SERS Reporters

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# Ultrasensitive Near-Infrared Raman Reporters for SERS-Based In Vivo Cancer Detection**

*Animesh Samanta, Kaustabh Kumar Maiti, Kiat-Seng Soh, Xiaojun Liao, Marc Vendrell, U. S. Dinish, Seong-Wook Yun, Ramaswamy Bhuvaneswari, Hyori Kim, Shashi Rautela, Junho Chung, Malini Olivo, and Young-Tae Chang**

Surface-enhanced Raman spectroscopy (SERS) has recently emerged as an alternative to fluorescence-based spectroscopy in bioimaging, as it can minimize photobleaching, peak overlapping, and low signal-to-noise ratio in complex biological systems.[1–3] SERS probes are based on the $10^{14}$–$10^{16}$-fold scattering enhancement caused by the proximity of Raman-active signature molecules to the surface of metal nanoparticles (NPs),[4–7] which can be modulated with molecular recognition motifs to render diagnostic tools for optical imaging and therapeutic studies.[8–12] However, the preparation of ultrasensitive SERS probes is hampered by the limited availability, sensitivity, and reproducibility of Raman-active compounds. This drawback is particularly important at the near-infrared (NIR) region, where the availability of reporters is restricted to a few Raman-active molecules. Herein, we report the first combinatorial approach to discover novel and highly sensitive NIR SERS reporters. The synthesis and screening of an 80-member tricarbocyanine library led to the identification of CyNAMLA-381 as a NIR SERS reporter with 12-fold higher sensitivity than the standard 3,3′-diethylthiatricarbocyanine (DTTC), and we validated its advantages for the construction of ultrasensitive in vivo SERS probes.

A major bottleneck in SERS probe discovery is the development of highly sensitive Raman reporters. Most of the commonly used Raman signature molecules are active in the UV/Vis range (e.g., crystal violet, malachite green isothiocyanate, rhodamine-6G, Nile blue, 2-napthalenethiol, TRITC (tetramethylrhodamine-5-isothiocyanate), and XRITC (X-rhodamine-5-(and-6)-isothiocyanate), and thus have a restricted potential for in vivo imaging.[13–16] The adequacy of the NIR region for in vivo studies has raised the interest in NIR surface-enhanced resonance Raman spectroscopy (SERRS)-active molecules. Although the cyanine derivative DTTC has been regarded as a standard in NIR SERRS studies,[9] it shows only a moderate Raman intensity, which limits the preparation of highly sensitive probes for in vivo applications.[17,18]

Since little is known about the correlation between the cyanine scaffold and its Raman intensity, we designed a library of structurally diverse tricarbocyanines with the aim of discovering novel NIR SERRS-active compounds that surpass the sensitivity of DTTC. The tricarbocyanine core is an accessible NIR structure, the central chlorine atom of which can be replaced with different nucleophiles.[19] We designed the synthesis of tricarbocyanine derivatives by substitution with different amines, and acetylated the resulting alkyl- or benzylamino groups to obtain compounds with NIR absorption properties and good chemical stability in aqueous media (CyNA).[20] To prepare compounds that could be chemisorbed on gold nanoparticles (AuNPs),[21] we prepared the scaffold **1** with an aminopropyl linker that could be later coupled to a disulfide-containing lipoic acid spacer (Scheme 1).

The amine group of **1** was Boc-protected prior to the derivatization of the central chlorine atom with 80 structurally different primary amines including heterocyclic, alkyl, and aromatic groups (for structures, see Chart S1 in the Supporting Information). After acetylation, the compounds were treated with an optimized TFA/dichloromethane (1:9) solution that overcame the lability of the tricarbocyanine core in acidic conditions.[22] The final coupling to a lipoic acid-activated ester resin yielded 80 derivatives (CyNAMLA) with an average purity of 90% (for data of HPLC-determined purities, see Table S1 in the Supporting Information).

CyNAMLA compounds proved to be remarkably NIR-active with absorbance maximum wavelengths around 800 nm (Table S1 in the Supporting Information). Their SERS

[*] A. Samanta, X. Liao, Prof. Y. T. Chang
Department of Chemistry & MedChem Program of Life Sciences Institute, National University of Singapore
117543 Singapore (Singapore)
Fax: (+65) 6779-1691
E-mail: chmcyt@nus.edu.sg
Homepage: http://ytchang.science.nus.edu.sg
Dr. K. K. Maiti, K. S. Soh, Dr. M. Vendrell, Dr. U. S. Dinish, Dr. S. W. Yun, S. Rautela, Prof. M. Olivo, Prof. Y. T. Chang
Singapore Bioimaging Consortium, Agency for Science, Technology, and Research (A*STAR)
138667 Singapore (Singapore)
R. Bhuvaneswari, Prof. M. Olivo
Division of Medical Sciences, National Cancer Centre
169610 Singapore (Singapore)
H. Kim, Prof. J. Chung
Department of Biochemistry and Molecular Biology and School of Medicine & Cancer Research Institute, Seoul National University
110799 Seoul (Republic of Korea)
Prof. M. Olivo
School of Physics, National University of Ireland
Galway (Ireland)

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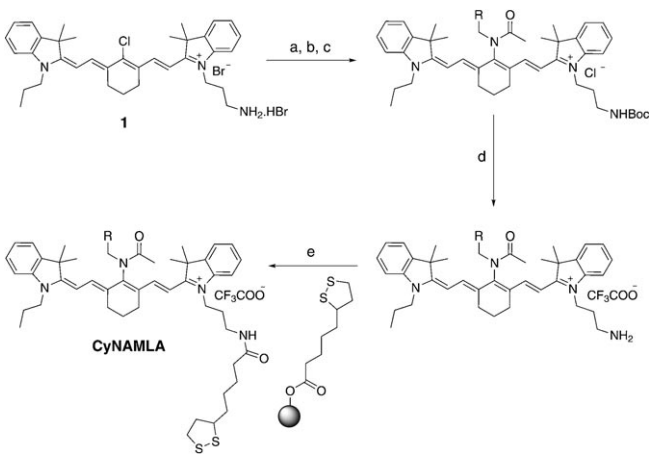



***Scheme 1.*** Synthesis of lipoic acid-containing amine acetylated tricarbocyanines (CyNAMLA). Reagents and conditions: a) $Boc_2O$, DIEA, $CHCl_3$, 60 °C, 4–6 h; b) R-$NH_2$, DIEA, $CH_3CN$, 60 °C, 20-90 min; c) $CH_3COCl$, DIEA, $CH_2Cl_2$, 0 °C, 5–10 min; d) TFA/$CH_2Cl_2$ (1:9), room temperature, 6 h; e) lipoic acid-activated ester resin, $CH_2Cl_2$/$CH_3CN$ (9:1), room temperature, 16 h. Boc = *tert*-butoxycarbonyl, DIEA = diisopropylethylamine, TFA = trifluoroacetic acid.

properties were examined under a compact Raman scanner upon incubation of every compound with citrate-stabilized AuNPs (size: 60 nm). Among metal NPs, AuNPs are particularly suitable for in vivo applications because of their low toxicity, adaptability to bioconjugation, and reproducible signal intensity and quantification.[23] This primary screening revealed that the SERS intensities of CyNAMLA compounds varied significantly throughout the library, thus indicating that the SERS properties depended on the amine structure. Notably, six derivatives containing mostly aromatic amines (CyNAMLA-80, 92, 221, 262, 381, and 478, represented in Figure 1 as A5, A6, C6, C8, E9, and G9, respectively) exhibited very high SERS intensities that exceeded the signal of DTTC, and were selected for further analysis.

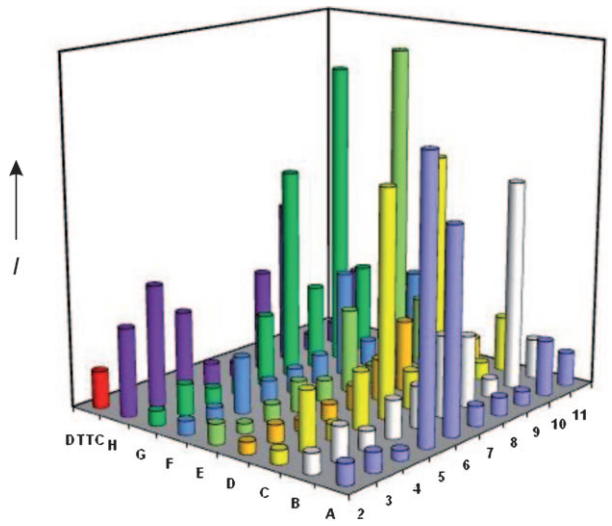
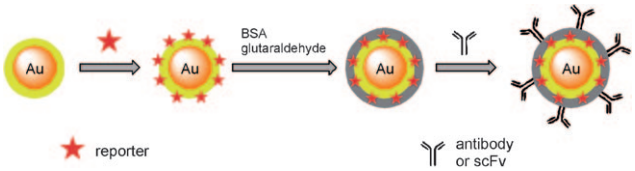



***Figure 1.*** Comparative SERS intensities of the whole CyNAMLA library (compound codes as indicated in the Supporting Information). SERS spectra were measured in a compact Raman scanner with excitation at 785 nm and 60 mW laser power. The intensity of the reference standard DTTC is plotted as a red bar.

The encapsulation of SERS-active NPs is a crucial step because it can prevent their aggregation and the desorption of Raman signature molecules from the NPs, and it can be used to introduce functional groups on their surface for bioconjugation.[24–27] To evaluate the long-term stability of the six selected CyNAMLA–AuNPs, we modified them with bovine serum albumin (BSA) and glutaraldehyde so that amine-containing molecules (antibodies) could be attached to the resulting cross-linked organic layer on the surface (Scheme 2).[28]




***Scheme 2.*** Preparation of BSA-stabilized and antibody- or single-chain variable fragment (scFv)-conjugated SERS nanotags.

The increased size (65–70 nm) of the BSA-encapsulated CyNAMLA–AuNPs was confirmed by transmission electron microscopy (TEM; Figure S12 in the Supporting Information), and we analyzed the stability of the SERS intensities for one month (Figures S4–S10 in the Supporting Information). Remarkably, the nanotags did not show any significant aggregation under ambient conditions and exhibited consistent SERS intensities over time, with a very low relative standard deviation (2 to 3 %). CyNAMLA-381 was chosen as the best Raman reporter in terms of both signal intensity and stability (Figures S3 and S8 in the Supporting Information), and it displayed around 12-fold higher sensitivity than the standard DTTC (Figure 2).

With the discovery of CyNAMLA-381 as a NIR highly sensitive SERS reporter molecule, we applied it to the preparation of SERS probes for cancer-cell detection and discrimination. The human epidermal growth factor receptor 2 (HER2) signaling pathway plays an important role in cell proliferation, and is upregulated in most breast cancers.[29] To prepare SERS nanotags that could selectively detect cancer cells expressing HER2 receptors, we conjugated

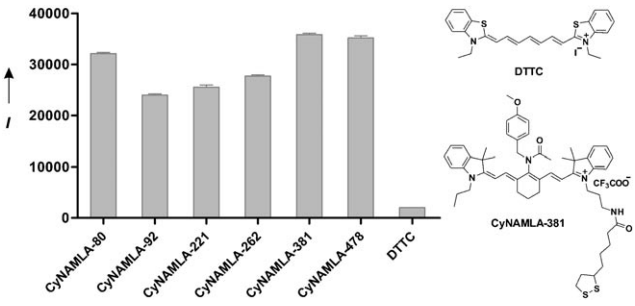



***Figure 2.*** SERS intensities of the selected BSA-encapsulated CyNAMLA–AuNPs. SERS spectra were measured in a Renishaw Raman microscope (excitation: 785 nm).

CyNAMLA-381–AuNPs to two HER2-recognition motifs: a full anti-HER2 monoclonal antibody (170 kDa) and an scFv anti-HER2 (26 kDa) antibody.[30] First we examined their in vitro specificity in SKBR-3 (HER2-positive) and MDA-MB231 (HER2-negative) cancer cells. Upon incubation of SKBR-3 cells with antibody-conjugated CyNAMLA-381–AuNPs, strong SERS signals were observed, whereas negligible signals were detected after incubating the same NPs with MDA-MB231 cells (Figure S13 in the Supporting Information). We also confirmed the target specificity of CyNAMLA-381–AuNPs in SKBR-3 cells by competition assays between antibody-conjugated nanotags and free HER2-recognition motifs: a 10- to 15-fold decrease of the SERS signals in the presence of the competing anti-HER2 antibodies was observed (Figure S14 in the Supporting Information). Interestingly, the signal intensities obtained with scFv-conjugated nanotags were 1.5 times stronger than those with the full-HER2 antibody. These data suggest that scFv-conjugated nanotags not only maintain the recognition properties but also improve the detection of full-size antibodies. Furthermore, the smaller size of scFv can significantly reduce the interstitial tumor pressure that impedes intratumoral distribution when using larger recognition motifs.[9]

We performed SERS mapping experiments in SKBR-3 and MDA-MB231 cells (Figure 3).[31] Mapping images of SKBR-3 cells after incubation with scFv-conjugated CyNAMLA-381 nanotags displayed high SERS intensities at representative frequencies of CyNAMLA-381 (i.e., 523 $cm^{-1}$). On the other hand, no distinguishable signals were observed in MDA-MB231 cells under the same experimental conditions. The mapping pictures confirmed that the interaction between scFv anti-HER2-conjugated nanotags and SKBR-3 cells was mainly localized at the cell surface, which corresponds well with the high expression of HER2 receptors at the plasma membrane of cancer cells.[32] Reflective-mode dark-field images[33] of SKBR-3 cells that were incubated with scFv-conjugated CyNAMLA-381 SERS nanotags also displayed a number of bright spots on the cell surface because of recognition of the receptor, whereas the same experimental conditions with MDA-MB231 cells showed a negligible scattering (Figure S14 in the Supporting Information). The corresponding SERS spectra showed that only intense SERS signals were observed from the particles located on the cell surface of the HER2-postive cells (points 2 and 3), and no SERS signals were detected in other regions of the SKBR-3 cells (point 1) nor in MDA-MB231 cells (points 4 and 5).

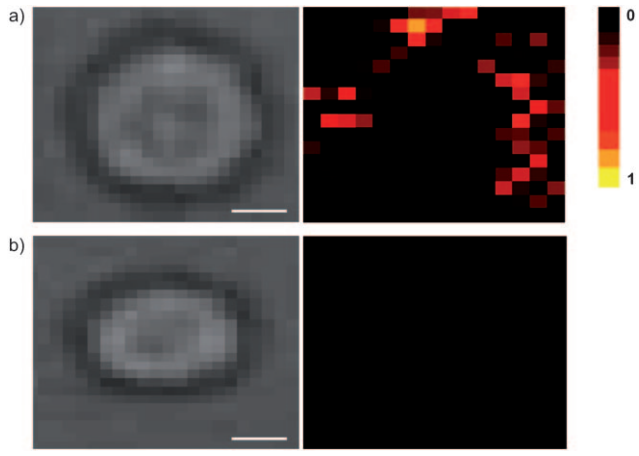



***Figure 3.*** Bright-field and SERS mapping images of cells treated with CyNAMLA-381 nanotags: a) SKBR-3 and b) MDA-MB231 cells. All mapping images (523 $cm^{-1}$) were scanned at an interval of 2 μm (785 nm excitation) and the intensities were normalized between the lowest (0) and the highest color (1) values. Scale bar: 10 μm.

Finally, to validate the optical detection by scFv-conjugated CyNAMLA-381 SERS nanotags in vivo, we injected them into nude mice bearing xenografts generated from SKBR-3 cells. Five hours after the tail-vein injection, we measured the SERS spectra of the tumor site through the skin with a NIR laser beam. The signal of the tumor site perfectly resembled the SERS spectra of the pure nanotag, whereas no SERS signal was detected from other anatomical locations (i.e., upper dorsal; Figure 4). On the other hand, we observed

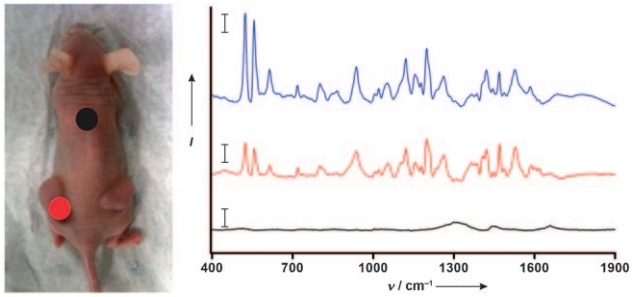



***Figure 4.*** In vivo detection of HER2-positive tumors with scFv-conjugated CyNAMLA-381 SERS nanotags: SERS spectrum of pure nanotags (blue), and SERS signals of the tumor location (red) and an upper dorsal area (black). Scale bar: 5000 cps.

that no significant SERS signal was detected after injecting the nanotags in xenograft models prepared with HER2-negative cancer cells (MDA-MB231; Figure S15 in the Supporting Information). Mapping experiments in SKBR-3 xenograft models revealed much higher SERS intensities (523 $cm^{-1}$) in the tumor region when compared to nontumor areas (Figure S16 in the Supporting Information). These results clearly indicate that the scFv-conjugated CyNAMLA-381 SERS nanotags were able to specifically detect HER2-positive tumors in vivo.

In summary, we have prepared a lipoic acid-containing NIR-active tricarbocyanine library (CyNAMLA), and screened the SERS properties after chemisorption in AuNPs. CyNAMLA compounds exhibited strong SERS intensities, and we identified CyNAMLA-381 as a highly sensitive NIR SERS reporter molecule with excellent signal stability and 12-fold higher sensitivity than the current standard DTTC. We further applied CyNAMLA-381 to the preparation of ultrasensitive SERS probes for in vivo cancer imaging by conjugating CyNAMLA-381–AuNPs to scFv anti-HER2 antibodies. These nanotags displayed very good SERS intensity and selectivity towards HER2-positive cancer cells under both Raman and dark-field microscopes. Furthermore,

we confirmed their in vivo application in HER2-positive and -negative xenograft models. The high sensitivity and tumor specificity of scFv-conjugated CyNAMLA-381 SERS nanotags proves their excellent potential as noninvasive diagnostic tools and opens up a new window for the development of SERS probes for cancer bioimaging.

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