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Supporting information

Constraining conformation of peptides with Au nanorods to construct multifunctional therapeutic agent with targeting, imaging, and photothermal ability

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MATERIALS AND METHODS

Materials

All chemical reagents (AR grade) for synthesis were used as received without further purification. $HAuCl_4 \cdot 3H_2O$ (≥ 49.0 % Au basis), sodium borohydride ($NaBH_4$) (≥ 98 %) were purchased from Sigma-Aldrich (USA). Cetyltrimethyl ammonium bromide (CTAB) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methoxy–PEG–thiol (mPEG–SH) (MW = 1000 Da) was purchased from Ponsure Biotechnology (Shanghai, China). LA-RGDK (MW = 662.83 Da, ≥ 95 %), *c*(CRGDC) (MW = 550.62 Da, ≥ 95 %) and LA-RGDK-LA (MW = 851.18 Da, ≥ 95 %) were purchased from GL Biochem Ltd. (Shanghai, China). Silver nitrate ($AgNO_3$) was purchased from Tianjin Day Feeling Chemical Technology Development Co., Ltd. (Tianjin, China). Ascorbic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The cell HepG2 were purchased from Keygen Biotec, and the cells MCF-7, MCF-7/ADR, and A549 were subcultured in our lab.

Methods

Synthesis of Au nanorod (AuNR) [1] and AuNR-PEG

Briefly, the seed solution was prepared by mixing $HAuCl_4$ (5 mL, 0.5 mM) with CTAB solution (5 mL, 0.2 M) and a freshly prepared ice-cold $NaBH_4$ solution (0.6 mL, 0.01 M). The seed solution was kept at room temperature for 2 h. The growth solution was prepared by adding $HAuCl_4$ (5 mL, 1mM), $AgNO_3$ (0.2 mL, 0.004 M), ascorbic acid (0.07 mL, 0.0788 M) into CTAB solution (5 mL, 0.2 M). After the resultant solution was mixed by stirring for 30 s, the seed solution (0.012 mL) was added rapidly. The reaction solution was mixed by gentle inversion for 2 mins and then kept at 30 °C overnight. Finally, AuNR was centrifuged at 10000 rpm for 15 mins and re-dispersed in water.

The AuNR solution (1 mL, 10 nM) obtained above was added to methoxy–PEG–thiol (mPEG–SH) (MW = 1000 Da, 250 µL, 0.4 mM) and stirred at room temperature for 24 h. Finally, AuNR-PEG was centrifuged at 8000 rpm for 15 mins and re-dispersed in

water.

Synthesis of AuNR-LA-RGDK

Synthesis of AuNR-LA-RGDK (5.0×10^3 molecules/per AuNR particle)

LA-RGDK (0.3314 g, 0.5 mmol) was dissolved in 2 mL water, equal molar amount of $NaBH_4$ (0.0189 g, 0.5 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface ligand exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the reduced LA-RGDK solution (125 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-LA-RGDK was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No LA-RGDK was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides LA-RGDK on each gold nanorod was assessed to 5000. [2]

Synthesis of AuNR-LA-RGDK (2.0×10^4 molecules/per AuNR particle)

LA-RGDK (0.3314 g, 0.5 mmol) was dissolved in 2 mL water, equal molar amount of $NaBH_4$ (0.0189 g, 0.5 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface ligand exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the reduced LA-RGDK solution (500 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-LA-RGDK was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No LA-RGDK was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides LA-RGDK on each gold nanorod was assessed to 20000. [2]

Synthesis of AuNR-*c*(CRGDC)

Synthesis of AuNR-*c*(CRGDC) (5.0×10^3 molecules/per AuNR particle)

c(CRGDC) (0.2753 g, 0.5 mmol) was dissolved in 2 mL water, equal molar amount of $NaBH_4$ (0.0189 g, 0.5 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface ligand exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the

reduced *c*(CRGDC) solution (125 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-*c*(CRGDC) was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No CRGDC was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides CRGDC on each gold nanorod was assessed to 5000. [2]

Synthesis of AuNR-*c*(CRGDC) (2.0×10^4 molecules/per AuNR particle)

c(CRGDC) (0.2753 g, 0.5 mmol) was dissolved in 2 mL water, equal molar amount of $NaBH_4$ (0.0189 g, 0.5 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface ligand exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the reduced *c*(CRGDC) solution (500 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-*c*(CRGDC) was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No CRGDC was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides CRGDC on each gold nanorod was assessed to 20000. [2]

Synthesis of AuNR-*c*(LA-RGDK-LA)

Synthesis of AuNR-*c*(LA-RGDK-LA) (5.0×10^3 molecules/per AuNR particle)

LA-RGDK-LA (0.4256 g, 0.5 mmol) was dissolved in 2 mL water, two times molar amount of $NaBH_4$ (0.0378 g, 1 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface ligand exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the reduced LA-RGDK-LA solution (125 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-*c*(LA-RGDK-LA) was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No LA-RGDK-LA was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides LA-RGDK-LA on each gold nanorod was assessed to 5000. [2]

Synthesis of AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR particle)

LA-RGDK-LA (0.4256 g, 0.5 mmol) was dissolved in 2 mL water, two times molar amount of $NaBH_4$ (0.0378 g, 1 mmol) was added to the solution and stirred at room temperature for 30 mins. The resultant mixture was directly used for surface exchange. AuNR-PEG solution (1 mL, 5 nM) obtained above was added to the reduced LA-RGDK-LA solution (500 μL, 0.2 mM) and stirred at room temperature for 24 h. Finally, AuNR-*c*(LA-RGDK-LA) was centrifuged at 8000 rpm for 15 mins and re-dispersed in water. No LA-RGDK-LA was detected in centrifugal supernatant with MS spectrometer, therefore the number of peptides LA-RGDK-LA on each gold nanorod was assessed to 20000. [2]

Cancerous cells uptake of AuNRs

HepG2 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in DMEM medium (containing 10 % FBS and 1 % antibiotics). A549, MCF-7/ADR and MCF-7 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in RPMI 1640 medium (containing 10 % FBS and 1 % antibiotics). Then the growth medium was removed, and 3 mL/well of AuNR-LA-RGDK, AuNR-*c*(CRGDC) and AuNR-*c*(LA-RGDK-LA) (5.0×10^3 or 2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL or 1.32 nmol/mL) were replaced on the dishes. After incubated at 37 °C, in 5 % CO_2 for 0.5 h or 3 h, the cells were washed with PBS solution for three times, and cells uptake behavior was acquired by using two-photon fluorescence microscope (OLYMPUS FVMPE-RS) (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm). The mean fluorescence intensity was acquired with ImageJ.

Competitive cell binding assay

A549 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in RPMI 1640 medium (containing 10 % FBS and 1 % antibiotics). Then the growth medium was removed, and 3 mL/well of 5 μg/mL *c*(RGDyK) was replaced on the dishes. After incubated at 37 °C, in 5 % CO_2 for 1 h, 3 mL/well of AuNR-LA-RGDK,

AuNR-*c*(CRGDC) and AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) were replaced on the dishes. After incubated at 37 °C, in 5 % CO_2 for 0.5 h, the cells were washed with PBS solution for three times, and cells uptake behavior was acquired by using two-photon fluorescence microscope (OLYMPUS FVMPE-RS) (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm). The mean fluorescence intensity was acquired with ImageJ.

Temperature increases in cells after irradiation with the laser 808 nm

HepG2 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in DMEM medium (containing 10 % FBS and 1 % antibiotics). A549 and MCF-7 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in RPMI 1640 medium (containing 10 % FBS and 1 % antibiotics). Then the growth medium was removed and 3 mL/well of AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 125 nmol/mL, peptide concentration is 3.3 nmol/mL) was replaced on the dishes to incubate at 37 °C, in 5 % CO_2 for 3 h, the cells were washed with PBS solution for three times. Then the cells were exposed to an 808 nm laser at a power of 2 W/cm^2, respectively. Temperature variations were recorded with infrared camera (FOTRIC 225-3).

In vitro phototoxicity

The phototoxicity of AuNR-*c*(LA-RGDK-LA) on cell viability was assessed using standard 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) analysis. A549, HepG2 and MCF-7 cells were seeded in each well of 96-well plates at a density of 5000 cells/well. Thereafter, growth medium was removed and 0.1 mL/well of AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 125 nmol/mL, peptide concentration is 3.3 nmol/mL) was replaced on the wells to incubate at 37 °C, in 5 % CO_2 for 3 h. After the cells were washed twice, a fresh cell culture medium was added. The photothermal therapy groups were irradiated with an 808 nm laser at a power of 2 W/cm^2. And then 20 μL 0.5 mg/mL MTT solution were added. After 4 h incubation, MTT solution was discarded and 100 μL DMSO was

added per well. The optical density was measured at 570 nm on PerkinElmer EnSpire 2300 Multilabel Plate Reader.

Time-resolved imaging after NIR photothermal therapy

A549 cells were seeded in 60 mm glass bottom dishes (1×10^5 cells/well) and cultured in RPMI 1640 medium (containing 10 % FBS and 1 % antibiotics). Then the growth medium was removed and 3 mL/well of AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 125 nmol/mL, peptide concentration is 3.3 nmol/mL) was replaced on the dishes. After incubated at 37 °C, in 5% CO_2 for 3 h, the cells were washed with PBS solution for three times, and propidium iodide (PI) was added to characterize the cell apoptosis. The cells were irradiated with a 20 mW 730 nm NIR laser, and the signal was collected within the 520 ~ 560 nm channel. The fluorescence of PI was excited with a laser at 830 nm, and the emission were collected within the 575 ~ 645 nm channel.

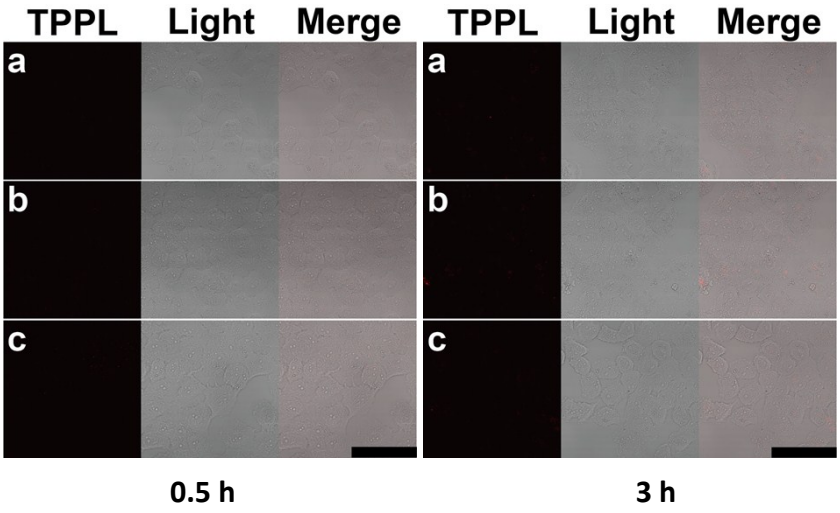

Figure S1. Two-photo photoluminescence (TPPL) images of MCF-7/ADR cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (5.0×10^3 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 100 μm.

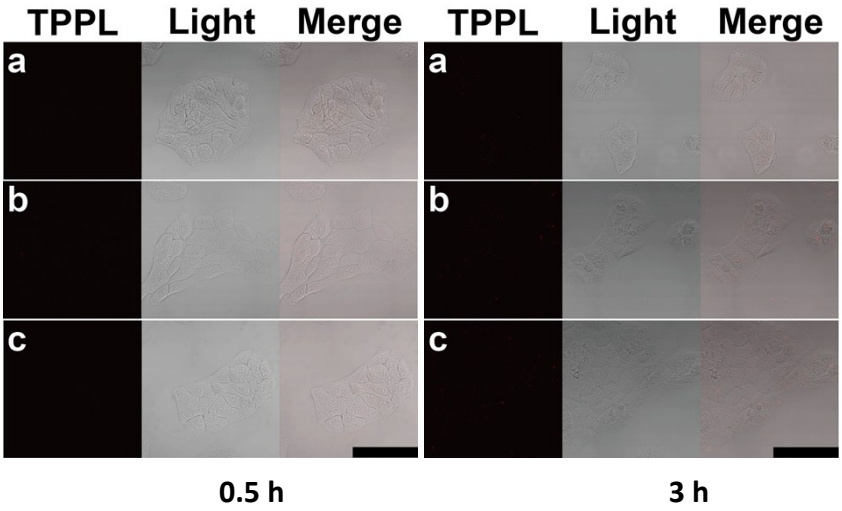

Figure S2. TPPL images of MCF-7 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (5.0×10^3 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 100 μm.

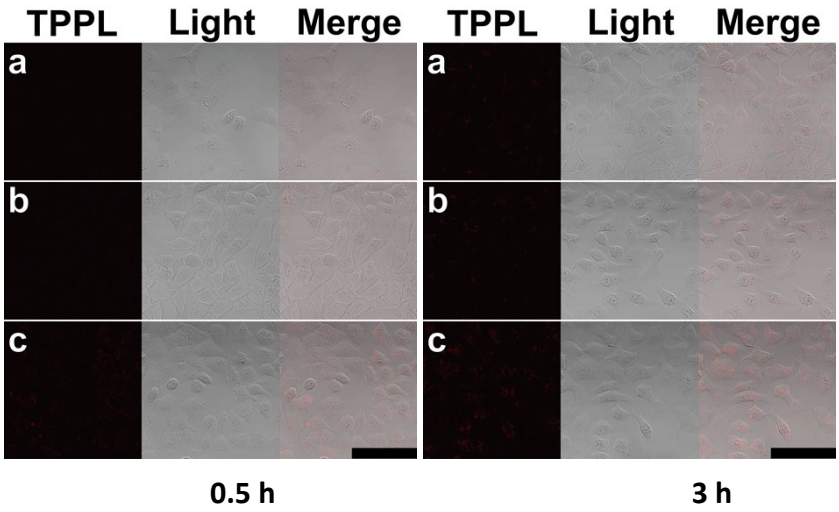

Figure S3. TPPL images of HepG2 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (5.0×10^3 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 100 μm.

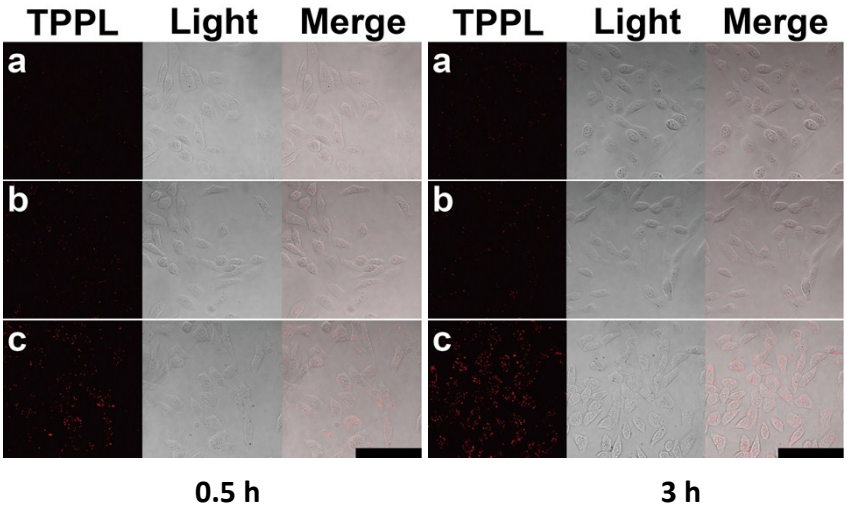

Figure S4. TPPL images of A549 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (5.0×10^3 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 100 μm.

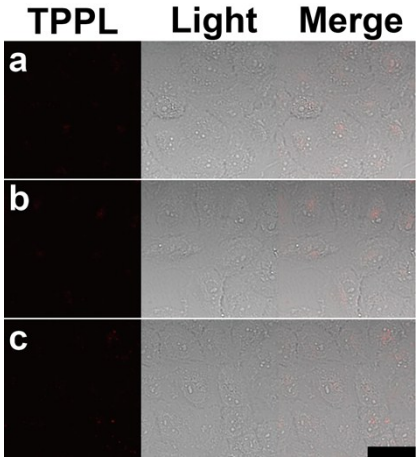

Figure S4. TPPL images of MCF-7/ADR cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 50 μm.

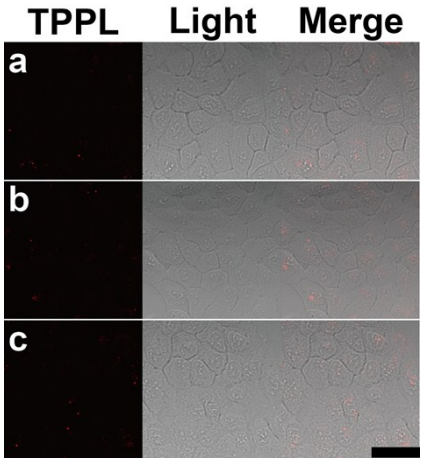

Figure S6. TPPL images of MCF-7 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 50 μm.

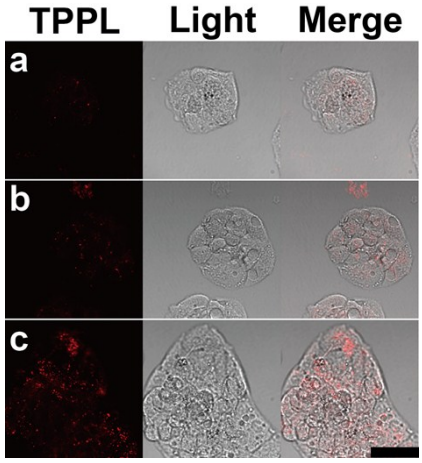

Figure S7. TPPL images of HepG2 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 50 μm.

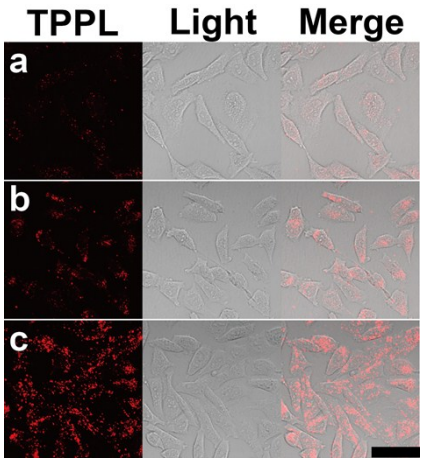

Figure S8. TPPL images of A549 cells incubated with AuNR-LA-RGDK (a), AuNR-*c*(CRGDC) (b) and AuNR-*c*(LA-RGDK-LA) (c) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) for 0.5 h and 3 h. The images from left to right correspond to AuNR fluorescence (λ_{ex} = 730 nm, λ_{em} = 520 ~ 560 nm), light field, overlay of the above images. Scale bars, 50 μm.

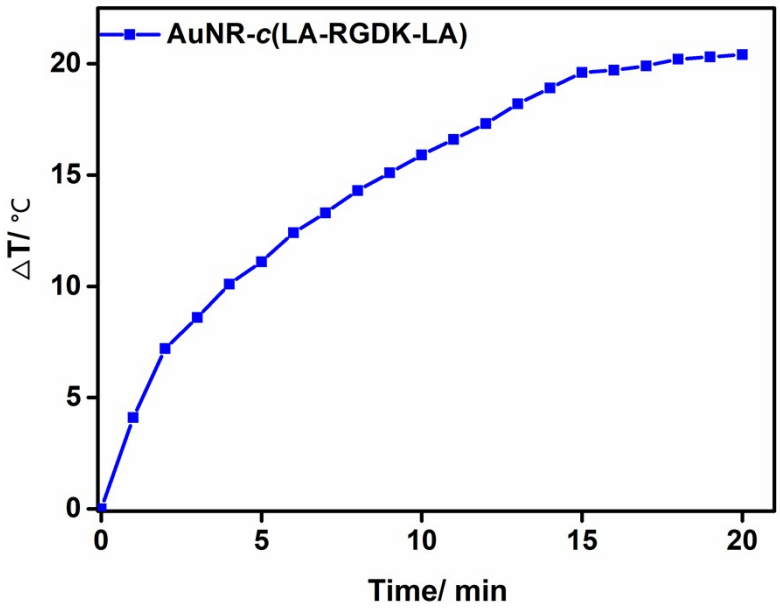

Figure S9. Temperature changes of AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 125 nmol/mL, peptide concentration is 3.3 nmol/mL) dispersed in water and irradiated with an 808 nm laser at a power density of 2 W/cm^2 as a function of time.

Table S1. Mean TPPL intensity ratio of AuNR-LA-RGDK, AuNR-*c*(CRGDC) and AuNR-*c*(LA-RGDK-LA) (5.0×10^3 or 2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 0.33 nmol/mL or 1.32 nmol/mL) incubating MCF-7/ADR, MCF-7, HepG2 and A549 cells for 0.5 h and 3 h.

		5.0×10^3 molecules/per AuNR				2.0×10^4 molecules/per AuNR			
		MCF-7/ADR	MCF-7	HepG2	A549	MCF-7/ADR	MCF-7	HepG2	A549
0.5 h	AuNR-*c*(CRGDC)/ AuNR-LA-RGDK	1.1	1.1	1.2	2.5	1.2	1.0	6.2	9.9
	AuNR-*c*(LA-RGDK-LA)/ AuNR-LA-RGDK	1.0	1.0	4.4	7.3	1.2	1.2	11.0	33.4
3 h	AuNR-*c*(CRGDC)/ AuNR-LA-RGDK	1.0	1.0	2.1	1.6	1.0	1.2	1.8	5.0
	AuNR-*c*(LA-RGDK-LA)/ AuNR-LA-RGDK	1.0	1.0	3.5	9.6	1.1	1.2	2.4	9.0

Table S2. Mean TPPL intensity ratio of before and after adding *c*(RGDyK) to block A549 cells incubated with AuNR-LA-RGDK, AuNR-*c*(CRGDC) and AuNR-*c*(LA-RGDK-LA) (2.0×10^4 molecules/per AuNR, gold concentration is 50 nmol/mL, peptide concentration is 1.32 nmol/mL) for 0.5 h.

	before blocking/ after blocking
AuNR-LA-RGDK	2.1
AuNR-*c*(CRGDC)	14.8
AuNR-*c*(LA-RGDK-LA)	28.5

References

1. B. Nikoobakht and M. A. El-Sayed, *Chem. Mater*., 2003, **15**, 1957–1962.
2. J. B. Song, X. Y. Yang, O. Jacobson, L. Lin, P. Huang, G. Niu, Q. J. Ma, X. Y. Chen, *ACS Nano,* 2015, **9**, 9199–9209.