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Revealing the Photophysics of Gold-Nanobeacons via Time-Resolved Fluorescence Spectroscopy

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We demonstrate that time-resolved fluorescence spectroscopy is a powerful tool to investigate the conformation states of hairpin DNA on the surface of gold nanoparticles (AuNPs) and energy transfer processes in Au-nanobeacons. Long-range fluorescence quenching of Cy5 by AuNPs has been found to be in good agreement with electrodynamics modelling. Moreover, time-correlated single-photon counting (TCSPC) is shown to be promising for real-time monitoring of the hybridization kinetics of Au-nanobeacons, with up to 60% increase in decay time component and 300% increase in component fluorescence fraction observed. Our results also indicate the importance of the stem and spacer designs for the performance of Au-nanobeacons.

Energy transfer between fluorophores and gold nanoparticles (AuNPs) provides a new paradigm with which to develop novel probes for bioassays.1 This is attributed to the unique physicochemical and biological properties of AuNPs, including tunable localized surface plasmon resonance (LSPR), facile surface modification, superior quenching capability, and good biocompatibility.2,3 Among the AuNP-based nanoprobe, of particular interest is the nanobeacon that comprises a AuNP and stem-loop (hairpin) oligonucleotides dually labeled with a fluorophore at one end and a thiol moiety at the other end.4,5 The potential advantage of the nanobeacon is that the fluorophore is still anchored to the AuNPs rather than being released into the cytoplasm upon hybridization. This makes it possible to acquire the spatial-temporal information about nucleic acid targets in living cells, offering great opportunities to understand the fundamental metabolism of cells.6,7

So far reports on the Au-nanobeacons have been mainly focused on steady-state fluorescence measurements.8,9,10,11 Fluorescence lifetimes are highly sensitive to the presence of energy transfer processes, which can provide invaluable information that is unavailable from steady-state fluorescence spectroscopy.12 In this letter, we took a novel aspect from time-resolved fluorescence spectroscopy to gain further insight into the sensing performance and energy transfer processes of Au-nanobeacons. Moreover, we demonstrated that TCSPC measurement could be used as a powerful tool to reveal the time evolutions of both fluorescence intensity and lifetime during the hybridization processes. The results also show important pointers to nanobeacon design in order to optimize their sensing performance.

The 13 nm AuNPs were synthesized by the sodium citrate reduction method.13 Three types of Cy5-labeled hairpin DNAs (Cy5-hpDNAs), which have the same loop sequence, but different stems and spacers (Table 1), were assembled on AuNPs through a salt-aging process.14 Note that hpCy5f consists of a five-nucleotide (5-nt) overhang at the 5’ end. The nanobeacons were purified by seven successive rounds of centrifugation (13,300 rpm, 15 min, 4 °C) using 10 mM phosphate buffer (pH 7.5). The concentrations of nanobeacons were determined by UV-vis spectrophotometry (λmax = 524 nm, ε = 2.7 × 10^4 L·mol⁻¹·cm⁻¹).

Table 1. DNA sequences used in this work.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Hairpin ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAhpCy5</td>
<td>Cy5-CTGACTTTG GTG AAG CTA AAG TTG AG CAA GTCGAA AA-SH</td>
<td>-6.84</td>
</tr>
<tr>
<td>hpCy5</td>
<td>Cy5-CCGGTGT GTG AAG CTA AAG TTG AG CACCGGTTT TT-SH</td>
<td>-5.85</td>
</tr>
<tr>
<td>hpCy5f</td>
<td>Cy5-AA TTT AAATTGAACTTG GTG AAG CTA AAG TTG AG CAAGTTCAATTT TT-TT TT-SH</td>
<td>-10.54</td>
</tr>
<tr>
<td>cDNAc</td>
<td>CTC AAG GTT AGC TTC AC</td>
<td></td>
</tr>
</tbody>
</table>

*The underlined bases represent the stem sequence.

a Free energy predicted at 25 °C in 137 mM [Na+] buffer by the UNAFold software (www.idtdna.com).

As depicted in Fig. 1 (a), the maximum optical absorption of AuNPs was shifted from 519 to 524 nm after conjugated with hpDNAs, due to the change in the surrounding refractive index. Additionally, there is an...
absorption shoulder at around 649 nm in all three nanobeacons corresponding to the absorption of Cy5, further confirming the presence of Cy5-hpDNAs on AuNPs. The surface loadings of hpDNA on AuNPs were quantitated to be 27 ± 6, 29 ± 3 and 19 ± 2 per nanoparticle for AuNP-AAhpCy5, AuNP-hpCy5 and AuNP-hpCy5f, respectively, according to a thiol exchange protocol. The decay times with amplitudes $\alpha_i$ are the decay times with amplitudes $\alpha_i$. The deviation of five measurements. Error bars are one standard deviation of five measurements.

The background fluorescence (i.e., without adding cDNA) of the nanobeacons showed an increasing trend in the following order: AuNP-AAhpCy5 < AuNP-hpCy5 < AuNP-hpCy5f (Fig. 1(b)), in line with the increasing spacer lengths used in the DNA designs (Table 1). Notably, all three nanobeacons experienced significant fluorescence recovery upon hybridization, showing comparable fluorescence intensities. This is not surprising for AuNP-AAhpCy5 and AuNP-hpCy5, as they have comparable surface coverage of hpDNAs and similar fully-extended DNA lengths (35 nt for AhpCy5 and 34 nt for hpCy5). For AuNP-hpCy5f, the situation is more complicated since it has the lowest surface loading of hpDNA but the longest length of oligonucleotide (i.e., 56 nt).

To gain further insight into the conformational change of hpDNA on AuNPs upon hybridization, and the fluorescence quenching effect of AuNPs, we performed time-resolved fluorescence measurements on all three nanobeacons using the TSCCP technique on a FluoroCube fluorescence lifetime system (Horiba Jobin Yvon IBH Ltd., Glasgow, UK). The system was equipped with an emission monochromator and a pulsed light-emitting diode (LED) of 638 nm operating at 1 MHz repetition rate as the excitation source. The fluorescence of Cy5 was detected at 680 nm with a 32-nm slit and a 670-nm longpass filter. Fluorescence decays were measured at a magic angle (54.7°) to eliminate polarization artifacts. The acquired data are shown in Figs. 2 (a), (c) and (e).

The fluorescence intensity decay curves were initially fitted to a multiexponential model using non-linear least-squares analysis:

$$I(t) = \sum \alpha_i \exp \left( -\frac{t}{\tau_i} \right),$$  \hspace{1cm} (1)

where $\tau_i$ are the decay times with amplitudes $\alpha_i$ and $\sum \alpha_i = 1$. The fractional contribution of each lifetime component to the steady-state intensity is represented by

$$f_i = \alpha_i \tau_i / \sum \alpha_k \tau_k ,$$ \hspace{1cm} (2)

The average lifetime $\bar{\tau}$ is calculated as

$$\bar{\tau} = \sum \alpha_i \tau_i .$$ \hspace{1cm} (3)

The retrieved lifetimes are summarized in Table 2.

The background intensity in Fig. 1 (b) can thus be corrected to be 3.14, 6.04 and 7.78 (a.u.) for AuNP-AAhpCy5, -hpCy5 and -hpCy5f, respectively. This is not surprising for AuNP-AAhpCy5 and AuNP-hpCy5, which contributes a fraction of ~70% to the steady-state emission intensity in all three nanobeacons, suggesting significant energy transfer from Cy5 to AuNPs. Moreover, the average lifetime of AuNP-hpCy5f in the absence of the target strands is significantly greater than those of AuNP-hpCy5 and AuNP-AAhpCy5. A short lifetime component (τ3 < 100 ps) is found in all three nanobeacons, which contributes a fraction of ~70% to the steady-state emission before hybridization, but progressively decreases after hybridization.

Despite small difference in the decay times of free Cy5-hpDNA in different designs, substantial reductions of lifetime are observed in all cases after Cy5-hpDNAs grafted on AuNPs, suggesting significant energy transfer from Cy5 to AuNPs. Moreover, the average lifetime of AuNP-hpCy5f in the absence of the target strands is significantly greater than those of AuNP-hpCy5 and AuNP-AAhpCy5. A short lifetime component (τ3 < 100 ps) is found in all three nanobeacons, which contributes a fraction of ~70% to the steady-state emission before hybridization, but progressively decreases after hybridization.

Upon hybridization, the fractional contributions of intermediate (τ2) and long (τ1) lifetime components significantly increase. It is striking to note that τ2 is shifted to a greater value, whereas τ1 and τ3 remained almost constant within the uncertainty. The presence of τ3 in the hybridized nanobeacons implies that there are still unhybridized hpDNAs on AuNPs. The presence of τ1 in the “as-prepared” nanobeacons suggests a small amount of unfolded or improperly folded hpDNAs on AuNPs. This should be excluded in order to quantitatively examine the influence of spacer design on the fluorescence background of the “as-prepared” nanobeacons. The background intensity in Fig. 1 (b) can thus be corrected to be 3.14, 6.04 and 7.78 (a.u.) for AuNP-AAhpCy5, -hpCy5 and -hpCy5f, respectively. Taking the surface coverage into account, it is apparent that the background fluorescence increases with increasing spacer length.
Table 2. Multiexponential analysis of fluorescence intensity decays

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\tau_1$ /ns</th>
<th>$\tau_2$ /ns</th>
<th>$\tau_3$ /ns</th>
<th>$\tau_4$ /ns</th>
<th>$f_1$</th>
<th>$f_2$</th>
<th>$f_3$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAhpCy5</td>
<td>1.436 ± 0.025</td>
<td>0.293 ± 0.006</td>
<td>0.321 ± 0.056</td>
<td>0.647 ± 0.010</td>
<td>0.590</td>
<td>0.410</td>
<td>0.000</td>
<td>1.056</td>
</tr>
<tr>
<td>AuNP-AAhpCy5</td>
<td>0.160 ± 0.021</td>
<td>0.251 ± 0.033</td>
<td>0.178 ± 0.038</td>
<td>0.710 ± 0.023</td>
<td>0.091</td>
<td>0.229</td>
<td>0.680</td>
<td>1.165</td>
</tr>
<tr>
<td>AuNP-AAhpCy5 + cDNA</td>
<td>0.485 ± 0.044</td>
<td>0.401 ± 0.033</td>
<td>0.110 ± 0.100</td>
<td>0.744 ± 0.256</td>
<td>0.243</td>
<td>0.648</td>
<td>0.109</td>
<td>1.625</td>
</tr>
<tr>
<td>hCy5</td>
<td>1.858 ± 0.022</td>
<td>1.110 ± 0.102</td>
<td>0.178 ± 0.010</td>
<td>0.743 ± 0.256</td>
<td>0.091</td>
<td>0.229</td>
<td>0.680</td>
<td>1.165</td>
</tr>
<tr>
<td>AuNP-hpCy5</td>
<td>0.178 ± 0.038</td>
<td>0.302 ± 0.038</td>
<td>0.320 ± 0.010</td>
<td>0.178 ± 0.038</td>
<td>0.091</td>
<td>0.229</td>
<td>0.680</td>
<td>1.165</td>
</tr>
<tr>
<td>AuNP-hpCy5 + cDNA</td>
<td>0.521 ± 0.038</td>
<td>0.412 ± 0.030</td>
<td>0.110 ± 0.100</td>
<td>0.744 ± 0.256</td>
<td>0.243</td>
<td>0.648</td>
<td>0.109</td>
<td>1.625</td>
</tr>
<tr>
<td>hpCy5f</td>
<td>1.718 ± 0.023</td>
<td>1.053 ± 0.089</td>
<td>0.320 ± 0.010</td>
<td>0.178 ± 0.038</td>
<td>0.091</td>
<td>0.229</td>
<td>0.680</td>
<td>1.165</td>
</tr>
<tr>
<td>AuNP-hpCy5f</td>
<td>0.329 ± 0.060</td>
<td>0.372 ± 0.051</td>
<td>0.110 ± 0.100</td>
<td>0.744 ± 0.256</td>
<td>0.243</td>
<td>0.648</td>
<td>0.109</td>
<td>1.625</td>
</tr>
<tr>
<td>AuNP-hpCy5f + cDNA</td>
<td>0.800 ± 0.029</td>
<td>0.569 ± 0.060</td>
<td>0.320 ± 0.010</td>
<td>0.178 ± 0.038</td>
<td>0.091</td>
<td>0.229</td>
<td>0.680</td>
<td>1.165</td>
</tr>
</tbody>
</table>

The retrieved lifetimes are presented with three standard deviations as error.

To reveal more information on the states of Cy5 in the nanobeacons, the fluorescence decays were reanalyzed using the maximum entropy method (MEM) (Pulse 5 software, MacEnt Ltd, Cambridge, UK). This generates a lifetime distribution without any a priori assumptions about the decay function. The lifetime distribution method (MEM) (Pulse 5 software, MacEnt Ltd, Cambridge, UK). The recovered lifetime distributions are depicted in Figs. 2 (b), (d) and (f). Clearly, four well-separated lifetime distributions of Cy5 are found in all three nanobeacons in the absence of target strands. Upon hybridization, both of the peaks in the intermediate time range are shifted to longer lifetimes, which becomes more obvious as the spacer and the fully-extended length of oligonucleotide increase. However, no significant shifts are observed for the shortest and longest lifetime distributions. This again suggests that not all hairpins on AuNPs are hybridized and that a small amount of unfolded hpDNAs are presented in the as-prepared nanobeacons, in line with the multiexponential analysis. As noted, there is a continuous lifetime distribution consisting of two broad peaks in the long time domain in the hybridized nanobeacons. The relatively broad lifetime distributions indicate that the Cy5 molecules in the open hpDNAs are likely in a range of distances from the surface of AuNPs due to the semi-flexibility of the unpaired nucleotides at the 3’ and 5’ ends.

Considering the biexponential decay of free Cy5-hpDNAs and two major states (i.e., closed or open) of hpDNAs on AuNPs, it is most likely that the two lifetime components in the long time domain and the two lifetime components in the long time domain are corresponding to the closed and open Cy5-hpDNAs, respectively. Accordingly, the energy transfer efficiencies in the Cy5-AuNP systems can be determined using eqn (5):18

$$E = 1 - \frac{\tau_{DA}}{\tau_{D}}. \quad (5)$$

where $\tau_{DA}$ and $\tau_{D}$ represent the amplitude-weighted lifetimes of Cy5 in the presence and absence of AuNPs, respectively. The calculated quantum efficiencies of energy transfer are plotted in Fig. 3 (a) as a function of Cy5-AuNP distance, considering the closed Cy5-hpDNAs in the as-prepared nanobeacons and the open Cy5-hpDNAs in the hybridized nanobeacons. The Cy5-AuNP separations are estimated by taking 43 Å per base for single-stranded DNA (ssDNA), 3.4 Å per basepair for double-stranded DNA (dsDNA), 1.5 Å for the C-C bond in the methylene linker and 2.3 Å for the Au-S bond.25-27 In addition, the ssDNA spacer in the “as-prepared” nanobeacons are assumed to be fully stretched where the extension of ssDNA is the contour length, while the ssDNA fragments in the hybridized hairpins are considered as semi-flexible chains whose length is approximately twice the radius of gyration.25,28 The radius of gyration is given as the square root of one-third of the product of contour length and persistence length (10.5 Å for ssDNA in PBS).26

![Fig. 3.](image-url) (a) Energy transfer efficiency plotted versus Cy5-AuNP separations obtained from the MEM analysis and two theoretical models: an NSET model (n = 4, d0 = 62 Å) and an electrodynamics model (n = 36, d0 = 102 Å). The horizontal error bar represent the flexibility of the 5-nt overhang in the closed-state hpCy5f. Inserted is the formula of distance-dependent energy transfer efficiency. (b) Measured ACFs for AuNPs and Au-nanobeacons. Solid lines show theoretical ACFs for monodisperse populations of particles with the diameter equal to the mean hydrodynamic diameter estimated from the initial decay.

Also plotted in Fig. 3 (a) are the theoretically distance-dependent energy transfer efficiencies from Cy5 to AuNPs derived from a nanometal surface energy transfer (NSET) model29 and an electrodynamics modeling fitting result of 10-nm AuNPs reported by Chhabra et al.30 respectively. In the NSET model, the characteristic distance length $d_0$ at which the energy transfer efficiency is 50% is calculated to be 62 Å, using a quantum yield of 0.235 and an angular frequency of $2.83 \times 10^{15}$ s$^{-1}$ for Cy5. It can be seen that the NSET model greatly underestimates the quantum efficiency of energy transfer between Cy5 and the 13-nm AuNPs, while the electrodynamics model shows a better agreement with the experiment data.

To validate these results based on beacon-intrinsic parameters with an independent measurement, we used dynamic light scattering (DLS) to measure the hydrodynamic radii of AuNPs and Au-nanobeacons. As depicted in Fig. 3 (b), the shift in the measured autocorrelation functions (ACFs) shows a clear increase in the mean hydrodynamic diameter from the AuNPs to the folded AuNP-AAhpCy5 and then, in the presence of cDNA, the nanobeacons extent further increasing the mean hydrodynamic diameter. A similar behaviour was found in AuNP-hpCy5f. The DLS results are consistent with the fluorescence
spectroscopic measurement and confirmed the mechanic function of the nanobeacons.

Furthermore, TCSPC measurements were conducted to simultaneously reveal the real-time changes of both fluorescence intensity and lifetime of Cy5 in all three nanobeacons during the hybridization processes. Sequential fluorescence decays were acquired with a data-collection time of 60 s. The data was fitted to the sum of three exponentials in a batch mode, recovering time-evolution fluorescence intensity and amplitude-weighted lifetime (Fig. 4). The relative background intensity ratios are consistent with the steady-state fluorescence measurements (Fig. 1 (b)). Significantly, all three nanobeacons responded to the addition of cDNA immediately, showing an increasing fluorescence intensity and amplitude-weighted lifetime. This again confirms the conformational switching of the nanobeacons and consistent with previous findings on molecular beacons.

In summary, we have demonstrated that time-resolved fluorescence spectroscopy is a versatile tool to investigate conformational switching and energy transfer processes of Au-nanobeacons. The long-range fluorescence quenching of Cy5 by AuNPs has been revealed, showing good agreement with electrodynamics modelling, TCSPC has been shown to be promising for real-time monitoring of hybridization events using Au-nanobeacons.

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