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Angewandte Chemie-International Edition

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<https://doi.org/10.1002/anie.202010089>

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How to cite:

International Edition: doi.org/10.1002/anie.202010089

German Edition: doi.org/10.1002/ange.202010089

Engineering the Protein Corona Structure on Gold Nanoclusters Enables Red-Shifted Emissions in the Second Near-infrared Window for Gastrointestinal Imaging

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Abstract: The application of NIR-II emitters for gastrointestinal (GI) tract imaging remains challenging due to fluorescence quenching in the digestive microenvironment. Herein, we report that red-shifting of the fluorescence emission of Au nanoclusters (AuNCs) into NIR-II region with improved quantum yields (QY) could be achieved by engineering a protein corona structure consisting of a ribonuclease-A (RNase-A) on the particle surfaces. RNase-A-encapsulated AuNCs (RNase-A@AuNCs) displayed emissions at 1050 nm with a 1.9% QY. Compared to rare earth and silver-based NIR-II emitters, RNase-A@AuNCs had excellent biocompatibility, showing >50-fold higher sensitivity in GI tract, and migrated homogeneously during gastrointestinal peristalsis to allow visualization of the detailed structures of the GI tract. RNase-A@AuNCs could successfully examine intestinal tumor mice from healthy mice, indicating a potential utility for early diagnosis of intestinal tumors.

Introduction

Barium swallow and endoscopic techniques are the mainstream techniques for gastrointestinal (GI) imaging.^[1] However, barium swallow is an X-ray based diagnosis technique with limited spatial resolution and notable radiation risk; endoscopy is an invasive diagnostic technique contraindicated in patients with suspected bowel stricture or obstruction. Therefore, non-invasive and X-ray-free imaging technologies are highly desired for early diagnosis of some severe GI diseases, especially tumors.^[2]

Fluorescence emissions in the second near infrared window (NIR-II, 1000–1400 nm) have attracted substantial research enthusiasms for bio-imaging.^[3] Since the excitation and emission wavelengths involved in obtaining NIR-II emissions already allow deep tissue penetration as display a strongly reduced autofluorescence and photon scattering, one-photon excitation was often used as laser source in NIR-II imaging. Compared to small molecular NIR-II emitters (e.g. IR1061,^[4] CH1055^[5] and BTC1070^[6]), nano-sized NIR-II emitters including quantum dots (QDs),^[7] rare earth nanoparticle (RENP),^[8] and single wall carbon nanotube (SWNT),^[9] show relatively higher quantum yields (QYs), and lower susceptibility to photobleaching. They have been exploited for liver,^[10] kidney,^[11] brain^[5] and lung^[12] imaging. However, few reports focus on NIR-II imaging of GI tract. As the acidic and enzymatic bio-contexts of GI tract may lead to fluorescence quenching of most nano-sized emitters, we proposed to synthesize inert metal (e.g. Au, Pt) based emitters for NIR-II imaging of GI tract.^[13]

In this study, we exploited a theory of ligand-metal-metal charge transfer (LMMCT)^[14] to synthesize Au-based nanoclusters (AuNCs) with emissions in NIR-II region. Ribonuclease-A (RNase-A) were therefore selected to encapsulate gold nanocluster (RNase-A@AuNC) for red-shifted emissions as it consists of a desired cocktail of thiol group (cysteine) and aromatic amino acids (histidine and tyrosine). The resulting RNase-A@AuNCs were exposed to GI tract simulated fluids and mammalian cells for stability and biosafety assessments. The in vivo imaging capability of RNase-A@AuNC in GI tract was examined in mice by oral administration. Finally, we employed an intestinal cancer model to justify the potential utility of AuNCs as an imaging agent for tumor diagnosis.

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<https://doi.org/10.1002/anie.202010089>.

Results and Discussion

Considering the biocompatibility and diversity of amino acids, we examined the feasibility of constructing aromatic amino acids on particle surfaces to reduce the fermi energy of AuNCs. A density functional theory (DFT) computations were performed to investigate the band energy between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of AuNCs. AuNCs binding with cysteine (Cys), histidine (His) and tyrosine (Tyr) showed band gaps at 2.308 eV, 0.038 eV and 0.104 eV, respectively (Figure 1A). These three amino acids were exploited to synthesize AuNCs by a microwave assisted method,^[15] including Au³⁺ binding with ligands, reduction by NaBH₄ and nucleation to form clusters. The emission wavelength and morphologies were examined to assess the impacts of Cys, His and Tyr (Table S1). While Cys could greatly facilitate the formation of Au clusters, introduction of His and Tyr was able to induce redshifts of the emission wavelengths. However, the resulting AuNCs had low quantum yields (QYs) probably due to low surface coverage.

We attempted to control the coverage of Cys, His and Tyr on particle surfaces by constructing a corona structure of RNase-A, which consists of desired number of Cys (8), His (4) and Tyr (6). For a comparison, a ligand library of glutathione, mercaptoethanol, dihydrolipoic acid, lipoic acid-based sulfo-betaine (LA-sulfo-betaine), and bovine serum albumin (BSA) was also included (Table S2). These ligands consist of different numbers of soft (HS-) and hard groups (e.g. Tyr and His) as well as charges, which may significantly impact the emissions of AuNCs based on LMMCT theory. Figure 1B depicted an illustration proposing the reaction path for synthesizing RNase-A@AuNCs. The globular RNase-A proteins were fully unfolded to form loose and expanded random-coil chains through cleavage of disulfide bonds by redox agents under strong basic conditions (Figure S1).

Figure 1. Band gaps of Cys/His/Tyr-Au composites and synthesis route of RNase-A@AuNCs. A) Band gaps of Cys-, His-, and Tyr-Au composites by DFT calculations. B) Schematic representation of the reaction path for the synthesis of RNase-A@AuNCs. The 3D arrangement of RNase-A dissociates in basic and reductive conditions. After nucleation of Au atoms under microwave irradiation, the dissociated peptide chains coordinate with Au atoms to facilitate the maturation of Au clusters.

Thereupon, the exposed functional groups (Cys, His and Tyr) along the peptide chains will coordinate with Au cations to assist the formation of AuNCs under microwave irradiation. The interaction of RNase-A with gold nanoclusters was further investigated by molecular dynamics simulation (MD). As shown in Figure S2, the Au₂₅NCs were stabilized by forming RNase-A@Au₂₅NC complexes at specific binding sites. In all three tested model, Cys, Tyr and His were found to bind on AuNC surface.

As expected, RNase-A encapsulated AuNCs were found to display a perfect Gaussian-shaped emission peak centered at 1050 nm with a full width at half-maximum (FWHM) of ≈ 205 nm (Figure 2A), which is relatively narrow compared to most reported novel-metal based imaging agents. The emission spectra of RNase-A@AuNCs had a nice overlap with the NIR transparency window of tissues (800–1400 nm).^[3] The morphology and primary size of RNase-A@AuNCs were examined by transmission electron microscopy (TEM). As shown in Figure 2B, RNase-A@AuNCs had an average diameter of 2.2 ± 0.1 nm and homogeneously distributed on copper mesh grids. The corresponding selected area electron diffraction (SAED) pattern in the diffraction rings was indicative of AuNCs that were randomly oriented and had face centered cubic Au structure (Figure 2C). The individual particles were further examined by high-resolution (HR) TEM and displayed a high crystalline structure with an

Figure 2. A) UV/Vis/NIR absorption and photoluminescence spectra of the RNase-A@AuNCs. 2 mg mL⁻¹ RNase-A@AuNCs were dissolved in water to examine their UV/Vis/NIR absorption by UV/Vis-NIR spectrophotometry. The photoluminescence spectra were acquired by fluorescence spectrophotometry, the solid red line is the Gaussian function fit from Origin Lab 8.0. B) TEM image, C) selected area electron diffraction (SAED) pattern, D) HR-TEM image, and E) AFM image of the as-synthesized RNase-A@AuNCs. The RNase-A@AuNCs at 2 mg mL⁻¹ were dispersed in water and dropped in Cu-grids or mica for dryness at room temperature. The inset of the TEM image shows size histogram of 100 particles. F) Plot of the integrated fluorescence spectra of both RNase-A@AuNCs and IR-26. A reference near-infrared fluorophore (IR-26) at five different concentrations was used to calculate the quantum yield of RNase-A@AuNCs by comparing the slopes of the linear fits.

inter-planar spacing of 0.25 nm (Figure 2D). Since RNase-A proteins are transparent under TEM, atomic-force microscopy (AFM) was exploited to assess the overall dimension of RNase-A@AuNCs at 3.5 ± 0.5 nm (Figure 2E). The thickness of corona structure on Au cluster was at 0.65 nm determined by equation 1 (supplementary methods). The quantum yield (QY) of RNase-A@AuNCs was 1.9% determined by comparison to a reference material (IR 26) with reported QY of 0.5%^[16] (Figure 2F). The QY of RNase-A@AuNCs was significantly higher than the values (0.012–0.6%) of most reported NIR-II emitting molecules.^[17]

Three subgroups of Au clusters (Au_{17} , Au_{20} and Au_{25}) could be detected in the as-prepared RNase-A@AuNCs by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure S3). Proteomic analysis was performed to examine the number of Cys, His and Tyr binding on AuNC surfaces (Figure S4, Table S3). As a result, each AuNC particle could bind with an average of 17 amino acids, including 8 Cys, 4 His and 5 Tyr. Since Au_{17} and Au_{20} theoretically bind less amino acids than Au_{25} , we speculated that Au_{25} might bind all Cys, His and Tyr of a RNase-A molecule and the number of amino acids binding on Au_{25} could come up to 18. This number is consistent with Zheng et al. report that Au_{25} provides 18 binding sites on the surface.^[18] The purity of RNase-A@AuNC was examined by gel separation. RNase-A@AuNCs merely displayed one band around the theoretical molecular weight of the prominent constitute (RNase-A@ Au_{25}) at ≈ 18.6 KD. RNase-A@AuNCs showed a significant migration shift compared to RNase-A proteins, suggesting a successful attachment of RNase-A on AuNC surfaces (Figure S5). The interactions of AuNCs with three amino acids could be evidenced by the amide II band of Fourier transform infrared (FTIR) spectra (Figure S6).

Since GI tract consists of acidic and enzymatic bio-contexts, it may digest NIR-II emitters to affect their optical activities and elicit hazard effects.^[19] To assess the stability in GI tract, RNase-A@AuNCs were exposed to a simulated gastric juice (SGJ)^[20] and PBS buffer to examine fluorescence changes and compared with two reported NIR-II nano-emitters including Ag_2S and $\text{NaYF}_4:\text{Er}/\text{Yb}$ (Figure 3A). As expected, RNase-A@AuNCs were robust to the acidic and enzymatic digestion process. While RNase-A@AuNCs showed only 25% decreases of fluorescence in 2 h, other emitters displayed more than 95% quenching effect in SGJ. The morphology and primary size of RNase-A@AuNCs, Ag_2S and $\text{NaYF}_4:\text{Er}/\text{Yb}$ in SGJ were examined by TEM. Ag_2S and $\text{NaYF}_4:\text{Er}/\text{Yb}$ underwent significant biotransformation in SGJ, evidenced by particle morphology and hydrodynamic size changes (Figure S7 and S8). Furthermore, the biosafety of RNase-A@AuNCs was assessed in two intestine epithelial cells (Caco-2, HCT116). As shown in Figure 3B, RNase-A@AuNCs showed extraordinary biocompatibility in two cell lines in a wide range of exposure doses ($0\text{--}500\text{ }\mu\text{g mL}^{-1}$), whereas $\text{NaYF}_4:\text{Er}/\text{Yb}$ and Ag_2S induced dose-dependent cytotoxicity. Furthermore, we used a LIVE/DEAD staining assay to visualize cell viability by confocal microscopy. As a result, substantial dead cells could be detected in two cell lines exposed to $\text{NaYF}_4:\text{Er}/\text{Yb}$, displaying massive red

Figure 3. A) Assessment of fluorescence stability of nanosized NIR-II emitters in SGJ and PBS buffer. Ag_2S , $\text{NaYF}_4:\text{Er}/\text{Yb}$, and RNase-A@AuNCs were dissolved in DI H_2O or SGJ at 2 mg mL^{-1} ($n=3$). Data are presented as mean values $\pm$ SD. Calculation of PL is described in supplementary methods. B) Cell viability test. Caco-2 and HCT116 cells were exposed to particles at concentrations of $0\text{--}500\text{ }\mu\text{g mL}^{-1}$ for 24 h. Cell viabilities were assessed by MTS assay ($n=3$ biologically independent cell samples). Data are presented as mean values $\pm$ SD. C) Fluorescence microscopy images by LIVE/DEAD staining. The cells were exposed to $500\text{ }\mu\text{g mL}^{-1}$ particles for 24 h. Then the cells were stained by a LIVE/DEAD staining kit for microscopy imaging. Scale bars = $100\text{ }\mu\text{m}$.

fluorescence of Ethidium homodimer-1 (EthD-1) binding with DNA (Figure 3C). This is probably because cellular internalization of RNase-A@AuNCs were limited except for a few associations with membranes (Figure S9).

The biokinetics of oral injected RNase-A@AuNC is determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) in different organs at 24 h post administration. The ICP-OES-based quantitative analysis indicated that fecal excretion ($\approx 91\%$) was the predominant pathway for the clearance of the injected nanoparticles, and 7% AuNCs were found in stomachs (Figure S10). The hematoxylin and eosin (H&E) staining images demonstrated that this AuNC probe had negligible toxicity to GI tract, heart, pancreas, liver and kidney (Figure S11). These results indicated that RNase-A@AuNCs had excellent biocompatibility and well sustained their NIR-II emission in GI tract microenvironment due to their negative surface charge and

high stability, denying cellular internalization as well as biotransformation-induced hazard effects.^[21]

Before tumor imaging, RNase-A@AuNCs were orally injected into healthy C57BL/6 mice to examine signal-dose relationships in GI tract, and compared with Ag₂S and NaYF₄:Er/Yb. As shown in Figure 4A, the fluorescence emissions of RNase-A@AuNCs got to a plateau at an administration dose of 10 mg kg⁻¹. The sensitivity of each emitter was determined by the slope of signal-dose curve. RNase-A@AuNCs displayed >50 higher sensitivity than Ag₂S and NaYF₄:Er/Yb. A dynamic movement process of AuNCs was observed in gastrointestinal peristalsis, including stomach duodenum, ileum and caecum (Figure 4B). Fluorescence intensity analysis of regions of interests (ROIs)^[16]

Figure 4. A) Signal-dose responses of NIR-II emitters in mice. The fluorescence was examined by InGaAs CCD camera (1000 nm LP, 200 ms, 808 nm excitation, 15 mWcm⁻²) 2 h after oral administration of emitters. The top panel shows representative NIR-II images of three nano-emitters in GI tract ($n=3$). Data are presented as mean values $\pm$ SD. B) NIR-II imaging of gastrointestinal peristalsis by RNase-A@AuNCs. Mice were exposed to RNase-A@AuNCs by oral administration at 2 mg kg⁻¹ for real-time monitoring of gastrointestinal peristalsis under 808 nm excitation.

indicated that AuNC based NIR-II imaging had a high resolution of 1.6 mm for GI tract, 4-fold higher than barium swallow (Figure S12).

Finally, we attempted to use RNase-A@AuNC for diagnosis of intestine cancer as the pathology changes may impede or block peristaltic movement, displaying signal changes in NIR-II imaging. At 2 h post oral administration, while RNase-A@AuNCs displayed consecutive distributions with uniform fluorescence in ileum of normal mice, dark craters were visualized at the forefront of ileum of intestine cancer mice, which were consistently present at the tumor nodules of intestine by ex vivo images as well as the H&E staining sections with abnormalities of glandular hyperplasia (Figure 5A and Figure S13). AuNC based NIR-II imaging was able to clearly examine intestinal tumor nodules with diameters as small as 2.5 mm, providing high imaging resolutions to abnormalities in GI tract. The valley areas of ROI curves of all tested mice were calculated for statistical analysis. As shown in Figure 5D, tumor-burden mice displayed 11-fold increments of valley areas than healthy mice. These data indicated that NIR-II imaging of GI tract by RNase-A@AuNCs could well differentiate tumor-burden mice from the healthy.

Figure 5. A) Mice with intestinal cancer and healthy mice were exposed to 2 mg kg⁻¹ RNase-A@AuNCs by oral administration. After 2 h, the NIR-II images were captured by InGaAs CCD camera. The dashed lines added on zoomed-in images represent the ROIs. The intensities of ROIs were measured by ImageJ 1.51. After NIR-II imaging, the animals were sacrificed to collect intestines for ex vivo examination. B) The animals were sacrificed to collect intestines for H&E staining (magnification: $\times 400$). C) The ROIs were calculated based on the dashed lines of NIR-II images of healthy and tumor-burden mice by ImageJ 1.51. D) Valley areas of ROI-distance curves were measured by Origin Lab 8.0. *** $p < 0.001$ compared to control group by two tailed Student's *t*-test.

Conclusion

In summary, red-shifted emissions of AuNCs at 1050 nm in NIR-II region could be achieved by engineering a unique protein corona on particle surface. The as-prepared RNase-A@AuNCs displayed a 1.9% QY of NIR-II emissions, high stability and excellent biocompatibility. Compared to two reported NIR-II emitters (Ag_2S and $\text{NaYF}_4:\text{Er}/\text{Yb}$), RNase-A@AuNC exhibited > 50-fold higher sensitivity in GI tract. Notably, this emitter could be used to examine intestinal tumor nodules with diameters as small as 2.5 mm. Overall, this study defined the first report of engineering protein corona on AuNCs for red shifts of emissions to NIR-II region and highlighted the promising utilities of RNase-A@AuNCs for diagnosis of intestinal tumors. Our findings provide new insights to engineer the surface coronas of nano-emitters for red-shifted emissions.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Number 21976126, 31671032), the grants from Key International Cooperation Projects of the Ministry of Science and Technology (2018YFE0120400), Key Project of social development of Jiangsu Province (BE2018653).

Conflict of interest

The authors declare no conflict of interest.

Keywords: fluorescent probes · gold nanoparticles · protein corona · red shift · tumor imaging

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Manuscript received: July 22, 2020

Accepted manuscript online: August 14, 2020

Version of record online: ■■■■■, ■■■■■

Communications

Fluorescent Probes

W. Wang, Y. Kong, J. Jiang, Q. Xie,
Y. Huang, G. Li, D. Wu, H. Zheng, M. Gao,
S. Xu, Y. Pan, W. Li, R. Ma, M. X. Wu, X. Li,
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Engineering the Protein Corona Structure
on Gold Nanoclusters Enables Red-
Shifted Emissions in the Second Near-
infrared Window for Gastrointestinal
Imaging

The precise control of the surface protein corona enabled the red-shifting of the emission of gold nanoclusters (AuNCs) into the NIR-II region. RNase-A-encapsulated AuNCs allowed the visualization of the detailed structures of the GI tract during gastrointestinal peristalsis, providing a non-invasive and X-ray-free technique to differentiate intestinal tumours.