, A., Jr. Near-Infrared Fluorescent Oligodeoxyribonucleotide Reporters for Sensing NF-κB DNA Interactions In Vitro. *Oligonucleotides* 2008, 18, 235−243.

(58) Zou, P.; Ting, A. Y. Imaging LDL Receptor Oligomerization during Endocytosis Using a Co-internalization Assay. *ACS Chem. Biol.* 2011, 6, 308−313.

(59) Anikeeva, N.; Sykulev, Y.; Delikatny, E. J.; Popov, A. V. Core-based lipid nanoparticles as a nanoplatform for delivery of near-infrared fluorescent imaging agents. *Am. J. Nucl. Med. Mol. Imaging* 2014, 4, 507−524.