Photochromic molecular switches
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Chapter 6

Towards a PALM Probe for Imaging Gold Nanoparticles in Living Cells

In this chapter, progress towards a photoswitchable fluorescent probe, which is suitable for gold surface functionalization, is described. The amphiphilic dithienylethene switch is fluorescent in its open form, while the fluorescence is quenched in the ring closed form. Although, suitable conditions for gold nanoparticle functionalization were not established, the probe could be visualized in living LAD2 cells. Furthermore, the fluorescence of the probe could be quenched and subsequently restored by irradiation of the cells. Additionally, it was shown that the amphiphile switches form worm-like micelles in aqueous solution. Upon irradiation of the shape of aggregates formed is altered.
6.1 Introduction

6.1.1 Gold nanoparticles

With the recent growth in interest in nanotechnology, interaction between engineered nanoparticles and organisms become ever more probable.\textsuperscript{1} It is therefore important to understand the interactions between these particles and cells, as well as understand how they impact cellular mechanisms. For example, gold nanoparticles with a diameter of 1.4 nm have been shown to trigger cell death through necrosis as a result of oxidative stress,\textsuperscript{2} whereas nanoparticles with a diameter of 1.2 nm trigger cell death through apoptosis.\textsuperscript{3}

The light scattering properties of gold nanoparticles allows particles to be detect with ±10^2 nm resolution with visible light microscopy.\textsuperscript{7} However, imaging of small nanoparticles (0.6-2 nm) in living cells or organisms is challenging. Confocal microscopy is limited by its spatial resolution of several hundred of nanometer (also see section 6.1.1), while only fixed cell samples can be used in electron microscopy. Therefore, time-dependent tracking of nanoparticles in cells is not possible with these microscopy techniques.\textsuperscript{4} Three-dimensional (3D) tracking of nanoparticles in cells can be achieved with fluorescence microscopy by using quantum dots\textsuperscript{5} to enable positional accuracy in the nanometer regime to be achieved. As a result of their plasmon band gold nanoparticles show strong light scattering.\textsuperscript{6} Scattering can therefore be used as an alternative method for direct observation of nanoparticles, with accuracy below the resolution limit of optical microscopy. Nevertheless, particles of 0.6-2 nm are too small to support a conduction band\textsuperscript{7} and therefore are difficult to detect via light scattering.\textsuperscript{8} Imaging and visualizing small gold nanoparticles in living cells in a facile way, could be achieved using particles labelled with photoswitchable fluorescent tags, which would enable the use of fluorescence microscopy techniques such as photo activated localization microscopy.

6.1.2 Photo activated localization microscopy

Photoactivated localization microscopy (PALM) has allowed for imaging of processes beyond the diffraction limit.\textsuperscript{9} In conventional fluorescence microscopy a fluorophore of several nm in diameter will be imaged by its fluorescence as an object of several hundred of nm as a consequence of the diffraction limit of light through optics. PALM is a fluorescence microscopy technique that enables one to record images at a resolution beyond the diffraction limit.\textsuperscript{10} The resolution is achieved taking advantage of two processes; 1) determination of the centroid of a fluorescence image of an object and 2) using fluorophores that are photoactivated consecutively. By assuming that fluorescence intensity is a point spread function, one can fit the intensity with a 2D Gaussian, from which the centroid can be determined with a 10-20 nm resolution. This
process however is limited to isolated fluorophores. In \textit{in vitro} fluorescent studies the fluorophores are often not isolated but clustered together, resulting in the overlap of the point spread functions of the fluorophores. Therefore, a 2D Gaussian cannot be adequately fitted and centroids of each fluorescent signal cannot be determined (Figure 6.1).

\textbf{Figure 6.1:} a) Schematic representation of the resolution of a 2.5 nm fluorophore using fluorescence microscopy. b) Localisation of the centroid of the point spread function by fitting of a 2D Gaussian. c) Illustration of the limitations of this technique, when the point spread function of several signals overlap. Parts of this illustration are reproduced with permission from ref. 11

In PALM the fluorophores are activated randomly by photoirradiation (Figure 6.2), this limits the chances of several fluorescent signals overlapping. The fluorescence of the activated fluorophores (on state) is recorded. Subsequently the activated fluorophores are photobleached, so that they are no longer fluorescent (off state). This procedure is repeated until all the fluorophores have been depleted (turned on and off). The images are accumulated and overlaid to form a single high resolution fluorescence image.
The following characteristics are important for an effective PALM probe:

1) Fluorescence of the PALM probes should have a high brightness, so that a large number of photons are detected before the probe is photobleached or reverts to its non-fluorescent state. The brightness is determined by the absorption coefficient ($\kappa$) and the fluorescence quantum yield ($\Phi_F$). An efficient probe has both a high $\kappa$ and $\Phi_F$. Some synthetic fluorophores, show excellent brightness with $\kappa$ of ~100,000 and a $\Phi_F > 0.90$. More conventional fluorescent proteins show much lower brightness; $\kappa$ ~15,000 - 85,000 and a $\Phi_F$ of 0.05 to 0.80.

2) In addition to the high brightness, high contrast between the fluorescent (on) states and the non-fluorescent (off) state is important. A high contrast results in a high signal to noise ratio.

3) Probes should be thermally stable over a range from 20 to 40°C in both the on- and off-state.

4) Fluorescence switching between the on- and off-state should be accompanied by large spectral changes.

5) Fluorophores should have a high fatigue resistance, i.e. a high number of switching cycles should be obtained before photodegradation occurs.
6) For PALM, it is of importance that fluorescence switching is achieved with control over the population of fluorophores in the on-state. For PALM only a small number of fluorophores should be in the on-state at any one time in order to assure that the fluorescent molecules are well spaced (Figure 6.1).
7) Furthermore, probes should be target specific, e.g. they should target a specific object, assembly or location of interest through labeling.

Although PALM is a powerful technique, it is largely dependent on the use of fluorescent proteins and as a result it is limited by several factors such as a low on/off contrast between the fluorescence of the two states, poor delivery as a result of solubility, and inability of a photobleached fluorophore to recover its fluorescence.

6.1.3 Photoswitchable fluorescent probes

Recently, small molecular fluorescent probes have received attention. Amongst these, probes based on photochromic switches are of great interest. The fluorescence of such fluorophores can be reversibly switched between the on and off state and are based upon the following principal. The emission is quenched as a result of energy transfer from the donor-part (fluorescent) to the acceptor-part (the switching unit) of the molecule (Figure 6.3). Energy transfer from the donor to the acceptor can occur via the Dexter or Förster resonance energy transfer mechanisms. In the Dexter mechanism the energy transfer is based upon double electron transfer and requires overlap between the wave functions of the donor and acceptor. An electron is transferred from the exited state of the donor to the lowest unoccupied molecular orbital. While simultaneously an electron is transferred from the highest occupied molecular orbital (HOMO) of the acceptor to the donor. In Förster resonance energy transfer (FRET) the energy is nonradiatively transferred from the exited donor to the acceptor via long-range dipole-dipole coupling. There has to be resonance between the oscillation of the two dipoles. When this occurs energy can be transfer through space from the donor exited state into the acceptor ground state without actual electron transfer. As the FRET mechanism occurs through space it exhibits a $6^{th}$ power dependence on the distance between the donor and acceptor in the range from 1.5 to 10 nm. In FRET the overlap in donor emission and acceptor absorption is essential (Figure 6.3).
There are several recent examples in which dithienylethene switches act as efficient quenchers for fluorophores, thereby enabling the switching of the chromophore between a fluorescent and a non-fluorescent state. Such systems can even be used for *in vivo* fluorescent imaging. Kim *et al.* described a system based on cyanine 3 (Cy3) fluorophores embedded in a 5th generation polyamidoamine dendrimer, which were cross-linked using hexa-fluoro dithienylethene switches. Cy3 act as a FRET donor, while in the ring closed isomer, dithienylethene switch acts as an efficient FRET acceptor, quenching the fluorescence (Figure 6.4). The fluorescence of the probe can be controlled via FRET through photoswitching of the dithienylethenes from the open (fluorescent) to the closed form (quenched).

Fluorescent dithienylethene switches can also be quenched upon ring closure of the switches. Huang and co-workers have developed a photoswitchable fluorescent probe based on amphiphilic dithienylethene switches (Figure 5). In this system the dithienylethene switch is both the fluorophore and the FRET acceptor. The amphiphilic switches form vesicles in aqueous solution with a critical aggregation concentration at ~1.2 × 10⁻⁶ M. In the open form the switches are green fluorescent at λ = 510 nm with a fluorescence quantum yield (Φ) of 0.44% at a concentration of 1.0 × 10⁻⁵ M. When the concentration is increased to 5.0 × 10⁻⁵ M the Φ increases to 0.73%. Upon irradiation at λ = 365 the fluorescence is 98% quenched as a result π-electron delocalization by an extension of the conjugated system.
Towards a PALM Probe for Imagining Gold Nanoparticles in Living Cells

Figure 6.4: Switching upon irradiation (365 nm) between the ring-open (1-o) and ring-closed (1-c) isomers of a diarylethene switch results in quenching of the fluorescence of a dendrimer bound Cy3 fluorophore in living Zebra fish. Irradiation at 590 restores the fluorescence. Reproduced from with permission ref 17. Copyright 2012 Wiley.

When Human Nasopharyngeal epidermal carcinoma cells (KB) are incubated in PBS buffer containing the switch 2, the switches are taken up by the cells. The fluorescence of switch 2 can be clearly observed with a high signal to noise ratio (I_1/I_2 > 50). Switching of the fluorescence can be observed in living cells, irradiation of the KB cells at λ = 405 nm results in a decrease in the fluorescence intensity. The fluorescence from the KB cells is completely recovered by irradiation at λ = 633 nm.

Figure 6.5: Switching of an amphiphilic dithienylethene upon irradiation. a) SEM image of a freeze dried sample of 2, indicating that 2 forms aggregates in water. b) Repetitive switching of the fluorescence of the probe in KB cells. The fluorescence of isomer 2 is quenched upon irradiation (365 nm) by switching to 2-c, while, irradiation at 590 nm results in reformation of 2-o and restores the fluorescent signal. Reproduced from ref 18 with permission. Copyright ACS 2008.
6.1.4 Towards a PALM probe for imaging gold nanoparticles in living cells

In this chapter progress towards a photo switchable fluorescent probe that enable visualization of small gold nanoparticles is described. The fluorescent probe is based on an amphiphilic dithienylethene photochromic switch and its fluorescence can be switched between the on- and off-state by irradiation with UV and visible light respectively. The aim is 1) to synthesize a photo responsive fluorescent probe based on an amphiphilic dithienylethene photochromic switch, which can subsequently be used to modify a gold surface through self-assembled monolayer (SAM) formation, 2) to establish switching of such a probe on gold nanoparticles in living cells.

![Figure 6.6: Photoswitching of the fluorescence from on a modified gold nanoparticle.](image)

6.1.5 Design

An amphiphilic pyridinium dithienylethene DTE switch\textsuperscript{18,19} was chosen as the probe, as this probe shows fluorescence at 510 nm with a \( \Phi_f \) of 0.73%. The ring closed form of 2 shows an absorption band at 650 nm. The change in conjugation results in loss of fluorescence (Figure 6.3). Furthermore DTE switches have been shown to be robust to multiple switching cycles and exhibit excellent thermal stability (Chapter 1, section 1.3.3). The probe was functionalized with a tetra ethylene glycol to facilitate internalization by living cells.\textsuperscript{18}

![Figure 6.7: Structures of switches 3 and 4.](image)
Furthermore, it was shown that DTE switches can be switched reversibly on gold nanoparticles.\textsuperscript{20} Even though gold nanoparticles could act as quenchers of the exited states of fluorophores,\textsuperscript{21,22} several functionalized gold nanoparticles bearing fluorescent groups have been reported.\textsuperscript{21,22,23}

6.2 Synthesis and characterization

6.2.1 Synthesis

Switches 3 and 4 were synthesized via asymmetric functionalization of dichloro substituted switch 5 using a Suzuki cross-coupling reaction. Initially it was attempted to couple a phenyl bromide bearing a C\textsubscript{11}-chain terminated with a \textit{t}-butyl protected thiol. The \textit{t}-butyl protecting group was chosen instead of the acetyl group as the latter is not stable under the conditions used in the Suzuki reaction.\textsuperscript{24} Although the \textit{t}-butyl thioether is stable under the reaction conditions, deprotection of this group using BBr\textsubscript{3} or TiCl\textsubscript{4} proved destructive to 3.

![Synthesis routes of photoswitchable probes 3 and 4.](image)

The trityl (-C\textsubscript{6}H\textsubscript{5}) protecting group was subsequently explored. The use of a trityl protecting group, in principal allows for milder deprotection conditions. However, the trityl protecting group is not robust under the Suzuki reaction conditions,\textsuperscript{24} resulting in the free thiol and subsequently leading to loss of reactivity of the palladium catalyst. To circumvent these issues the synthetic route described in figure 6.9 was employed using the Suzuki cross-coupling and the Mitsunobu reaction as key steps.\textsuperscript{25}
Dichloro substituted switch 5 was synthesised following a procedure reported by Lucas et al.\textsuperscript{26} Switch 5 was coupled to 11-(4-bromophenoxy)undecan-1-ol 6 under Suzuki cross-coupling conditions. For probe 5, the alcohol of switch 6 was converted to a bromo substituent using Apple reaction conditions. Subsequently the bromo functionality was substituted with a \textit{t}-butylthiol, resulting in thioether 10.

**Figure 6.9:** Synthesis routes of photoswitchable probes 3 and 4.
For the synthesis of probe 4 the terminal alcohol was protected as tetrahydropyranyl ether (THP) 9 prior to the second Suzuki cross-coupling was protected as tetrahydropyranyl ether (THP) 9 to prevent deprotonation during the preparation of the boronic ester of 7. Switches 9 and 10 were subsequently coupled with 4-bromopyridine hydrochloride under Suzuki cross-coupling conditions, resulting in 11 and 12 respectively. Upon deprotection of the THP ether 12, the protonated pyridine was obtained as a tosylate salt 13. This salt was dark green, where the dithienylethene switches are usually colourless in their open form or purple in their closed form.

Treatment of the salt under basic aqueous conditions liberates the pyridine moiety and 14 were obtained. A thiol group was introduced to allow to self-assembly of the fluorescent switch on gold substrates. The sulfur moiety was added as a triphenylmethanethiol under Mitsunobu reaction conditions. The OEG₄ moiety was subsequently introduced to switches 11 and 12 to complete amphiphilic fluorescent switches 3 and 4 respectively.

6.2.2 Preparation and characterization of functionalized Au nanoparticles

Amphiphilic Au nanoparticles bearing [11-(methylcarbonylthio)-undecyl]tetra-(ethylene glycol) were prepared via a procedure described by Brust and coworkers. Synthesized NPs bearing OEG₄-C₁₁-thiols were found to have diameters of 2-4 nm, with an average diameter of 2.7 nm ± 0.6 nm by cryo-TEM (Figure 6.10).

The intensity of the SP band in the UV/Vis (± 520 nm) absorption spectrum increases with increase of the average core diameter of the gold nanoparticles. UV/Vis absorption spectroscopy of a solution of OEG₄-C₁₁-S-Au in EtOH shows no distinct absorption band for the surface plasmon resonance for the Au nanoparticles (Figure 6.10).

![Figure 6.10](image_url)
6.11), indicating NP’s of a size below 3 nm. This observation is consistent with the particles size found by cryo-TEM (Figure 6.9b). The SP band of the gold is obscured as a result of scattering by the nanoparticles. The SP band absorption of the OEG4-C11-S-Au NP’s can be extracted by correction with a Mie-scattering model (Figure 6.11b).

Figure 6.11: a) UV/Vis absorption spectrum of OEG4-C11-S-Au NP’s in EtOH. b) Mie corrected UV/Vis absorption spectrum of OEG4-C11-S-Au NP’s in EtOH.

Comparison of the 1H-NMR and FTIR-spectra of OEG4-C11-SH and OEG4-C11-S-Au in CDCl3, Figure 6.12 and Figure 6.13 respectively, revealed peak broadening of all OEG4-C11 signals for the Au-NP sample. The signal of the S-H hydrogen at 2.92 ppm is absent in the Au-NP sample and the signal of the CH2 positioned α to the S moiety is shifted up-field. IR-spectroscopy shows an increase of intensity of the OH stretching at 3365 cm⁻¹ upon assembly of OEG4-C11-SH on Au-NP’s. While, the strong band at 1102 cm⁻¹ can be ascribed to the C-OH and the C-O-C modes, indicate that the Au-NP’s were functionalized with OEG4-C11 thiols.

Figure 6.12: 1H-NMR spectroscopy of a) OEG4-C11-SH and b) OEG4-C11-S-Au-NP’s in CDCl3.
Towards a PALM Probe for Imaging Gold Nanoparticles in Living Cells

Figure 6.13: FTIR-ATR spectroscopy of a thin film of OEG$_{4}$C$_{11}$-SH (---) and OEG$_{4}$C$_{11}$-S-Au-NP’s (-----) in CDCl$_{3}$.

It was attempted to synthesis Au-nanoparticles bearing probe 4 via the Burst method.$^{27,30}$ Switch 4 was deprotected with TFA using TIPS as a carbocation scavenger, following a methodology used for the functionalization of Au-NPs bearing cationic OEG terminated alkanethiols.$^{31}$ The volatiles were removed and the resulting thiol was kept under argon, deprotected 4 was directly used in the synthesis in gold nanoparticles, however, nanoparticles were not observed to form. This is probably the result of the poor solubility of 4 under the reaction conditions (2-propanol/methanol/acetic acid 10:1:0.1). The addition of 2.5% DCM as a co-solvent also did not result in the formation of gold nanoparticles. In the future a two-phase reduction of auric acid in the presence of an excess thiol or disulfide in the organic phase such as described by Roth et. al. might proves more successful$^{32}$ or via a place-exchange on gold nanoparticles of n-butyl thiols by the thiol of DTE the switch.$^{31}$

6.2.3 Characterisation of the photochemical properties of 3 and 4

Photoreversible switching of 3 in EtOH was demonstrated by UV/Vis absorption spectroscopy (Figure 6.14). Irradiation ($\lambda = 365$ nm) of an argon purged solution of open-3 ($2.54 \times 10^{-5}$ M in EtOH) led to the appearance of a new absorption band at $\lambda_{\text{max}}$ 688 nm in the UV/Vis absorption spectrum (Figure 6.14). This band is indicative of the formation of the ring closed form of dithienylethene switches.$^{18}$ Isosbestic points were maintained at 263, 402 and 447 nm indicating that side reactions did not occur during the photoisomerisation. Changes in the UV/Vis absorption spectrum could be recovered by irradiation at $\lambda > 600$ nm. The sample containing 3 could be repeatedly switched between the ring-opened and -closed form without significant degradation (Figure 6.14, insert).
Chapter 6

Figure 6.14: Changes in UV/Vis spectroscopy of 3 in EtOH. a) As a result of ring closure upon irradiation with $\lambda = 363$ nm. b) As a result of ring closing ($\lambda = 363$ nm) and ring opening ($\lambda = 600$ nm), insert shows switching cycles.

The photo-stability of 3 was confirmed by $^1$H-NMR spectroscopy (Figure 6.15). Irradiation at $\lambda = 365$ nm of sample of open-3 in CDCl$_3$ resulted in ring closure of the dithienylethene core, which is accompanied by an up-field shift for most signals in the
aromatic region (Figure 6.4, middle). Upon reaching the photostationary state (PSS), 43% of open-3 was converted to closed-3. Irradiation of the PSS mixture at > 600 nm resulted in the recovery of the original 1H-NMR spectrum (Figure 6.15, bottom). Switch 4 shows similar behavior, however, at the PSS 61% of the ring closed isomer is obtained. Continued irradiation of a sample of 4 resulted in the formation of a byproduct which precipitates out of the solution.

Amphiphilic switches 3 and 4 aggregate in aqueous media, which might result in changes in the photochemical behavior of the switch.18,33 The switching behavior of 3 was studied in water and PBS buffer (pH 7.4). Changes in the UV/Vis absorption spectra upon irradiation of samples containing 3 in water (Figure 7.16) and PBS buffer (Figure 6.17) are similar to those in EtOH (Figure 6.14). However, repetitive switching of 3 in deoxygenated aqueous solutions results in degradation of the switch. Degradation of dithienylethenes is often associated with photochemical rearrangement of the ring closed form (see Chapter 1, Section 1.3.3).34 The open form of switch 3 in water shows fluorescence ($\lambda_{\text{max}}$ 556 nm), and at the PSS a decrease in fluorescence is observed as expected. However, irradiation with visible light to open-3 was accompanied by a blue shift in the emission maximum ($\lambda = 525$ nm), but not to a recovery of the intensity of fluorescence.

Figure 6.15: Changes in the 1H-NMR spectroscopy of 3 in CDCl3. Initial sample containing open-3 (---), PSS mixture (57:43) of open- and closed-3 (——) upon irradiation (365 nm). Recovery of the original 1H-NMR spectrum (——) upon visible irradiation (> 600 nm).
Huang and coworkers observed (Figure 6.5) structures with a spherical morphology ($\varnothing = 50-200$ nm) by SEM in freeze-dried samples of piridinium dithienylethene switches.\(^{18}\) However, preparation of SEM samples can potentially damage structures where water is an integral part of the structure.\(^{35}\) Aggregation of 4 in water was studied using cryo-transmission electron microscopy (TEM). In Cryo-TEM, samples are frozen rapidly allowing the imaging of specimens in their native environment. Cryo-TEM of a sample of 4 in water (1 mg/mL, $9.0 \times 10^{-4}$ M) confirmed that the ring opened form of 4 forms worm-like micelles instead of vesicles with an average diameter of $\pm 7$ nm. The calculated length of switch 4 is $\pm 4.8$ nm, (calculated with the RM1 semi-empirical method using Firefly 8.0.1,\(^{36}\) which is based partially on GAMESS US source code).\(^{37}\) The data suggests that the aliphatic chains might be interdigitated in the micelles as the radius of the micelles is $\pm 3.5$ nm and the modeled length of 4 corresponds to $\pm 4.8$ nm.
Figure 6.18: Cryo-TEM images of ring open 4 in H2O, forms worm-like micelles of several hundred nm in length and an average diameter of ± 7 nm.

Figure 19: Cryo-TEM images of ring open 4 in H2O, forms worm-like micelles of several hundred nm in length. Upon irradiation λ = 365 nm smaller micelles are formed. Irradiation 550 nm) results in the formation of vesicles.
6.2.4 Fluorescence switching of 1 in LAD2 cells

The switching of the fluorescence of 6 was performed using cells from a leukocyte adhesion deficient type 2 cell line (LAD2). Cells were loaded with the fluorescent probe, by introducing 6 to the medium, and were subsequently cultured for 30 min (for details see section 3.6.4). The uptake was spontaneous and 6 could not be removed by rinsing the cells.

![Figure 6.20](image)

**Figure 6.20:** Microscopy images of a LAD2 cell containing 6. a) Wide field optical microscopy image and b) fluorescence microscopy image at 60× magnification.

Comparison of the images obtained by optical and fluorescence microscopy confirm the uptake of 6 by the cells (Figure 6.20). It can also be observed that 6 is concentrated in the granules of the LAD2 cells and that as a result these areas show higher fluorescence intensity. A fluorescence spectrum of 6 could be directly obtained from LAD cells, once the position of a cell is established (Figure 6.16a) an image from the fluorescence intensity (λ_{Ex} = 405 nm) can be obtained (Figure 6.21b). These data are subsequently used to obtain a fluorescence spectrum (Figure 6.22).
Cells that were not loaded with fluorescent switch 4 showed weak background fluorescence at the same intensity as the Raman scattering from the immersion oil, with a sharp signal at ± 475 nm (Figure 6.22a). The fluorescence intensity originating from cells containing switch 4 (Figure 6.22b) is considerable stronger with an emission maximum at 560 nm, the Raman scattering at ± 475 nm can no longer be observed. Photochemical ring closure of 4 upon prolonged exposure at 405 nm leads to loss in emission intensity at 560 nm (Figure 6.23). However, the fluorescence was not completely depleted upon completion of the irradiation, this is the result of ring closed 4 still present in the sample. Irradiation (λ = 488 nm) of the ring closed enriched mixture of switch 4 in LAD2 cells results in the recovery of the fluorescence intensity. However, signal recovery is accompanied by a red shift in the fluorescence emission (λ_{max} ± 595 nm).
Figure 6.23: a) Photochemical ring closing ($\lambda = 405$ nm) and ring opening ($\lambda > 488$ nm) of 4. b) Fluorescence spectra ($\lambda_{ex} = 405$ nm) of LAD2 cells loaded with 4 in PBS. Initial fluorescent state (---), quenching of the fluorescence upon irradiation 405 nm (----) and recovery of the fluorescence intensity upon irradiation 488 nm (-----).

Figure 6.24: a) Graphical representation of the fluorescence intensity from a LAD2 cell containing fluorescence switch 4 at $\lambda_{Em} = 560$ nm. On-off switching of the fluorescence occurs as a result of irradiation $\lambda_2$ ($\lambda = 405$ nm). Recovery occurs at 488 nm ($\lambda_1$). b) Fluorescence microscopy images of the LAD2 cell at set time intervals during the experiment.

To completely recover 4 to the ring opened form the LAD2 cells were irradiated for 20 sec at $\lambda_{Ro}$ 488 nm (Figure 6.23a). Subsequently the fluorescence emission of 4 in LAD2 cells was monitored at $\lambda_{Em}$ 560 nm. At this wavelength no significant fluorescence was observed. Subsequent, irradiation of LAD2 cells at 405 nm, results in emission ($\lambda_{max}$ 560 nm). Continued irradiation (25-100 s) at 405 nm leads to a decrease in the
fluorescence intensity as a result of photobleaching (Figure 6.17). Alternated irradiation between the two wavelengths results in a loss of the fluorescence at approximately 20, 100 and 180 s. Repetition of the sequential irradiation at 488 nm and 405 nm results in a second switching cycle (Figure 6.25).

Figure 6.25: Changes in the fluorescence intensity of 4 in LAD2 Cells as a result of switching at $\lambda =$ 405 and 488 nm.

6.3 Discussion

In this chapter a fluorescent amphiphilic DTE probe for gold surface functionalization was developed. Towards this objective, a $t$-butyl (3) and a trityl group (4) protected thiols were synthesized. The $t$-butyl thioether could not be deprotected to the corresponding thiol without damaging the probe. Efforts to functionalize gold nanoparticles with in-situ deprotected 4 were unsuccessful, even though gold nanoparticles could be successfully functionalized with amphiphilic OEG$_2$-C11-thiols. The lack of nanoparticle formation is probably due to the poor solubility of 4 under the reaction conditions (2-propanol/methanol/acetic acid 10:1:0.1). The addition of 2.5% DCM as a co-solvent also did not result in nanoparticle formation.

Irradiation of 3 and 4 with UV-light results in 43 and 61% of the ring closed isomer, respectively. Ring closure of 3 and 4 is accompanied by a loss in the fluorescence, subsequent irradiation with visible light results in the partial recovery of the fluorescence spectrum and of the UV/Vis absorbance. This is associated with the formation of a side product as a result of the photoirradiation. Irie and co-workers have established that prolonged irradiation of dithienylethene switches results in a condensed system, which bears two 6-membered heterocyclic rings.\textsuperscript{38} It is thought that the side product is formed from the ring closed isomer by radical migration in the $\pi$-conjugated system. The condensed product is stable under irradiation. Introduction of methyl
substituents on the 4 and 4′ positions of the thiophene rings prevents the second photoisomerisation from occurring, furthermore it would also result in an increase in the quantum yield of ring closure.\textsuperscript{34} In the future a hexa-fluoro-DTE switch should be considered as a photoswitchable quenching unit as they are generally have higher fatigue resistance than their hexa-hydro-counterparts. However, the hexa-fluoro DTE switch could have a significantly different solubility.

The results in this chapter show that pyridinium functionalized DTE switches can be used as photoswitchable fluorescent probe, however, in photo activated localization microscopy the brightness of a fluorescent probe is of key importance. The switches described in this chapter exhibit weak fluorescence, and for similar systems fluorescent quantum yields of 0.44\% have been reported.\textsuperscript{18} A more efficient PALM probe might be obtained by introducing a commonly used fluorescent tag such as Alexa fluor 488 which has a $\Phi_0$ of 0.92 and $\varepsilon$ 71000.

![Figure 26: Schematic structure of a more efficient photoswitchable fluorescent probe.](image)

The combination of a bright fluorophore in combination with the 4- and 4′- methyl substituted hexa-fluoro-DTE switching core could result in a promising PALM probe.

### 6.4 Conclusion

In conclusion the synthesis and photochemical characterization of a photoswitchable fluorescent probe is described. In the ring open form switches 3 and 4 show fluorescence around 560 nm; upon ring-closure by UV irradiation the intensity of the fluorescence is reduced. Subsequent irradiation with visible light can restore the intensity of the fluorescence, however, after several switching cycles photobleaching becomes apparent. Functionalization of gold nanoparticles with the free thiol of switch 4 using the Burst method\textsuperscript{27} was unsuccessful as a result of the poor solubility of 4 under the reaction conditions. The addition of a co-solvent did not result in the formation of gold nanoparticles.

As with other previously reported amphiphilic DTE switches, 4 forms aggregates in water \textit{i.e.} worm-like micelles. Preliminary studies indicate that irradiation of these micelles results in a change in size and shape of the aggregates (Figure 18). These are interesting properties for drug delivery systems\textsuperscript{39} and investigations will be continued in
Towards a PALM Probe for Imagining Gold Nanoparticles in Living Cells

Amphiphilic switch 4 was shown to be internalized by LAD2 using fluorescence microscopy. Furthermore, the fluorescence of the internalized switches could be switched from the on to the off state and vice-versa.

6.5 Experimental section

General remarks

For general information of the experimental work see chapter 2.5 experimental section.

Synthesis

1,2-bis(5-chloro-2-methylthiophen-3-yl)cyclopent-1-ene (5). Switch 5 was synthesized on a 22 g scale following a 3 step procedure reported by L. N. Lucas et.al. and was obtained in an overall yield of 13%. $^1$H NMR (300 MHz, CDCl$_3$) δ: 6.56 (s, 2H), 2.70 (t, $J$ = 7.5, 4H), 2.05-1.95 (m, 2H), 1.86 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ: 135.0 (C), 134.6 (C), 133.5 (C), 126.9 (CH), 125.4 (C), 38.6 (CH$_2$), 23.0 (CH$_2$), 14.4 (CH$_3$). m/z (EI): 327.9 (100.0%).

11-(4-bromophenoxy)undecan-1-ol (6). 11-bromoundecan-1-ol (29.0 g, 115.6 mmol) was added to a mixture of 4-bromophenol (20.0 g, 115.6 mmol), K$_2$CO$_3$ (47.9 g, 347.0 mmol) and NaI (17.3 g, 115.6 mmol) in acetonitrile (600 mL). The reaction mixture was heated to reflux and stirred for 72 h. After which the reaction was allowed to attain rt and water (300 mL) and ethyl acetate (1000 mL) was added. The organic layer was washed with water (3 × 300mL) and brine (300 mL) and subsequently dried over MgSO$_4$. The organic solute was removed under reduced pressure, resulting in a white solid (39.2 g, 114.2 mmol 99% yield). $^1$H NMR (300 MHz, CDCl$_3$) δ: 7.36-7.28 (m, 2H), 6.77-6.69 (m, 2H), 3.87 (t, $J$ = 6.6, 2H), 3.59 (t, $J$ = 6.6, 2H), 1.82 (s, 1H), 1.77-1.68 (m, 2H), 1.57-1.48 (m, 2H), 1.47-1.22 (m, 14H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ: 158.4 (C), 132.3 (CH), 116.4 (CH), 112.7 (C), 68.4 (CH$_2$), 63.1 (CH$_3$), 32.9 (CH$_2$), 29.7 (CH$_2$), 29.7 (CH$_2$), 29.6 (CH$_2$), 29.5 (CH$_2$), 29.3 (CH$_2$), 26.1 (CH$_2$), 25.9 (CH$_3$). m/z (APCI pos.) = 345 (+H$^+$); HRMS (EI): calcd. for C$_{17}$H$_{27}$BrO$_2$: 345.12467 found 345.1275. Elemental Analysis calculated: C, 59.48; H, 7.93. Measured: C, 59.48; H, 7.98.
11-(4-(2-(5-chloro-2-methylthiophene-3-yl)cyclopent-1-en-1-yl)-5-methylthiophen-2-yl)phenoxy)undecan-1-ol (7). To a solution of 5 (1.45 g, 4.40 mmol) in THF (20 mL) n-BuLi (1.6 M, 3.0 ml, 4.84 mmol) was added drop-wise at 0°C under an atmosphere of argon. The temperature was allowed to attain rt, while stirring was continued for 1 h. Subsequently, the reaction mixture was cooled to 0°C and tributyl borate (1.10 g, 4.84 mmol) was added dropwise. The resulting mixture was allowed to attain rt and was stirred for 1.5 h. The resulting solution of boronic ester was added without further purification to a solution of 6 (0.75 g, 2.20 mmol), Na2CO3 in H2O (20 ml, 2M) and ethylene glycol 20 drops in THF (20 mL) under an argon atmosphere. The reaction mixture was heated to reflux and stirred for 48 h. Upon completion the reaction was allowed to attain room temperature and DCM (200 mL) was added. The organic layer was washed with H2O (3 × 100 mL) and brine (100 mL). The organic layer was separated, dried over MgSO4 and subsequently concentrated under reduced pressure. 7 was further purified using flash chromatography (SiO2, pentane/ethyl acetate 10:1, Rf = 0.12). 7 was obtained as a colourless oil, 1.81 g, 3.25 mmol 74% yield. 1H NMR (400 MHz, CDCl3): 7.40 (d, J = 8.6, 2H), 6.86 (d, J = 7.8, 3H), 6.63 (s, 1H), 3.94 (t, J = 6.5, 2H), 3.62 (t, J = 6.6, 2H), 2.77 (dt, J = 25.7, 7.2, 4H), 2.04 (dd, J = 14.9, 7.6, 2H), 1.98 (s, 3H), 1.88 (s, 3H), 1.83 – 1.70 (m, 2H), 1.55 (dd, J = 13.4, 6.7, 2H), 1.44 (d, J = 7.5, 2H), 1.31 (s, 14H). 13C NMR (101 MHz, CDCl3): 158.6 (C), 140.0 (C), 136.4 (C), 135.6 (C), 135.4 (C), 133.7 (C), 133.5 (C), 133.6 (CH2), 133.4 (C), 133.3 (C), 127.2 (C), 127.0 (CH), 126.6 (CH), 125.0 (C), 122.7 (CH), 114.9 (CH), 68.2 (CH2), 63.1 (CH2), 38.6 (CH2), 38.5 (CH2), 32.9 (CH2), 29.7 (CH2), 29.7 (CH2), 29.7 (CH2), 29.6 (CH2), 29.5 (CH2), 29.4 (CH2), 26.2 (CH2), 25.9 (CH2), 23.0 (CH2), 14.4 (CH3), 14.3 (CH3). m/z (DART-TOF) 557.1 [M + H]. HRMS (ESI): calcd. for C32H41ClO2S2H+: 557.2309 found 557.2281 [M + H].

5-(4-((11-bromoundecyl)oxy)phenyl)-3-(2-(5-chloro-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-2-methylthiophene (8) A mixture of 7 (0.57 g, 1.10 mmol), triphenylphosphine (2.37, 9.04 mmol) and tetrabromomethane (2.48 g, 7.49 mmol) in CH2Cl2 (20 mL) was stirred overnight at rt. The volatiles were removed under reduced pressure and 8 was loaded onto Celite. Compound 8 was further purified using column chromatography (SiO2, pentane/ethyl acetate 10:1, Rf = 0.77) and was obtained as a brown oil (0.56 g, 0.90 mmol, 82%). 1H NMR (400 MHz, CDCl3): 7.40 (d, J = 8.2, 2H), 6.87 (m, 3H), 6.62 (s, 1H), 3.95 (t, J = 6.2, 2H), 3.40 (t, J = 6.8, 2H), 2.77 (d, J = 25.0, 4H), 2.11-2.01 (m, 2H), 1.98 (s, 3H), 1.88 (s, 3H), 1.87-1.71 (m, 4H), 1.44 (s, 4H), 1.30 (s, 10H). 13C NMR (101 MHz, CDCl3): 158.6 (C), 140.0 (C), 136.4 (C), 135.6 (C), 135.4 (C), 133.7 (C), 133.5 (C), 133.4 (C), 127.3 (C), 127.0 (CH), 126.7 (CH), 125.1 (C), 122.8 (CH), 115.0 (CH), 68.3 (CH2), 38.7 (CH2), 38.5 (CH2), 34.2 (CH3).
(CH₂), 33.0 (CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.6 (CH₂), 29.4 (CH₂), 29.0 (CH₂), 28.4 (CH₂), 26.2 (CH₂), 23.1 (CH₂), 14.5 (CH₃), 14.4 (CH₃). HRMS (ESI): calcd. for C₃₂H₄₁BrClO₃: 619.1465 found 619.1460.

5-(4-((11-(tert-butylthio)undecyl)oxy)phenyl)-3-(2-(5-chloro-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-2-methylthiophene (10). Under an argon atmosphere a solution of 8 (0.57 mg, 0.92 mmol) in ethanol/bezene 1:1 (3 mL) was added to a solution of NaOH (53 mg, 1.32 mmol), H₂O (0.6 mL) and 2-methylpropane-2-thiol (456 mg, 1.65 mmol) in ethanol/bezene 1:1 (3 mL). The resulting mixture was stirred overnight at rt and was subsequently poured onto a saturated NaHCO₃ solution (10 mL). The aqueous layer was extracted with DCM (3 × 15 mL), the organic layers were combined and washed with brine (1 × 20 mL). Subsequently the organic layer was dried over MgSO₄ and concentrated under reduced pressure. 10 was loaded onto Celite and further purified by column chromatography and was obtained as a brown oil (394.3 mg, 0.63 mmol, 68%). ¹H NMR (300 MHz, CDCl₃) δ: 7.40 (d, J = 8.7 Hz, 2H), 6.86 (t, J = 4.3 Hz, 3H), 6.62 (s, 1H), 3.94 (t, J = 6.5 Hz, 2H), 2.77 (dt, J = 17.1, 7.2 Hz, 4H), 2.52 (t, J = 7.4 Hz, 2H), 2.02 (dd, J = 13.4, 5.8 Hz, 3H), 1.97 (s, 3H), 1.89 (d, J = 6.9 Hz, 3H), 1.81-1.71 (m, 3H), 1.56 (dd, J = 14.8, 7.5 Hz, 3H), 1.35 (m, 28H). ¹³C NMR (101 MHz, CDCl₃) δ: 158.5 (C), 140.0 (C), 136.2 (C), 135.5 (C), 135.3 (C), 133.6 (C), 133.3 (C), 133.3 (C), 127.2 (C), 126.9 (C), 126.6 (C), 126.0 (C), 125.0 (C), 122.7 (CH), 114.9 (CH), 68.1 (CH₂), 41.8 (C), 38.6 (CH₂), 38.5 (CH₂), 31.1 (CH₃), 30.0 (CH₂), 29.7 (CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.4 (CH₂), 29.4 (CH₂), 28.4 (CH₂), 26.2 (CH₂), 23.0 (CH₃), 14.4 (CH₃), 14.3 (CH₃). HRMS (ESI): calcd. for C₃₆H₅₀ClO₃: 629.2707 found 629.2673.

4-(4-(4-((11-(tert-butylthio)undecyl)oxy)phenyl)-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methylthiophen-2-ylpyridine (11). To a solution of 10 (373.9 mg, 0.59 mmol) in THF (20 mL) n-BuLi (1.6 M, 0.56 ml, 0.89 mmol) was added drop-wise at 0°C under an atmosphere of argon. The temperature was allowed to attain rt, while stirring was continued for 1 h. Subsequently, the reaction mixture was cooled to 0°C and tributyl borate (205.0 mg, 0.89 mmol) was added dropwise. The resulting mixture was allowed to attain rt and was stirred for 1.5 h. The resulting boronic ester solution was added without further purification to a solution of 4-bromopyridine hydrochloride, Na₂CO₃ in H₂O (3 mL, 2M) and ethylene glycol 16 drops in THF (15 mL) under an argon atmosphere. The reaction mixture was heated to reflux and stirred for 24 h. After which the reaction was allowed to attain room temperature and DCM (15 mL) was added. The organic layer was washed with H₂O (3 × 10 mL) and brine (10 mL). The organic layer was separated, dried over MgSO₄ and subsequently concentrated under reduced pressure. 13 was
Chapter 6

4-(4-(2-(5-(4-((11-(tert-butylthio)undecyl)oxy)phenyl)-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-methylthiophen-2-yl)-1-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)pyridin-1-ium bromine (5). 2-(2-(2-(2-bromoethoxy)ethoxy)-ethoxy)ethanol (141.4 mg, 0.55 mmol) was added to a solution of 13 (230.0 g, 0.34 mmol) in acetonitrile (3 mL) was stirred at rt for 3 d.

was loaded onto Celite and purified on a Grace Reveleris flash chromatograph system (SiO2, starting (2 min) at pentane/dichloromethane/methanol 1:4:0 to 0:10:1 over 15 min, the sample was eluted with the final solvent mixture for an additional 10 min. The organic solvent was removed under reduced pressure and 6 was subsequently freeze dried, resulting in a green powder (17 mg, 0.02 mmol, 6% yield). **1H NMR** (400 MHz, CDCl3) δ: 9.22 (d, J = 6.6, 2H), 7.79 (d, J = 6.6, 2H), 7.55 (s, 1H), 7.35 (d, J = 6.6, 2H), 6.83 (d, J = 7.0, 3H), 5.03 (s, 2H), 4.20–4.14 (m, 1H), 4.01 (s, 2H), 3.92 (t, J = 6.5, 2H), 3.76 (t, J = 6.0, 1H), 3.60 (ddd, J = 17.0, 9.9, 4.8, 11H), 3.42 (t, J = 6.0, 1H), 2.82 (t, J = 6.9, 4H), 2.48 (t, J = 7.5, 2H), 2.13–2.04 (m, 5H), 2.00 (s, 1H), 1.93 (s, 3H), 1.77–1.70 (m, 2H), 1.57–1.48 (m, 2H), 1.45–1.21 (m, 29H). **13C NMR** (101 MHz, CDCl3) δ: 158.8 (C), 145.39, 149.2 (C), 145.4 (CH), 140.7 (C), 140.0 (C), 137.6 (C), 136.0 (C), 133.5 (C), 132.8 (CH), 132.7 (C), 132.5 (C), 126.9 (C), 126.7 (CH), 122.5 (CH), 121.2 (CH), 115.0 (CH), 71.31 (CH2), 70.7 (CH2), 70.5 (CH2), 70.5 (CH2), 70.4 (CH2), 69.9 (CH2), 69.3 (CH2), 68.3 (CH2), 63.6 (CH2), 60.1 (CH2), 41.94 (C) 38.8 (CH2), 38.6 (CH2), 31.2 (CH3), 30.9 (CH2), 30.1 (CH2), 29.7 (CH2), 29.7 (CH2), 29.7 (CH2), 29.6 (CH2), 29.5 (CH2), 29.4 (CH2), 28.5 (CH2), 26.2 (CH2), 23.1 (CH2), 15.5 (CH3), 14.6 (CH3).
2-((11-(4-(4-(2-(5-chloro-2-methylthiophen-3-yl)cyclopent-1-en-1-yl)-5-

methylthiophen-2-yl)phenoxy)undecyl)oxy)tetrahydro-2H-pyran (9). 3,4-Dihydro-2H-
pyran (5 mL, 54.8 mmol), Pyridinium p-toluenesulfonate (122 mg, 0.48 mmol) and 7 (1.28 g, 2.3
mmol) were added to DMC (7 mL). The resulting reaction mixture was stirred o.n. at rt. Upon completion
DCM was added (20 mL) and the organic layer was extracted with H2O (2 × 10mL) and
a saturated Na2CO3 solution (2 × 10mL). The organic layer was subsequently dried over
MgSO4 and concentrated in vacuo. 9 was further purified using flash chromatography
(SiO2, pentane/ethyl acetate 10:1, Rf = 0.52). 9 was obtained as a colourless oil, 1.31 g,
2.0 mmol 93% yield. 1H NMR (400 MHz, CDCl3) δ: 7.39 (d, J = 8.7, 2H), 6.86 (s, 1H),
6.85 (d, J = 7.3, 2H), 6.62 (s, 1H), 4.63-4.52 (m, 1H), 3.94 (t, J = 6.5, 2H), 3.91-3.83
(m, 1H), 3.74 (dt, J = 9.5, 6.9, 1H), 3.52-3.47 (m, 1H), 3.38 (dt, J = 9.5, 6.7, 1H), 2.77
(dt, J = 25.7, 7.3, 4H), 2.07-1.99 (m, 2H), 1.97 (s, 3H), 1.88 (s, 3H), 1.86-1.67 (m, 4H),
1.64-1.40 (m, 8H), 1.30 (m, 12H). 13C NMR (101 MHz, CDCl3) δ: 158.6 (C), 140.0 (C),
136.3 (C), 135.6 (C), 135.3 (C), 133.6 (C), 133.4 (C), 127.2 (C), 127.0 (CH),
126.6 (CH), 125.0 (C), 122.7 (CH), 114.9 (CH), 98.9 (CH), 68.2 (CH3), 67.8 (CH3),
62.4 (CH2), 38.6 (CH2), 38.5 (CH2), 30.9 (CH2), 29.9 (CH2), 29.7 (CH2),
29.7 (CH2), 29.6 (CH2), 29.5 (CH2), 29.4 (CH2), 26.4 (CH3), 26.2 (CH3), 25.7 (CH2),
23.0 (CH2), 19.8 (CH2), 14.4 (CH3), 14.3 (CH3). m/z (DART-TOF) 642.2 [M + H].

To a solution of 9 (1.31 g, 2.04 mmol) in THF (20 mL) n-BuLi (1.6 M, 1.5 ml, 2.54 mmol) was
added drop-wise at 0°C under an atmosphere of argon. The temperature was allowed to attain rt, while stirring
was continued for 1 h. Subsequently, the reaction mixture was cooled to 0°C and tributyl borate (0.52 g, 2.24 mmol) was added dropwise.

The resulting boronic ester solution was added without further purification to a solution of 4-
bromopyridine hydrochloride, Na2CO3 in H2O (20 mL, 2M) and ethylene glycol 20
drops in THF (20 mL) under an argon atmosphere. The reaction mixture was heated to
reflux and stirred for 48 h. Upon completion the reaction was allowed to attain room
temperature and DCM (50 mL) was added. The organic layer was washed with H2O (3 × 50 mL) and brine (50 mL). The organic layer was separated, dried over MgSO4 and
subsequently concentrated under reduced pressure. 12 was further purified using flash
chromatography (SiO2, pentane/ethyl acetate). 12 was obtained as a colourless oil, 1.11
g, 1.63 mmol, 80% yield. 1H-NMR (400 MHz, CDCl3) δ: 7.39 (d, J = 8.7, 2H), 6.85 (d,
J = 7.3, 3H), 6.62 (s, 1H), 4.63-4.52 (m, 1H), 3.94 (t, J = 6.5, 2H), 3.91-3.83 (m, 1H),
3.74 (dt, J = 9.5, 6.9, 1H), 3.49 (dd, J = 10.9, 4.9, 1H), 3.38 (dt, J = 9.5, 6.7, 1H), 2.77
(dt, J = 25.7, 7.3, 4H), 2.07-1.99 (m, 2H), 1.97 (s, 3H), 1.88 (s, 3H), 1.86-1.67 (m, 4H),
1.64-1.40 (m, 8H), 1.30 (s, 12H). Subsequently switch 12 (1.11 g, 1.63 mmol) was deprotected with pyridinium p-toluenesulfonate (1.31 g, 5.22 mmol) in MeOH (40 mL). Upon completion the organic solvent was evaporated and the resulting solid was dissolved in CHCl₃. The organic layer was extracted with a saturated NaHCO₃ solution (2 × 50 mL). The organic layer was dried over Na₂SO₄ and was concentrated under reduced pressure. The tosylate salt of 13 was obtained as a green oil. The salt was dissolved in THF (15 mL) Na₂CO₃ (0.71 g, 6.72 mmol) and H₂O (5 mL) was added. The reaction mixture was refluxed for 1 h, after which CHCl₃ (30 mL) was added and the organic layer was washed with water (2 × 25 mL). The organic phase was collected, dried over Na₂SO₄ and evaporated *in vacuo*. 14 was further purified using flash chromatography (SiO₂, ethyl acetate, *Rf* = 0.4), 14 was obtained as a purple oil (0.91 g, 1.52 mmol, 74% yield). ¹H NMR (400 MHz, CDCl₃) δ: 8.49 (dd, *J* = 4.7, 2H), 7.38 (d, *J* = 8.7, 2H), 7.32 (dd, *J* = 4.7, 1.5, 2H), 7.20 (s, 1H), 6.87 (s, 1H), 6.84 (d, *J* = 8.8, 2H), 3.93 (t, *J* = 6.6, 2H), 3.62 (t, *J* = 6.6, 2H), 2.82 (t, *J* = 7.4, 4H), 2.11-2.05 (m, 2H), 2.02 (s, 3H), 1.95 (s, 3H), 1.79-1.71 (m, 2H), 1.59-1.50 (m, 2H), 1.47-1.38 (m, 2H), 1.28 (s, 13H). ¹³C NMR (101 MHz, CDCl₃) δ: 158.67 (C), 150.02 (CH), 141.94 (C), 140.12 (C), 137.8 (C), 137.6 (C), 136.4 (C), 136.3 (C), 135.9 (C), 134.0 (C), 133.5 (C), 127.3 (C), 126.9 (CH), 126.7 (CH), 122.9 (CH), 119.5 (CH), 115.0 (CH), 68.3 (CH₂), 63.1 (CH₂), 38.6 (CH₂), 38.6 (CH₂), 33.0 (CH₂), 29.8 (CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.4 (CH₂), 26.2 (CH₂), 26.0 (CH₂), 23.2 (CH₂), 14.9 (CH₃), 14.5 (CH₃). HRMS (APCI): calcld. for C₃₇H₄₆NO₂S₂: 600.2965, found: 600.2955.

4-(5-methyl-4-(2-(2-methyl-5-(4-((11-(tritylthio)undecyl)oxy)phenyl)thiophen-3-yl)cyclopent-1-en-1-yl)thiophen-2-yl)pyridine (15). To a solution of switch 14 (386.2 mg, 0.64 mmol), triphenylmethanethiol (410.9 mg, 1.60 mmol) and triphenylphosphine (390.2 mg, 1.49 mmol) in dry THF (10 ml) at 0°C, DIAD (0.29 mL, 2.31 mmol) was added drop-wise. The mixture was allowed to attain rt overnight under stirring. The organic solvent was removed under reduced pressure and the resulting solid was loaded onto celite. 15 was further purified by column chromatography (SiO₂, pentane/ethyl acetate 4:1, *Rf* = 0.31) obtaining a 1:1 mixture of 15 and diisopropyl hydrazine-1,2-dicarboxylate. Diisopropyl hydrazine-1,2-dicarboxylate was removed by distillation under reduced pressure (145 °C, 1.5 × 10⁻² mbar) obtaining 15 as a waxy solid (273.7 mg, 0.32 mmol, 50% yield). ¹H-NMR (400 MHz, CDCl₃) δ: 8.54 (s, 2H), 7.45 (d, *J* = 7.7, 6H), 7.42 (s, 1H), 7.36 (s, 2H), 7.30 (t, *J* = 7.6, 6H), 7.23 (dd, *J* = 15.3, 7.8, 5H), 6.93 (s, 1H), 6.88 (d, *J* = 8.6, 2H), 3.96 (t, *J* = 6.5, 2H), 2.87 (t, *J* = 7.4, 4H), 2.17 (t, *J* = 7.4, 2H), 2.15-2.08 (m, 2H), 2.06 (s, 3H), 1.99 (s, 3H), 1.83-1.75 (m, 2H), 1.43 (dd, *J* = 15.1, 7.8, 4H), 1.38-1.15 (m, 15H). ¹³C NMR (101 MHz, CDCl₃) δ: 158.7 (C), 149.5 (CH), 145.2 (C), 142.3 (C), 140.2 (C), 138.1 (C), 137.7 (C), 136.4 (C), 136.1 (C), 135.9 (C), 133.8 (C), 133.5 (C),
Towards a PALM Probe for Imagining Gold Nanoparticles in Living Cells

129.8 (CH), 128.1 (CH), 127.9 (CH), 126.7 (CH), 126.6 (CH), 122.8 (CH), 119.5 (CH), 115.0 (CH), 68.3 (CH2), 38.7 (CH2), 38.5 (CH2), 32.2 (CH2), 29.7 (CH2), 29.5 (CH), 29.5 (CH2), 29.4 (CH2), 29.3 (CH2), 29.2 (CH2), 28.8 (CH2), 26.2 (CH2), 23.2 (CH2), 14.9 (CH3), 14.6 (CH3). m/z (DART-TOF) 858.3 [M + H]. HRMS (ESI): calcld. for C56H59NOS3: 858.3832, found: 858.3847.

1-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)-4-(5-methyl-4-(2-(2-methyl-5-((11-(tritylthio)undecyl)oxy)phenyl)thiophen-3-yl)cyclopent-1-en-1-yl)pyridin-1-ium (4). 2-(2-(2-(2-bromoethoxy)ethoxy)ethoxy)ethanol (153.3 mg, 0.15 mmol) was added to a solution of 15 (126.5 g, 0.60 mmol) in acetonitrile (1 mL) was stirred at rt for 3 d. The organic solvent was removed under reduced pressure. Subsequently 4 was loaded onto Celite and purified on a Grace Revealeris flash chromatograph system (SiO2, starting (2 min) at pentane/dichloromethane/methanol 1:4:0.4 to 0:10:1 over 15 min, the sample was eluted with the final solvent mixture for an additional 10 min. The organic solvent was removed under reduced pressure and 4 was subsequently freeze dried, resulting in a green powder (111 mg, 0.12 mmol, 80% yield). 1H NMR (600 MHz, CDCl3) δ: 8.97 (s, 2H), 7.82 (s, 2H), 7.60 (s, 1H), 7.39-732 (m, 8H), 7.21 (t, J = 7.6, 6H), 7.14 (t, J = 7.2, 3H), 6.81-6.80 (m, 3H), 4.84 (s, 2H), 3.98 (s, 2H), 3.89 (t, J = 6.5, 2H), 3.67-3.52 (m, 12H), 2.80 (t, J = 7.2, 4H), 2.07 (dd, J = 15.5, 8.0, 2H), 2.03 (s, 3H), 1.90 (s, 3H), 1.70 (dd, J = 14.3, 6.9, 2H), 1.40-1.06 (m, 18H). 13C NMR (126 MHz, CDCl3) δ: 158.7 (C), 149.1 (C), 145.4 (C), 145.2 (C), 140.5 (C), 139.9 (C), 137.3 (C), 136.0 (C), 133.4 (C), 132.8 (CH), 132.7 (C), 132.6 (C), 129.7 (CH), 128.8 (CH), 127.9 (CH), 127.0 (CH), 126.9 (C), 126.6 (CH), 122.4 (CH), 121.3 (CH), 114.9 (CH), 72.6 (CH2), 70.4 (CH2), 70.2 (CH2), 70.0 (CH2), 69.6 (CH2), 68.3 (CH2), 66.5 (C), 61.3 (CH2), 60.2 (CH2), 46.3 (CH2), 38.7 (CH2), 38.5 (CH2), 32.1 (CH2), 29.8 (CH2), 29.6 (CH2), 29.6 (CH2), 29.5 (CH2), 29.3 (CH2), 29.3 (CH2), 29.1 (CH2), 28.7 (CH2), 26.1 (CH2), 23.1 (CH2), 15.4 (CH3), 14.5 (CH3). HRMS (ESI): calcld. for C64H76NO5S3: 1034.4880, found: 1034.4892.

Preparation of [11-(Methylcarbonylthio)-undecyl]tetra(ethylene glycol) nanoparticles

Amphiphilic nanoparticles were prepared according to the following general procedure: HAuCl4 (33.5 mg, 0.10 mmol) was dissolved in a mixture of 2-propanol (50 mL) and acetic acid (0.5 mL). [11-(Methylcarbonylthio)-undecyl]tetra(ethylene glycol) (9.5 mg, 0.02 mmol) was added to the solution under stirring. Rapid addition of a premixed solution of NaBH4 (94.4 mg, 2.5 mmol) in MeOH (5 mL) led to a color change of the solution from yellow to dark red. The resulting mixture was stirred for 3 h at rt and was subsequently poured into hexane (200 mL). The supernatant was removed and the
precipitate was washed with diethyl ether (50 mL) by centrifugation (14,000 rpm, 10°C, 30 min) the supernatant was removed and precipitate was taken up in EtOH. The EtOH solution was centrifuged (14,000 rpm, 10°C, 30 min) the supernatant was removed and the precipitate was taken up in H2O (40 mL) using centrifugation. Resulting in Au-NP’s with a diameter of 2-4 nm (average diameter 2.7 nm ± 0.6 nm, over 102 NP’s).

**Cryo-TEM imaging**

The cryo-TEM samples were prepared as follows: From a 1 ml/mL (9.0 × 10⁻⁴ M) stock solution in CHCl3 a thin film was prepared under vacuum. The film was subsequently hydrated with double distilled water to a concentration of 1mg/ml. The sample was three times; stirred under vortex, sonicated and freeze-thaw degassed. Images were recorded on a Philips CM 120 electron microscope operating at 120 keV, fitted with a Gatan cryostage (model 626).

**Preparation of fluorophore loaded LAD2 cells**

The LAD2 cells loaded with 4 were prepared using the following method. The LAD2 cells were cultured in a PBS solution (pH 7.4) at 37°C under an atmosphere of 5% CO₂ with a 95% relative humidity. The cell culturing medium replaced with PBS buffer (pH 7.4) containing 20 μM of 4 for 30 min. The culturing medium containing 4 was removed of by centrifugation (12 000 rpm). To remove 4 adhered on the outside of the LAD2 cells, the cells were washed with PBS buffer, followed by removal of the buffer using centrifugation (12 000 rpm). Subsequently, the LAD2 cells were resuspended in fresh PBS buffer (pH 7.4) for analysis using wide field confocal microscopy.

**Microscopy imaging of LAD2 cells**

Wide field fluorescence microscopy Images were obtained using an REVOLUTION xD Laser Control System for two solid state lasers (405 and 488 nm), LC-401A Laser Modulator and Combiner Unit, PCUB-110 Precision Control Unit and an AOTF for excitation, an iXon DU-897E EMCCD mounted on a Shamrock300i spectrograph coupled directly to a Nikon Ti-E microscope and 60 times confocal oil immersion lens. Semrock Filter sets were used LF405-A-NTE and LF488-A-NTE. Andor Solis was used for image acquisition.
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6.7 References

35 V. Klanga, C. Valenta and N. B. Matsko, Micron, 2013, 44, 45