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Poly(ethylene glycol) with Observable Shedding

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PEG with Observable Shedding**

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Abstract

A novel FRET-bearing poly(ethylene glycol) (PEG) conjugate fluoresces at 520 nm when it is cleaved off from nanoparticles (NPs). When the NPs were targeted to cancer cell lines, the reducing redox of the endosomal compartment caused disulfide bond cleavage and shedding of the PEG layer. The fluorescence emission can be suppressed by *N*-ethylmaleimide to inhibit disulfide cleavage and restored by dithiothreitol, a disulfide cleavage reagent, indicating a direct correlation between fluorescence emission and PEG shedding.

Keywords

FRET; PEG; disulfide; shedding; nanoparticles

The surface functionalization of nanoparticle (NP) drug delivery systems with *poly*(ethylene glycol) (PEG) has become a preferred coating strategy to increase NP circulation time by reducing protein opsonization and macrophage uptake.[1, 2] Indeed, several clinically-approved therapeutics rely on PEG for improved *in vivo* profiles, including PEG-liposomes (e.g. Doxil®), PEG-drug conjugates (e.g. Oncaspar®) and polymeric NPs (e.g. Genexol-PM®).[3] However, it has been reported that the benefit from using PEG to increase circulation half-time may be compromised by PEG which may cause steric and electrostatic hindrance to the entry of NPs at target sites.[4] Even after the NPs are endocytosed at these target sites, the PEG layer can still act as a diffusion barrier to NPs for the efficient release of their payloads.[5] To overcome this 'PEG dilemma', it has been proposed that PEG-sheddable NPs may be formulated by crosslinking PEG using disulfide bond that is cleaved in response to the reducing conditions.[6]

Previous research has shown that some NP delivery systems, when equipped with disulfide-linked sheddable PEG, show cell-responsive dynamics and improved delivery of therapeutic

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molecules. For example, Takae *et al.* reported *poly*(aspartamide) based plasmid gene delivery NPs that showed enhanced gene expression by using cleavable disulfide bonds to crosslink the stealth PEG layer.[7] In another example, Cerritelli *et al.* observed faster cytoplasm release of calcein from NPs formed using PEG-SS-*poly*(propylene sulfide) co-polymers when compared with a non-cleavable formulation.[8] However, so far, assays to evaluate PEG shedding are end-point focused and yield no information of PEG shedding process during NP-cell interactions. In addition, these functional assays are unique to the delivery of the specific payloads, and thus may not be generalized across NP systems. In an effort to rationally design PEG-sheddable NPs for efficient and environment-sensitive delivery, it would be desirable to develop a method to directly observe PEG-shedding across various NP systems and conditions.

To achieve this goal, we designed and synthesized a PEG molecule that bears a disulfide-based fluorescence resonance energy transfer (FRET) pair, containing an Oregon Green-488 (OG-488) as donor and a Dabcyl molecule as quencher (Figure 1). When the disulfide bond is intact, only minimal fluorescence from OG-488 can be detected due to intramolecular quenching. However, if the PEG chain is shed due to disulfide cleavage, intramolecular quenching is abolished and increased fluorescence can be measured.[9] Notably, our design preserves a reactive amine group in the FRET-bearing PEG molecule, which allows for conjugation to different NP systems. In this study, we choose to conjugate the FRET-bearing PEG molecule with *poly*(lactic-co-glycolic acid) (PLGA) to form PLGA-(FRET)-PEG NPs as a model polymeric NP platform. Using this NP formulation, we are able to directly visualize endosomal PEG shedding during NP endocytosis.

The chemical synthesis to form the FRET-bearing PEG molecule is illustrated in Scheme 1. Specifically, Dabcyl is introduced by conjugating BOC-Lysine(COOH)-Dabcyl (**1**) to PEG-hydrazide (**2**) using EDC/NHS coupling. After standard BOC deprotection using trifluoroacetic acid (TFA), a disulfide bond is introduced by a heterofunctional crosslinker, *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), which allows for subsequent disulfide coupling to thiol-containing molecules. The products in each conjugation step are purified using dialysis and gel filtration. Next, we adopt solid phase synthesis to assemble a small molecule (**9**) that contains a free thiol, amine and OG488 dye. Here, Fmoc-Cys(Trt)-OH is first conjugated to Bis-(2-aminoethyl)-ether trityl resin through pyBOP/HOBt coupling, followed by Fmoc deprotection using 20% (v/v) piperidine in DMF. Both conjugation and deprotection reactions are confirmed by the ninhydrin test. The resin is then reacted with amine-reactive OG488 dyes to assemble (**8**). Since the thiol of cystine is protected by a trityl group, thiol deprotection and resin cleavage can thus be carried out in a single TFA treatment. After the solvent is removed by vacuum, the molecule (**9**) is immediately mixed with (**4**) in water to avoid potential thiol oxidation. The coupling reaction between thiol and pyridyl disulfide results in the formation of the final FRET-bearing PEG molecule (**10**). Notably, the amine group preserved in the final molecular structure provides a functional end-group for further conjugation of FRET-bearing PEG to different NP systems.

To confirm the conjugation reactions in scheme 1, matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry is used to analyze key products **1**, **3**, **4** and **10,** and plotted in Figure 2(a). The molecular weight of each product measured from the spectra matches the predicted values (*see supporting materials*). UV-Vis spectroscopy provides a simple and direct method to further confirm our MALDI-TOF analysis. As shown in Figure 2b, the initial mPEG-hydrazide molecule (**1**) does not show obvious absorption at 300 – 700 nm. After conjugation with BOC-Lysine-(COOH)-Dabcyl (**2**), an absorption maximum at 460 nm was observed which matches the adsorption of the Dabcyl molecule. Further conjugation with SPDP leads to the formation of (**4**), which shows

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a distinct absorption peak at 280 nm, originating from the 2-pyridyldithio group of the SPDP molecule (*see supporting materials*). The final reaction between (**4**) and (**9**) forms product **(10)** through the replacement of 2-pyridyldithio by the thiol group in (**9**). The spectrum of **(10)** reveals an additional peak at 490 nm, which is consistent with the absorption wavelength of OG488. This double-peak feature of the UV absorption spectrum confirms FRET pair formation in the final product (**10**).

After the synthesis of FRET-bearing PEG (**10**), we conjugated this molecule with PLGA to formulate PLGA-(FRET)-PEG NPs. PLGA-PEG NPs are well-studied polymeric drug delivery systems, which we can now use to observe the PEG-shedding effect.[10–12] To form PLGA-(FRET)-PEG copolymers, carboxyl-terminated PLGA (PLGA-COOH) was first activated using EDC/NHS to form PLGA-NHS, and then conjugated to the free amine of the FRET-bearing PEG molecule (**10**). By using the nanoprecipitation method as previously reported, we formulated PLGA-(FRET)-PEG NPs with sizes that are approximately 100 – 120 nm.[10] The NP structure is depicted in Figure 3a, where the hydrophobic PLGA core is stabilized by the surrounding hydrophilic PEG layer, and the disulfide-based FRET pair is located at the interface of hydrophobic and hydrophilic sections.

We proceeded to examine the fluorescent emission of PLGA-(FRET)-PEG NPs in response to a reducing stimulus. Initially, analysis by fluorescence emission spectroscopy shows a weak absorption at 520 nm (Figure 3b). After the addition of 1 mM dithiothreitol (DTT, disulfide cleavage reagent), the emission at 520 nm increases nearly 6-fold. This increase in fluorescence emission at 520 nm results from disulfide cleavage which abolishes intramolecular FRET quenching by the Dabcyl molecule. To further confirm that the disulfide cleavage leads to PEG shedding, NP sizes were monitored after the addition of 1 mM DTT. As shown in Figure 3c, DTT rapidly induces an increase in the size of PLGA-(FRET)-PEG NPs. In non-cleavable PLGA-PEG NP controls, a similar size increase was not observed. This NP size increase results from particle destabilization and aggregation upon DTT addition, confirming that disulfide cleavage leads to the loss of the stabilizing PEG layer.[7]

To demonstrate that our molecular design makes it possible to directly measure PEG shedding during NP interaction with target cells, we formulate folate-targeted NPs by blending PLGA-(FRET)-PEG with 10 wt% of PLGA-PEG-folate copolymers, followed by nanoprecipitation and self-assembly of NPs. NPs formulated using this method show preferential targeting to the folate receptor on human nasopharyngeal epidermoid carcinoma (KB) cells and exhibit enhanced cell uptake through receptor-mediated endocytosis (*see supporting materials*).

PEG-shedding during NP endocytosis in KB cells was examined by confocal microscopy. Images of KB cells without NP treatment were taken as background controls (Figure 4a). After the cells were incubated with 1 mg/mL NPs for 4 hrs, green fluorescence signals can be observed in the perinuclear region, as shown in Figure 4b. The visible green fluorescence indicates that PEG shedding has taken place after the NP endocytosis in KB cells. As a positive control to confirm that the fluorescence is due to endosomal disulfide cleavage and not un-quenched OG488 emission, the cells were further treated with 100 μM DTT after NP incubation. After DTT treatment, the fluorescent image (Figure 4c) shows similar intensity of green fluorescence as Figure 4b, confirming that the positive readout is indeed caused by disulfide cleavage and the consequent emission of fluorescence at 520 nm. Our microscopic observations were confirmed by flow cytometry analyses (Figure 4d). The increase in green fluorescence intensity in cells incubated with NPs compared to cells without NPs indicates that disulfide cleavage and PEG shedding has taken place after NP endocytosis. Likewise, the intensity of green fluorescence remains the same after DTT treatment.

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To simulate a non-reducing intracellular environment, KB cells were incubated with NPs in the presence of 10 μM *N*-ethylmaleimide (NEM), an irreversible and membrane-permeable thiol reagent. It has been found that NEM inhibits the ability of KB cells to cleave disulfide bonds.[13] Therefore, NEM treatment serves as a negative control to examine endosomal PEG shedding. Fluorescence images of cells treated with 10 μM NEM were first taken as background controls (Figure 4e). After the addition of 1 mg/mL NPs, no fluorescence above the background level can be measured (Figure 4f), suggesting that PEG shedding through disulfide cleavage is completely abolished. However, the green fluorescent signal becomes visible after the removal of NEM and the addition of 100 μM DTT (Figure 4g). Flow cytometry analysis again confirmed this microscopic observation as shown in Figure 4h. The spectrum of cells incubated with NPs overlaps with the background spectrum when the cells were NEM-treated, but shifts to higher intensity if cells were subsequently incubated with DTT. Thus, the microscopic measurements and flow cytometry analyses prove a direct correlation of the PEG-shedding effect with fluorescence emission from NPs. Further studies using PEG-sheddable NPs indicate that the PEG shedding largely takes place at the early stage after endocytosis (*see supporting materials*). Taken together, these results imply that FRET-bearing PEG can serve as a sensor to report the uptake and accumulation of NPs upon PEG shedding.

Fluorescence-labeling and tracking of NP distribution *in vitro* and *in vivo* is limited by the inability to readily differentiate intracellular vs. extracellular localization of NPs while creating high background signals. For example, using whole-body fluorescence imaging techniques *in vivo*, one may not be able to distinguish between NPs in extracellular compartments from NPs that have been taken up by target cells. With this FRET-based PEG technique, NPs can be observed only after intracellular PEG shedding, making it possible to directly equate NP fluorescence with the reducing environment of intra-cellular compartments.

In summary, herein we report to our knowledge the first direct observation of endosomal PEG shedding during NP endocytosis by synthesizing a disulfide-based FRET-bearing PEG molecule and applying it to a targeted PLGA-PEG NP model system. More broadly, the sheddable PEG designed in this study is applicable to NPs where PEG functionalization is needed, including liposomes, polymeric micelles, lipoplexes and polyplexes, and can be tested under various conditions. Therefore, the FRET-bearing PEG molecule described here may provide a useful tool to study the PEG shedding process and aid the design of environmentally sensitive NP systems for improved drug delivery.

Experimental Section

Reagents and measurements

PEG reagents (mPEG-NH-NH_2 and tBoc-NH-PEG-NH_2) were purchased from LaysanBio Inc. SPDP was purchased from Thermo Fisher Scientific Inc. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (pyBOP), *N*-Hydroxybenzotriazole (HOBt), and Boc-Lys(Dabcyl)-COOH were purchased from Anaspec Inc. OG488 was purchased from Invitrogen Inc. *O*-Bis-(aminoethyl) ethylene glycol trityl resin was purchased from EMD Chemicals Inc. All other chemicals including folic acid, 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), and *N*,*N*-diiso-propylethylamine (DIEA) were purchased from Sigma-Aldrich.

Dialysis was performed using 2K Slide-A-Lyzer® cassettes and the gel filtration was carried out on D-Salt Dextran desalting columns, both from Thermo Fisher Scientific. In gel filtration, the first yellow-orange band that eluted from the column was collected. MALDI-TOF analysis was performed on an Applied Biosystems Model Voyager DE-STR instrument

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in the Biopolymer Laboratory at the Massachusetts Institute of Technology (MIT). UV-Vis measurements were performed using a Varian Cary 100 UV-Vis spectrophotometer. The NP size (diameter, nm) was obtained from three repeat measurements by quasi-elastic laser light scattering with a ZetaPALS dynamic light scattering detector (15mW, λ = 676 nm; Brookhaven Instruments Corp., Holtsville, NY).

Synthesis of (4) mPEG-(Dabcyl)-NH_2

Boc-Lys(Dabcyl)-COOH (23 mg, 0.046 mmol), NHS (26 mg, 0.23 mmol) and EDC (44mg, 0.23 mmol) were dissolved in 1 mL DMF, to which 0.5 mL DMF containing 29 mg mPEG-$NHNH_2$ (0.0058 mmol) and 20 μL DIEA (0.11 mmol) was added. The mixture was stirred in the dark at room temperature. After 14 hrs, the mixture was precipitated in 50 mL diethyl ether to produce a bright red solid, followed by washing using diethyl ether. After a standard BOC deprotection using TFA, the product was purified by dialysis and gel filtration. After lyophilizing, the compound (**4**) was collected as a bright yellow powder. The conjugation was confirmed using MALDI-TOF.

Synthesis of (5) mPEG-(Dabcyl)-OPSS

SPDP (7.8 mg, 0.025 mmol) was dissolved into DMF (0.2 mL). The solution was then added to 25 mg mPEG-(Dabcyl)-NH_2 (**4**, 0.005 mmol). After the solid was dissolved, 10 μL DIEA (0.055 mmol) was added. The mixture was stirred in the dark at room temperature. After 14 hrs, the reaction was terminated by adding 2 mL water. After dialysis and lyophilization, the compound (**5**) was collected as a brown powder. The conjugation was confirmed by MALDI-TOF.

Synthesis of (10) OG488-Cys(Trt)-ONH-$CH_2CH_2OCH_2CH_2O$-NH-trityl resin

Bis-(2-aminoethyl)-ether trityl resin (30 mg) was loaded into an solid phase reactor and pre-swelled in DMF for 4 hrs at room temperature. Fmoc-Cys(Trt)-OH (94 mg, 0.16 mmol), pyBOP (124.8 mg, 0.24 mmol) and HOBt (37 mg, 0.24 mmol) were dissolved into 0.5 mL dry DMF and added to the resin together with DIEA (20 μL, 0.11 mmol). The reaction was carried out in the dark for 16 hrs, followed by DMF wash and pyperidine treatment (1 mL, 20% in DMF) to deprotect the amine of the cystine. The conjugation and deprotection were confirmed by the ninhydrin test. After washing in DMF, OG488-NHS (10mg, 0.02 mmol) and DIEA (20 μL, 0.11 mmol) in 0.5 mL DMF was added to the resin. The mixture was stirred in the dark for 16 hrs, followed by DCM wash. The functionalized resin was then dried under vacuum.

Synthesis of (12) mPEG-(Dabcyl)-SS-(OG488)-NH_2

30 mg functionalized resin (**10**) was treated with the mixture of 0.1 mL TFA, 0.4 mL DCM, 30 μL water and 10 μL triisopropylsilane for 2 hrs. The solvent was evaporated at room temperature under vacuum. Water (2 mL) was added to the dried product to extract the cleaved molecule. The extraction was then added to 25 mg (**2**). The solution was stirred in the dark at room temperature for 12 hrs. The product (**12**) was purified by dialysis and gel filtration, followed by lyophilization, and was collected as a dark brown powder. The conjugation was confirmed by MALDI-TOF.

Synthesis of PLGA-(OG488)-SS-(Dabcyl)-mPEG

PLGA-COOH was activated to form PLGA-NHS through EDC/NHS chemistry as reported before.[10] Briefly, 100 mg PLGA-COOH was dissolved into DCM (1mL), to which 0.5mL DCM containing EDC (5mg, 0.027mmol) and NHS (3mg, 0.027mmol) was added. The mixture was stirred at room temperature for 2 hrs. PLGA-NHS was then precipitated by addition of cold methanol (50mL) and dried under vacuum. The collected polymer was

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dissolved in 1mL dry DMSO, to which 25 mg mPEG-(Dabcyl)-SS-(OG488)-NH_2 (compound **12**, ~0.0042 mmol) and 10 μL DIEA (0.055 mmol) was added. The mixture was stirred in the dark for 16 hrs. The product was precipitated by the addition of 50 mL cold methanol. As a final purification, the polymer was repeatedly dissolved in DMF followed by precipitation in cold methanol, and finally dried in vacuum.

Nanoparticle formulation

To synthesize folate-targeted NPs by nanoprecipitation and self-assembly, PLGA-PEG-folate (1.5 mg in 0.15 mL acetonitrile), PLGA-(FRET)-PEG (1.5 mg in 0.15 mL acetonitrile) and acetonitrile (0.2 mL) were mixed and added dropwise into a 7 mL vial of DI H_2O under gentle stirring. The organic solvent phase was allowed to evaporate in the dark for 16 hrs under gentle stirring. The NPs were washed with ultra pure water using 100 kDa Amicon Ultra centrifugal filters and concentrated to 0.2 mL. The NPs were transferred into buffered solutions by a slow addition of 0.022 mL 10× PBS before the NPs were added to cells.

Cell experiments

KB cells were purchased from ATCC and cultured in RPMI medium (RPMI-1640 containing 1 mg/L FA, Invitrogen) with 10 % fetal bovine serum, 2 mM glutamine, 50 units/mL penicillin and 50 ug/mL streptomycin at 37 °C and 5% CO_2. Cells were routinely passaged by treatment with trypsin (0.25%) / EDTA. Before the cells were incubated with NPs, cells were cultured overnight in FAfree RPMI medium with the same additives.

For fluorescence microscopy studies, KB cells were seeded on a 4-well chamber slide with 15,000 cells / well and incubated at 37 °C and 5% CO_2 for 24 hrs. Next, the cells were incubated in fresh complete medium with NPs, followed by a 6-hr incubation at 37 °C and 5% CO_2. After the incubation, the cells were washed three times in PBS and fixed in 4% paraformaldehyde at room temperature. For imaging, slides were mounted with a coverslip using H-1000 Vectorshield mounting medium with DAPI. Fluorescent analysis was carried out using an Applied Precision DeltaVision Spectris Microscope.

Flow cytometry analysis was performed on a BD Biosciences FACSCalibur HTS at the MIT Koch Institute flow cytometry core facility. KB and HUVEC cells were seeded on 6-well plates with a density of 200,000 cells / well and incubated overnight. To allow nanoparticle uptake, cells were incubated with 0.5 mg / mL NPs for 4 hrs at 37 °C and 5% CO_2. Before flow cytometry analysis, cells were washed with PBS, trypsinized, and re-suspended in PBS containing 0.4% BSA. Each flow cytometry result was collected from 12,000 live cells.

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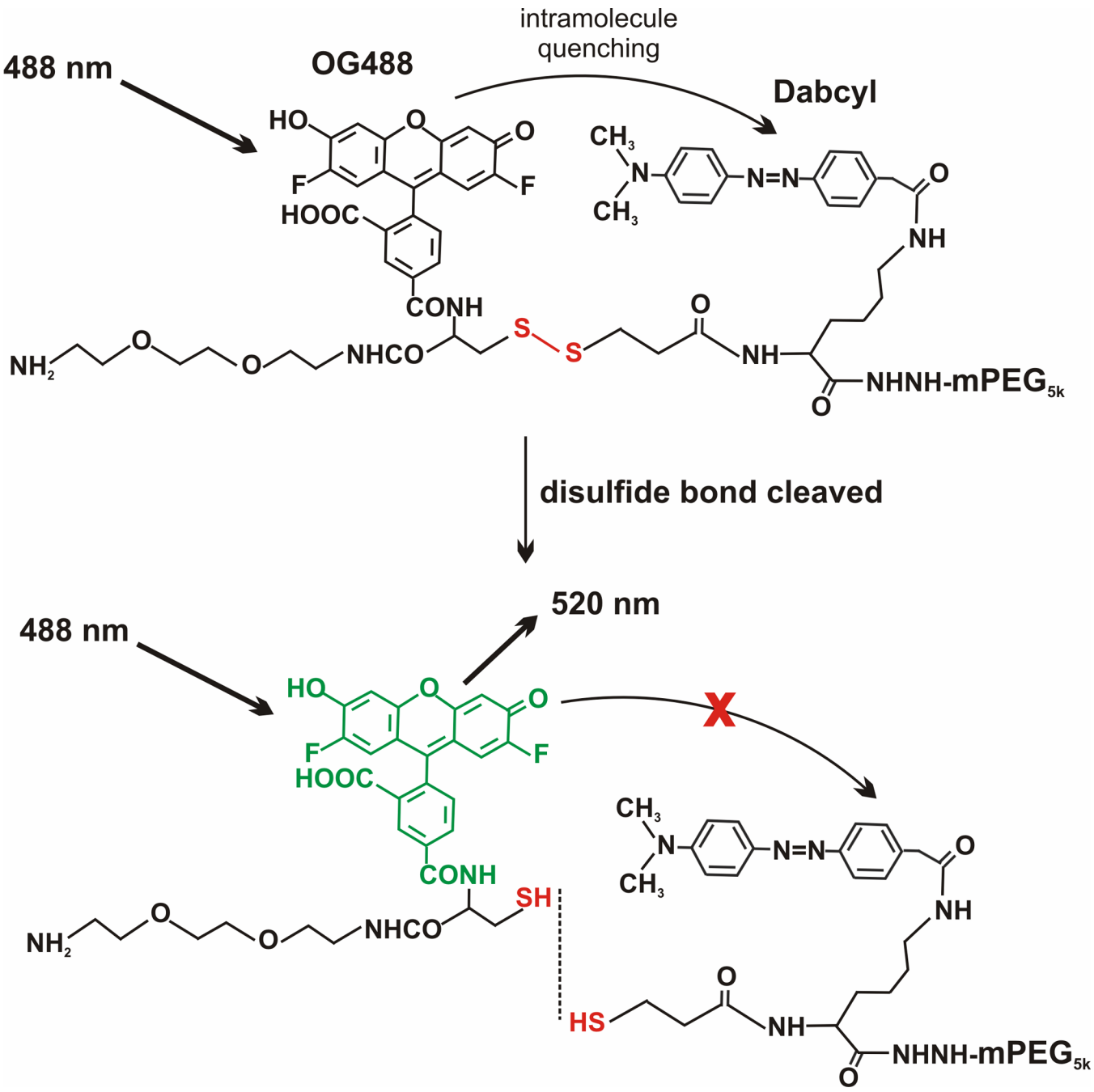

Figure 1.
The design of FRET-bearing PEG that allows the direct observation of its shedding.

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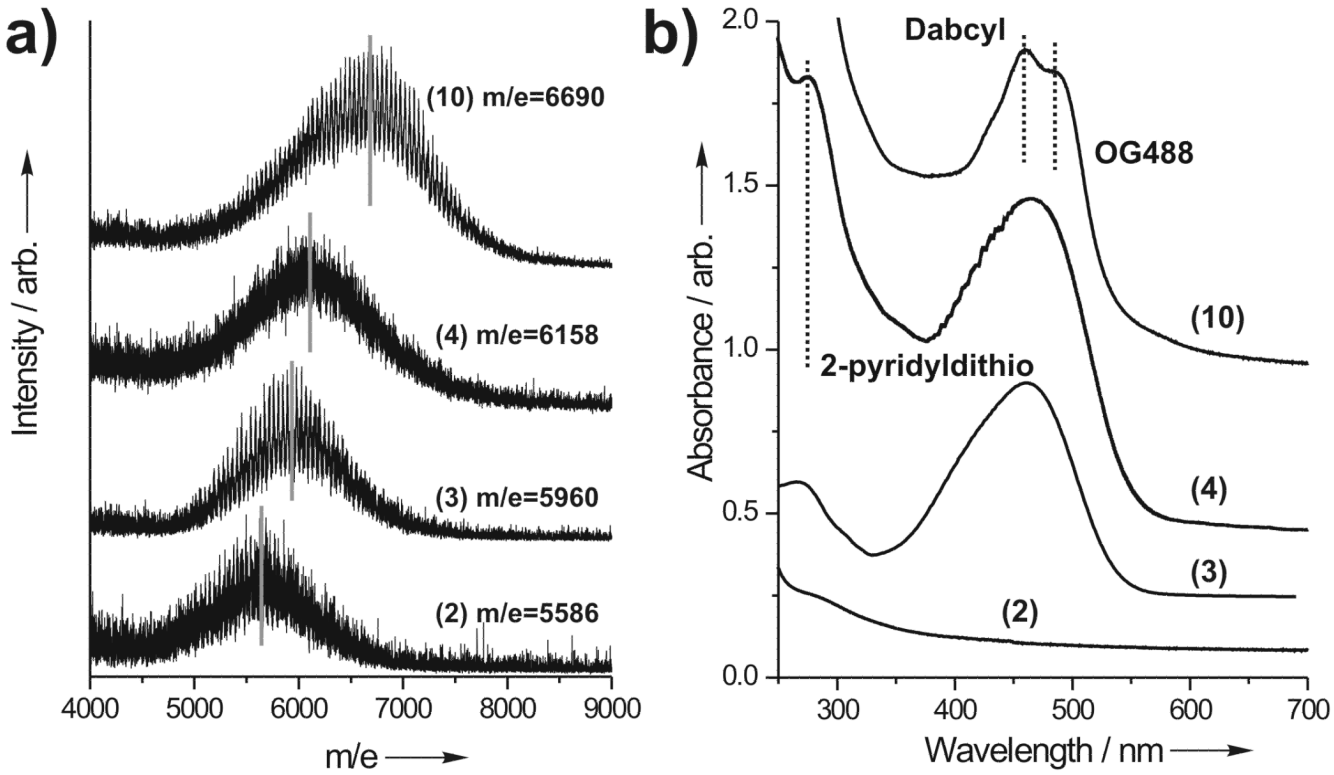

Figure 2.

(a) MALDI-TOF analysis and (b) UV-Vis spectroscopy of conjugation products to form the FRET-bearing PEG.

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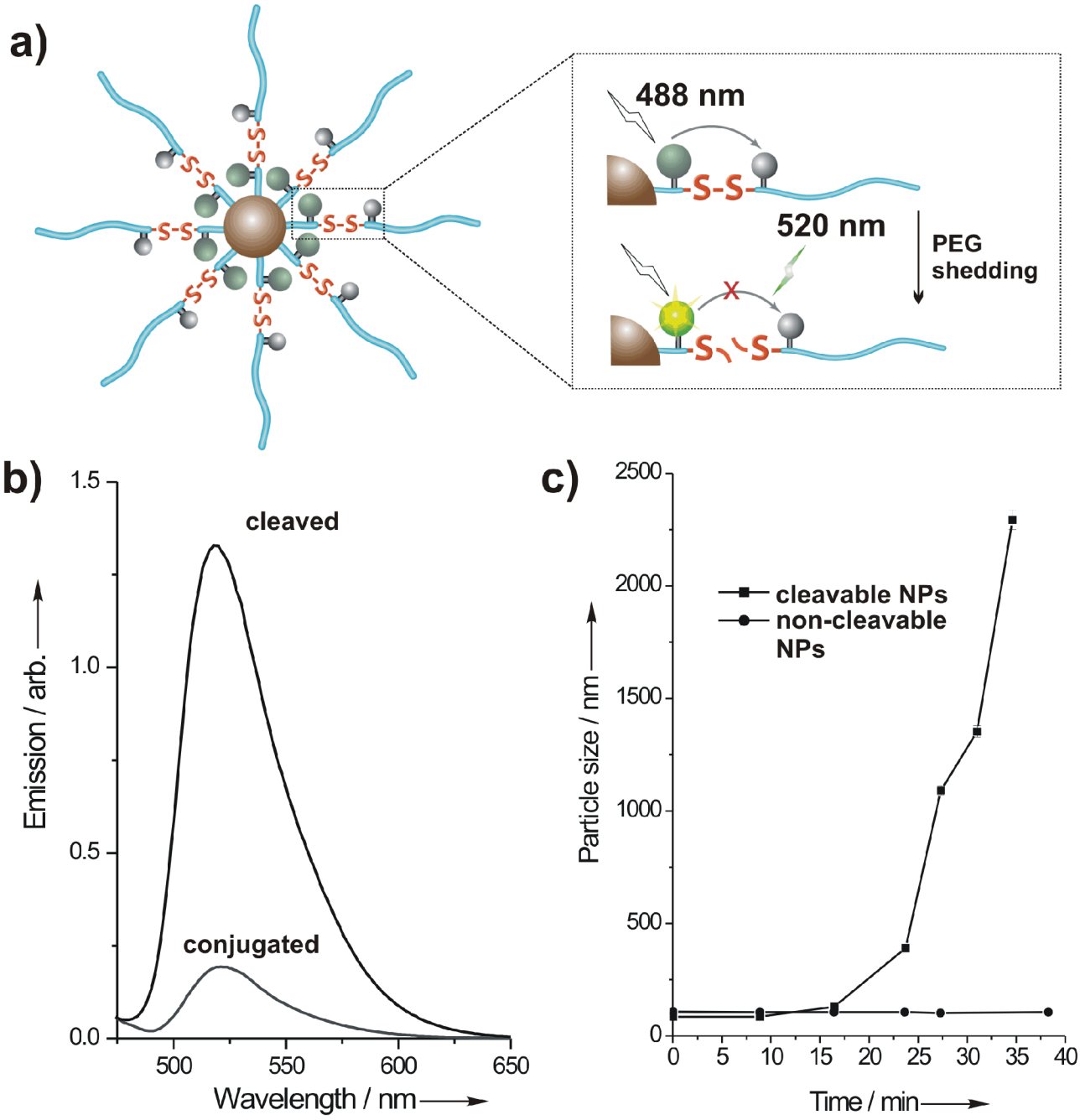

Figure 3.

(a) NP structure using PLGA-(FRET)-PEG copolymer through nanoprecipitation. (b) Fluorescence emission spectroscopy shows that the disulfide cleavage induces a nearly 6-fold increase in 520 nm emission compared to un-cleaved NPs. (c) The disulfide cleavage induces PEG shedding and increase in NP size.

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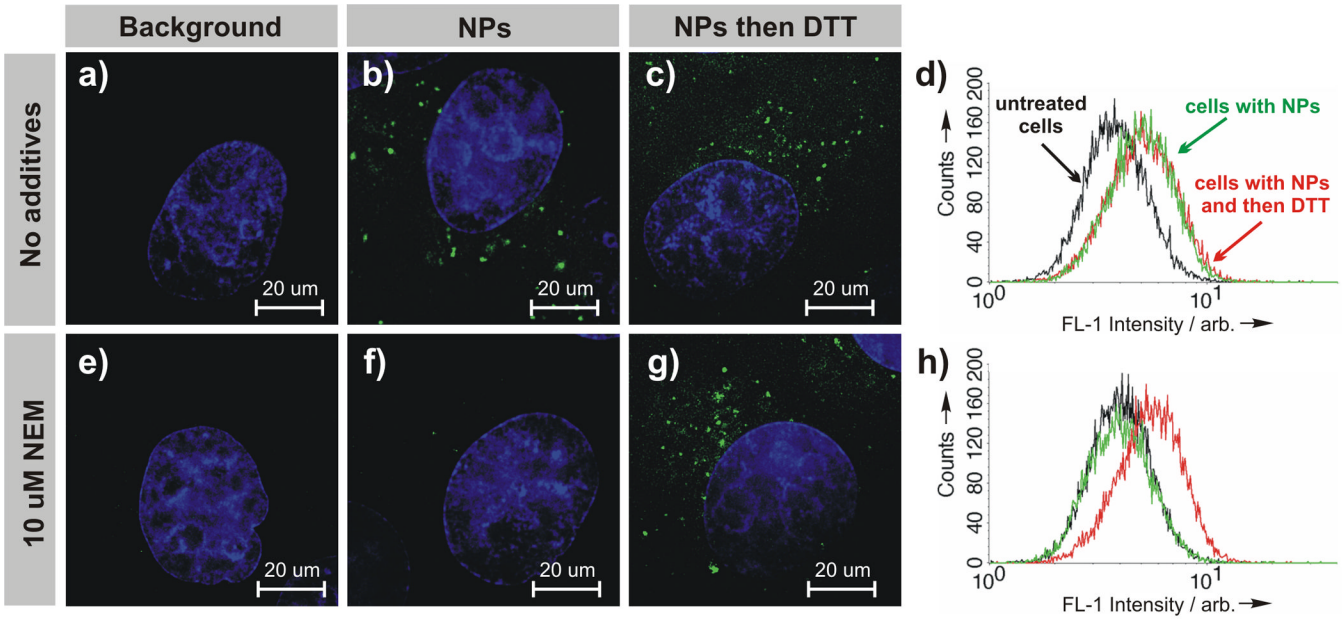

Figure 4.

Observation of endosomal PEG shedding by fluorescent microscopy and flow cytometry in: (a)–(d) KB cells with no NEM and (e)–(h) KB cells with addition of NEM. In each row, the fluorescent intensity at 520 nm is compared among cell-only controls, cells incubated with NPs, and cells incubated with NPs followed by DTT treatment. In all experiments, the NP concentration is 1 mg/mL. The DTT concentration is 100 μM and the NEM concentration is 10 μM.

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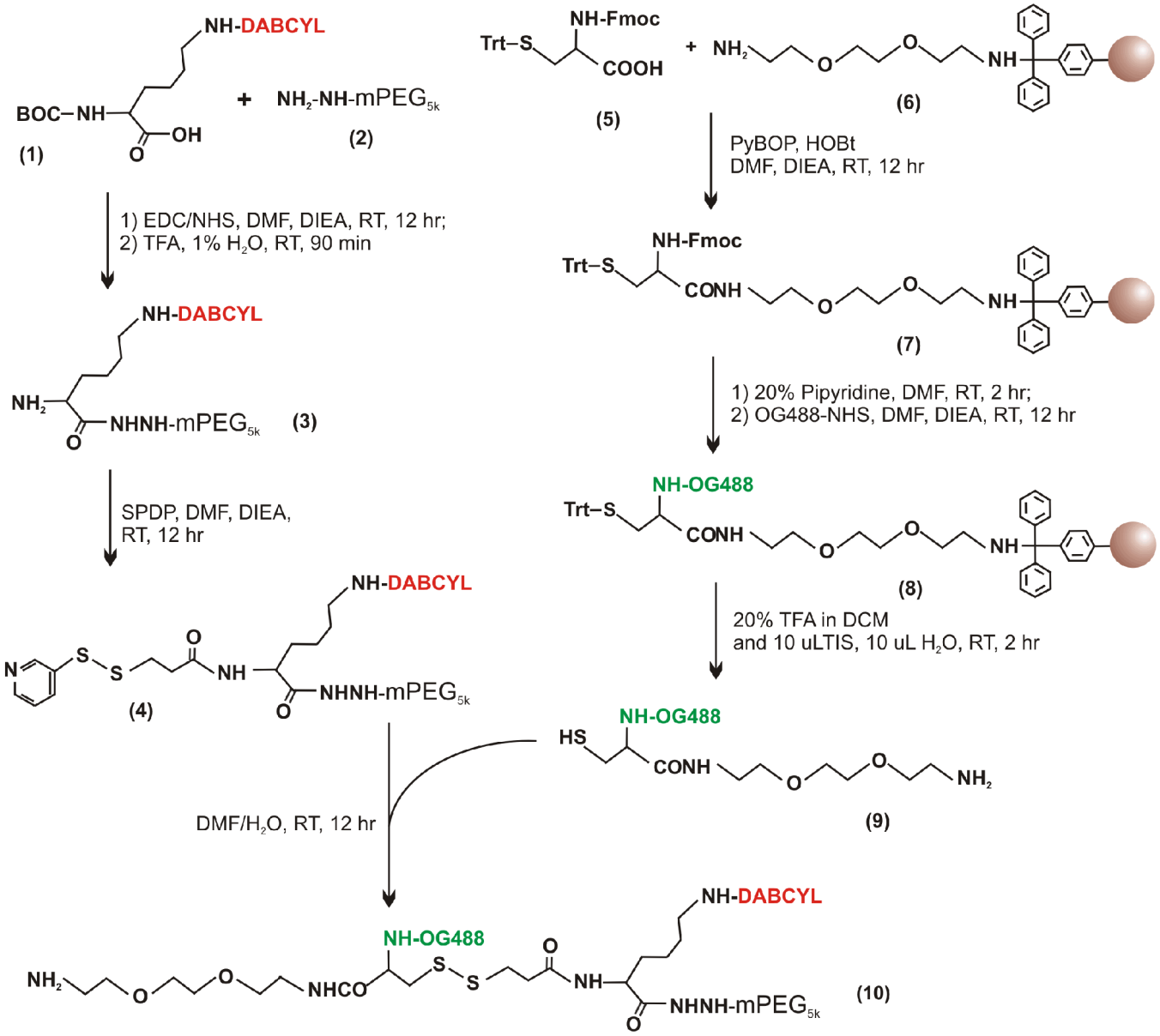

Scheme 1.
Chemical synthesis of FRET-bearing PEG.