Competitive Reaction Pathway for Site-Selective Conjugation of Raman Dyes to Hotspots on Gold Nanorods for Greatly Enhanced SERS Performance

Hao Huang, Jia-Hong Wang, Weihong Jin, Penghui Li, Ming Chen, Han-Han Xie, Xue-Feng Yu,* Huaiyu Wang, Zhigao Dai, Xiangheng Xiao, and Paul K. Chu*

1. Introduction

Surface-enhanced Raman scattering (SERS), a viable alternative to fluorescence in ultrasensitive optical biodetection and bioimaging due to merits such as large multiplexing ability, high spatial resolution, high signal-to-noise ratio, and non-photobleaching,[1–10] takes advantage of the dramatically enhanced Raman scattering yield from molecules adsorbed on a nanostructured metal surface[11] caused by the enhanced electromagnetic (EM) field around the noble metal nanostructure.[12] Extensive research has been performed to produce various types of noble metal nanostructures with strong EM field to boost the Raman signals.[13–20] In particular, gold nanorods (GNRs) with a strong longitudinal surface plasmon resonance (LSPR) band in the near-infrared (NIR) region are promising NIR SERS substrates in a number of biosensing and bioimaging applications.[13,18,21–27]

Anisotropic metal nanostructures with protrusions, for instance, those with a rod-like morphology, exhibit larger SERS enhancement than spherical metal nanostructures.[13,20,28] The big SERS enhancement observed from anisotropic nanostructures is attributed to the strong EM field concentrated in very small regions with high curvatures known as hotspots.[29,30] The surface EM field near the ends of GNRs is much stronger than that on the sides and it is generally called the lightning rod effect.[13,31] Consequently, site-selective adsorption of target molecules onto hotspots can further enhance the SERS
signals[32–34]. Current methods to prepare SERS probes generally rely on random conjugation of Raman dyes onto metal nanostructures but unfortunately, most of the Raman dyes are not located at the hotspots and contribute little to the SERS signals. In fact, the difficulty to specifically position Raman dyes at hotspots on metal nanostructures limits the enhancement and hinders SERS-based applications. Herein, we describe site-selective conjugation of Raman dyes onto the hotspots on GNRs through a synergistic reaction involving gold overgrowth and dye conjugation. These bright dye-GNR conjugates boast SERS enhancement factors that are dozens of times bigger than those from conjugates synthesized by conventional techniques.

2. Results and Discussion

The original GNRs are fabricated by a seed-mediated method in a cetyltrimethylammonium bromide (CTAB) solution as reported previously.[35–39] The bilayer of the CTAB surrounding the nanorod is responsible for the rod shape and colloidal stability. A gold overgrowth reaction was established to induce transverse overgrowth of the GNRs (Figure 1). When the stock solution of the GNRs reacts with the growth solution containing HAuCl₄, L-ascorbic acid (AA), CTAB, and NaOH, the GNRs become wider and their lengths stay unchanged, and their LSPR band wavelength blue shifted. These results indicate a transverse overgrowth in which the gold layer grows on the side surface of the GNRs. It is known that the direction of gold overgrowth on the GNRs is influenced by many factors such as the amounts of chemicals, pH, and so forth.[40–42] In our experiments, the relative amounts of the reagents in the solution are carefully regulated to achieve transverse overgrowth. Although the gold layer is not uniform, it can be observed that the reaction mainly takes place on the rod side surface. In fact, uniform overgrowth of the gold layer on the side surface of the GNRs is extremely difficult due to the nonuniform surface chemistry of the GNRs.[42]

Based on above gold overgrowth reaction, a new strategy for synthesizing dye-GNR conjugates was established (Figure 2). Two typical and popular NIR Raman dyes, 3,3′-diethylthiadicarbocyanine iodide (DTDCI) and 3,3′-diethylthiatricarbocyanine iodide (DTTCI),[1,26,43–47] were employed to study the efficacy in our study (refer to Figure S1, Supporting Information, for their molecular formula, absorption spectra). In a conventional conjugation reaction to prepare dye-GNR conjugates,[1,26,43–47] the nanorod stock solutions react directly with the Raman dyes (DTDCI or DTTCI) and the dyes are sequestered into the CTAB bilayer along the nanorod surface forming a layer composed of CTAB and dye.[1,26,43–47] Herein, in order to control the conjugation site on the Raman dyes, a competitive reaction between the conventional conjugation reaction and aforementioned overgrowth reaction is established in which the GNRs react with the Raman dyes (DTDCI or DTTCI) and the dyes are sequestered into the CTAB bilayer along the nanorod surface forming a layer composed of CTAB and dye.[1,26,43–47] Herein, in order to control the conjugation site on the Raman dyes, a competitive reaction between the conventional conjugation reaction and aforementioned overgrowth reaction is established in which the GNRs react with the Raman dyes and solution simultaneously. The solution also contains HAuCl₄, L-ascorbic acid (AA), CTAB, and NaOH, same as those used in the gold overgrowth reaction. To keep the notations simple, the dye-GNR conjugates synthesized by the conjugation reaction and competitive reaction are designated as dye/GNRs and dye@GNRs, respectively.

Figure 3 illustrates the effects of the gold overgrowth reaction on the dye (DTDCI) conjugation site by comparing the corresponding products of DTDCI/GNRs and DTDCI@GNRs. Compared to the DTDCI/GNRs with the almost unchanged morphology and diameter (about 16.5 ± 2 nm) of the original GNRs, obvious transverse gold overgrowth can be observed from the DTDCI@GNRs after the competitive reaction, resulting in the larger diameter (about 18.6 ± 3 nm) and almost unaltered length of the GNRs (Figures 3a and 3b). The corresponding high-resolution TEM (HR-TEM) image of the DTDCI@GNRs is exhibited in Figure S2 (Supporting Information). The variations of the NR morphology and diameter in the competitive reaction are similar to that in the overgrowth reaction, suggesting that transverse overgrowth
is more competitive than dye conjugation to the side surface of the GNRs. As shown in Figures 3c and 3d, the average hydrodynamic diameter of the DTDCI@GNRs is 66.5 nm, which is just a little bigger than that of the DTDCI/GNRs (65.0 nm). The results agree with the increased rod diameter in the competitive reaction and demonstrate that the competitive reaction does not lead to aggregation of the GNRs.

The absorption spectra of DTDCI/GNRs and DTDCI@GNRs are shown in Figure 3e. Compared to the original GNRs, the conjugation reaction results in a slightly blue-shifted LSPR peak wavelength of the DTDCI/GNRs due to insertion of the dyes into the CTAB bilayer. In contrast, the competitive reaction results in a large blue-shifted LSPR peak wavelength (≈43 nm) of the DTDCI@GNRs, which confirm the transverse overgrowth of the GNRs in the competitive reaction.

The zeta potential is measured to determine the surface charge variations of the products in the conjugation and competitive reactions. As shown in Figure 3f, the zeta potential of the original CTAB-coated GNRs is 37.4 mV, while that of the DTDCI/GNRs decreases to 25.4 mV due to the coating of the DTDCI molecules onto the whole surface of the GNRs. In contrast, after the competitive reaction, the zeta potential of the DTDCI@GNRs only decreases to 32.9 mV, which is closer to that of the original CTAB-coated GNRs. It demonstrates that the competitive reaction does not alter the surface chemistry of the GNRs significantly, which is probably due to the prevention of the coating of DTDCI on the side surface of the GNRs.

To further compare the loaded DTDCI amount onto the GNRs in the conjugation reaction and competitive reaction, the residual DTDCI amount in the supernatants of these two reactions were examined by testing the adsorption intensity of DTDCI in an aqueous solution using a 785 nm NIR laser as the excitation source (Figure 4). The peaks at 783, 847, 1131, 1246, 1295, 1340, 1465, and 1580 cm⁻¹ in the SERS spectra agree well with the DTDCI SERS peaks reported previously.26,47,48 It is noted that the Raman signals from DTDCI@GNRs are about 50 times stronger than those from the DTDCI/GNRs. Considering that the same amount (1.67 µm) of Raman dyes is used in these two reactions and only some of the Raman dyes are actually conjugated to the GNRs in the competitive reaction, the actual degree of SERS enhancement is more impressive than that illustrated in Figure 4. These results demonstrate that the competitive reaction is an efficient method to synthesize the conjugated dye-GNRs showing strong SERS intensity.

The mechanism of Raman enhancement induced by the competitive reaction is presented. It is well known that the original GNRs are covered by at least a bilayer of CTAB49 and are pentatetrahedral twins with the {111} faces of gold at the ends and [100] faces along the length of the rods. The CTAB preferentially binds to the [100] faces along the length of the rods compared to the end [111] Au faces due to the...
size of the CTAB head group.\cite{33,49–51} In the conjugation reaction, the hydrophobic molecules of DTDCI or DTTCI are generally sequestered into the CTAB bilayer and bind to the GNRs by the Au–S or Au–N interactions with high efficiency.\cite{26,43–47} Hence, most of them are generally conjugated to the side surface of the GNRs in the simple conjugation reaction. Since the Raman signal from DTDCI is very weak under the 785 nm NIR laser excitation, the observed Raman signal is mainly due to the SERS enhancement from the GNRs with a strong surface EM field. The surface EM field near the ends of the GNRs is much stronger than that on the sides under NIR light excitation\cite{18,52} and therefore, most of the Raman dyes conjugated to the GNR side surface through the simple conjugation reaction contribute little to the SERS signal. In the competitive reaction, the transverse gold overgrowth is more competitive than dye conjugation to the GNR side surface, thus precluding the Raman dyes from conjugating to the side surface (Figure 3c,f). Therefore, the Raman dyes are preferentially coated onto the ends of the GNRs, which support the strongest EM field inducing the dramatic SERS enhancement. These results suggest a site-selective conjugation mechanism to produce the obtaining dye-GNR conjugates with highly efficient SERS enhancement.

The applicability of the competitive reaction for site-selective adsorption of Raman dyes is further assessed using another Raman dye: DTTCI (see Figure 5). The peaks at 633, 783, 847, 1017, 1131, 1240, 1296, 1407, 1503, and 1580 cm\(^{-1}\) in the SERS spectra agree well the DTTCI SERS peaks reported previously.\cite{26,47,53} Similar to the DTDCI@GNRs, the DTTCI@GNRs synthesized through the competitive reaction with 1.67 \(\mu\)M DTTCI show an overgrown gold layer on the side surface (Figure 5a,b), and their SERS intensity is dozens of times larger than the DTTCI/GNRs synthesized through the simple conjugation reaction (Figure 5d), illustrating that the competitive reaction scheme can be extended to other Raman dyes. It is known that the Raman spectra of DTDCI and DTTCI in solutions can hardly be acquired under 785 nm NIR light excitation and so the enhancement factor cannot be estimated in these dye-GNRs conjugates as reported in the literatures.\cite{43,46,47,53}

The influence of the amount of the Raman dyes on the SERS enhancement is further investigated (Figures 5c–e). With increasing dye amounts, the SERS intensity from the DTTCI/GNRs increases, demonstrating that the dye amount (1.67 \(\mu\)m) is not excessive in coating the whole GNR surface. Furthermore, the better SERS enhancement effect can be obtained by decreasing the dye amount in the dye@GNRs compared to dye/GNRs. Typically, when the dye amount is reduced to 0.33 \(\mu\)m which is 1/5 of the above value, the competitive reaction can produce over two orders of magnitude enhancement compared to the simple conjugation reaction. When the dye amount is reduced from 1.67 to 0.33 \(\mu\)m which is much less for the whole GNR surface, a larger proportion of them can coat on the GNR end surface by the competitive reaction, while only the same proportion of them can coat on the GNR end surface by the simple conjugation reaction. Therefore, comparing to that of the dye/GNRs, the relative SERS enhancement of dye@GNRs is even better with the decrease of the dye amount. The results suggest that this competitive reaction strategy for SERS enhancement is particularly suitable for single molecule detection.

Since the SERS enhancement may be due to many reasons, other possible mechanisms are analyzed. Firstly, aggregation of GNRs can generally induce significant SERS enhancement of the Raman dyes. However, in our experiments, both the TEM images (Figures 3a,b) and hydrodynamic diameter measurements (Figures 3c,d) reveal that the dye/GNRs and dye@GNRs have similar dispersability in the aqueous solution, demonstrating that GNR aggregation is not the reason for the SERS enhancement. Secondly, the altered morphology of the GNRs with enhanced EM field can generally yield better SERS enhancement. However, in our experiments, no obvious increase in the EM intensity can be observed under 785 nm light excitation after the competitive reaction (Figure S3, Supporting Information). Thirdly, it is known that Raman dyes can be embedded into the gold cores and overgrown metal layers to improve SERS.\cite{16,54} However, in our experiments, there is no gap between the nanorod and overgrown gold layer can be observed in the dye@GNRs (Figure 3b and Supporting Information Figure S2), suggesting that embedding of Raman dyes does not occur here. Furthermore, if the overgrowth reaction is carried out after the conjugation reaction, the SERS intensity from the dye-GNR conjugates decreases (Figure S4, Supporting Information). Indeed, only when the gold overgrowth and conjugation reaction take place simultaneously can such dramatic SERS enhancement be observed thereby confirming the competitive reaction mechanism.

To further fathom the competitive reaction mechanism responsible for the SERS enhancement, control experiments are performed and the possible influence of the chemicals (HAuCl\(_4\), AA, CTAB, and NaOH) in the growth reaction is investigated. Since CTAB influences the surface chemistry of the GNRs, it is taken into first consideration. As shown in Figure 6a, if the nanorod stock solution reacts with the Raman dyes and the CTAB at the same concentration of 0.005 m in the solution, no obvious SERS enhancement can
be observed. It demonstrates that the addition of CTAB is not the reason for the SERS enhancement in the competitive reaction and the results are similar for the other three chemicals. However, CTAB can preferentially bind to the side surface in lieu of the end surface on account of the size of the CTAB head group\cite{33,49,50} thus suggesting possible influence on the conjugation site of the Raman dyes. Based on this consideration, another competitive reaction between the dye conjugation and CTAB coating (with greatly increased amount) to the GNRs is conducted. As shown in the inset TEM image in Figure 6b, after the GNR stock solution reacts simultaneously with the Raman dyes at a higher CTAB concentration of 0.13 M, a dense bilayer of CTAB can be observed from the nanorod side surface by TEM using phosphotungstic acid hydrate as a staining agent, whereas it is increasingly sparse at the ends of the GNRs (see Supporting Information Figure S5 for the comparison of the GNRs before and after staining). The CTAB coating is preferred over dye conjugation to the nanorod side surface, thus precluding the Raman dyes from conjugating to the side surface except the ends of the GNRs. As shown in Figure 6b, the dye-GNR conjugates synthesized by the competitive reaction produce Raman signals that are several times more intense than those from common conjugates. The results confirm the proposed competitive reaction pathway for the better SERS enhancement and suggest that the pathway can be extended to other systems simply by adopting different competitive reactions against dye conjugation.

Furthermore, an opposite competitive reaction is established to further illuminate the mechanism for the SERS enhancement (Figure 7). When the GNRs react with the Raman dyes and solution containing HAuCl4, NaOH, and CTAB with the same amount of the growth solution (just without AA), an interesting phenomenon can be observed. As shown in Figure 7b, the nanorod length diminishes obviously but the diameter does not change, implying that an end etching reaction takes place. Such etching reaction is due to the presence of CTAB and Au3+ ions. Under certain reaction conditions, the redox reaction between Au0 and Au3+ ions occurs in the presence of CTAB micelles and meanwhile, the CTAB micelles approach the nanoparticles preferentially at the tips leading to spatially directed oxidation.\cite{55} It should be emphasized that the products result in even lower SERS intensity compared with the conjugates by commonly conjugation reaction (Figure 7d). These results provide an opposite competitive reaction between end etching and dye conjugation, in which etching can prevent the Raman molecules from conjugating to the end surface except the nanorod side, where only small SERS enhancement is expected.
3. Conclusion

A simple but powerful simple strategy to accomplish site-selective conjugation of Raman dyes to SERS-active hotspots on GNRs based on the competitive reaction between gold overgrowth and dye conjugation is described. The dye-GNR conjugates exhibit SERS enhancement that is dozens of times larger than that observed from conventional materials, in addition to almost unaltered surface properties of the GNRs as well as very high stability in aqueous solutions (Figure S6, Supporting Information). In comparison with common SERS enhancement strategies such as controlled assembly of GNRs, these SERS probes are mono-dispersed and the SPR properties are only slightly modified. Therefore, they are more suitable for biological applications because the multi-functionality of the GNRs is preserved. For example, the dye-GNR conjugates not only can be used as the probes for Raman imaging of cells, but also exhibit the good photothermal ability that is almost the same as that of the original GNRs (Figure S7–S9, Supporting Information). In addition, this strategy can be extended to other metal nanostructures with Raman-intense EM hotspots and bodes well for in-depth SERS-based biological imaging and single molecule detection applications.

4. Experimental Section

Materials: Chloroauric acid (HAuCl₄·4H₂O, 99.99%), silver nitrate (AgNO₃, 99.8%), L-ascorbic acid (AA, 99.7%), sodium hydroxide (NaOH), hydrochloric acid (HCl, 36–38%), and phosphotungstic acid hydrate (H₃O₄PW₁₂·xH₂O) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Sodium borohydride (NaBH₄, 96%) was supplied by Aldrich (America) and CTAB (99.0%) by Amresco Inc. (America). DTCI (99%) was obtained from Acros Organics (America) and DTDCI (99%) was purchased from Alfa Aesar (America). All the chemicals were used as received without purification.

Synthesis of GNRs: The original GNRs were synthesized by the seed-mediated method in CTAB solutions as reported previously. In brief, the 3–4 nm gold seed particles were prepared by mixing 5 mL of 0.5 mM HAuCl₄ with 5 mL of 0.2 M CTAB. The solution was stirred vigorously followed by dropwise addition of 600 µL of freshly prepared ice-cold 10 mM of NaBH₄. The seed solution was left for more than 2 h before use. In the GNR synthesis, 18 mL of 5 mM HAuCl₄ and 180 µL of 0.1 M AgNO₃ were added to 90 mL of 0.2 M CTAB and then 180 µL of 1.2 M HCl and 10.5 mL of 10 mM AA were added and gently swirled as the color changed from dark orange to colorless. After the color had changed, 150 µL of the CTAB-stabilized gold seed solution was rapidly injected. The resulting solution was gently mixed for 10 s and left undisturbed overnight. Finally, the GNR solution was centrifuged at 10 000 rpm for 10 min to stop the reaction. The supernatant was removed and precipitate was resuspended in ultrapure water. The GNR concentration was estimated to be about 0.65 nm according to extinction coefficient at the LSPR wavelength.
Gold Overgrowth Reaction: The growth solution was prepared by mixing 6 mL of 0.2 M CTAB, 1.2 mL of 5 mM HAuCl₄, 30 µL of 1 M NaOH, and 1 mL of 10 mM AA. In the overgrowth reaction, an appropriate amount of the solution was added to 1 mL of GNR solution with a certain volume of ultrapure water. The final volume of the solution was 3 mL (for example, 300 µL of growth solution was added to 2.7 mL solution containing 1 mL of the GNR solution and 1.7 mL of ultrapure water). The solution was kept at 30 °C undisturbed for 15 h. Afterwards, the solution was centrifuged at 10 000 rpm for 10 min and resuspended in ultrapure water in the same volume.

Dye Conjugation Reaction: For obtaining dye@GNRs, the growth solution was prepared according to the method described above. Afterwards, 80 µL of the solution was added to 2.92 mL of the solution containing 1 mL GNRs and 1.92 mL ultrapure water, followed by the addition of 5 µL of 1 mM Raman dyes (DTTCI or DTDCI) immediately. The resulting solution was kept at 30 °C undisturbed for 15 h, centrifuged at 10,000 rpm for 10 min, and resuspended in ultrapure water in the same volume.

Competitive Reaction between Gold Overgrowth and Dye Conjugation: For obtaining dye@GNRs, the growth solution was prepared according to the method described above. Afterwards, 80 µL of the solution was added to 2.92 mL of the solution containing 1 mL GNRs and 1.92 mL ultrapure water, followed by the addition of 5 µL of 1 mM Raman dyes (DTTCI or DTDCI) immediately. The resulting solution was kept at 30 °C undisturbed for 15 h, centrifuged at 10,000 rpm for 10 min, and resuspended in ultrapure water in the same volume.

Characterization: The TEM images were acquired on a JEOL 2010 HT transmission electron microscope and the HR-TEM images were obtained on a JEOL-JEM-2100F field-emission transmission electron microscope. The absorption spectra were recorded by UV–Vis–NIR spectrophotometry (Cary 5000, Varian). Raman scattering was performed on a Jobin Yvon LabRAM HR800 micro-Raman spectrometer equipped with a 100 mW, 785 nm laser at room temperature. 300 µL of an aqueous sample in 96-well plates were detected. The exposure time was 10 s and the Raman signals were collected by a 10× objective lens.[44] The zeta potential and hydrodynamic distribution of the samples was determined using a Zeta sizer (Nano ZS90, Malvern Instruments, UK) at 25 °C. The samples were dispersed in deionized water with concentration of 0.23 mM.

Coating with a Polyoxometalate Contrast Agent for TEM Imaging: In order to localize the low contrast CTAB molecules on the GNRs, phosphotungstic acid hydrate was used as a staining agent. Since phosphotungstic acid hydrate is electron-dense, they are commonly used as a negative stain in imaging of biological samples by TEM. Here, it could electrostatically bind to the positively charged head of the outer CTAB layer around the GNRs. The staining process is as follows. 10 µL of the purified dye-GNR solution was dropped onto one micro-grid which was left standing overnight. Prior to TEM observation, 10 µL of a 3.0 wt% phosphotungstic acid hydrate solution (pH adjusted to 7 by 1 M NaOH) was dropped onto the micro-grid.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Supporting Information

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Figure S1. Molecular formula and absorption spectra: DTDCI and DTTCI.
**Figure S2.** HR-TEM image of one typical overgrown gold nanorod (dye@GNR) after the competitive reaction.
Figure S3. FDTD simulation of electric field distribution of the (a) normal GNR and (b) dye@GNR synthesized by the competitive reaction for central cross section at a wavelength of 785 nm. The FDTD simulation is performed using FDTD Solutions 8.5 developed by Lumerical Solutions, Inc. The dielectric constants of gold are taken from the Handbook of optical constants of solids of Palik.[1] In the calculation, the mesh around the Au nanostructure is 1 nm * 1 nm * 1 nm. Because the gold nanorods are dispersed in aqueous solutions, the refractive index of the medium is taken to be 1.333.
Figure S4. (a, b) TEM images and (c) SERS spectra of the dye-GNR conjugates synthesized by the conjugation reaction without and with the following overgrowth reaction.
Figure S5. TEM images of GNRs before and after using phosphotungstic acid hydrate as a staining agent.
Figure S6. SERS spectra of the dye@GNRs before and after deposition in the aqueous solution for 3 months at room temperature (25 °C), revealing that the Raman signals are essentially unchanged and good stability in the aqueous solution.
Figure S7. (a, d) Bright field images, (b, e) SERS spectra, and (c, f) Fast SERS mappings of PC-9 cells incubated with pure buffer solution (control, up) and dye@GNRs (down). The scale bar is 4 μm. Bright field optical imaging of the fixed cells is performed to locate the cells for subsequent confocal Raman imaging of the cells targeted with the dye@GNRs using a 785 nm laser as the excitation source. The intensity map of the 783 cm⁻¹ peak of DTTCI reveals the distribution of the dye@GNRs on the cell surface enabling delineation of the cell shape. The cell without the probe shows no Raman signal. Representative spectra from various spots across the cell show typical Raman signals from the dye@GNRs, indicating that the dye-GNR conjugates are efficient probes in in vitro SERS mapping and yield large signal-to-noise ratios. Cell SERS detection and mapping: PC-9 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum and incubated at 37 °C and 5% CO₂. The PC-9 cells were seeded on 24-well plates at a density of 2 × 10⁴ per well and grown for 16 h at 37 °C in a 5% CO₂ incubator in the cell uptake experiments. The fresh prepared dye-GNR conjugates (dye@GNRs) were washed twice by DI water and resuspended in DMEM. The culture medium was replaced with 500 μL of the fresh medium and dye-GNR conjugates (230 pM) to incubate for 2 h. The cells were rinsed with PBS and fixed with paraformaldehyde. In the cell mapping, The Raman band at 783 cm⁻¹ was chosen as the standard band to construct the maps. The map size was 50 μm × 50 μm, exposure time was 1 s, and objective lens was 50 X.
Figure S8. MTT assay showing the cytotoxicity of dye-GNR conjugates. The cytotoxicity of dye-GNR conjugates on Cal-27 cells is assessed by the MTT assay. Dye-GNR conjugates are washed twice by DI water before diluting with DMEM containing 10% FBS to achieve a series of equivalent concentrations of 0, 0.03, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.55 nM, respectively. The Cal-27 cells are distributed on 96-well plates at $1.5 \times 10^4$ cells per well for 24 h to attach overnight. For the dye-GNR conjugates treated group, the culture medium is replaced with different concentrations of dye-GNR conjugates solution as described above and incubated at 37 °C for 4 h. For the untreated control group, the fresh culture medium without dye-GNR conjugates is added to the wells. After 4 h, the above different medium is removed and the cells are rinsed twice with PBS buffer three times. Then the wells are refilled with the complete medium and the cells are incubated for another 24 h. Subsequently, the cell viability is measured by the MTT assay as described previously. The cell viability is calculated by the following formula: $(OD_{treated}/OD_{control}) \times 100\%$ and three independent experiments are performed.
Figure S9. Temperature increase observed from water, orginal GNRs, and dye-GNR conjugates under 810 nm light irradiation.
