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Affibody-Functionalized Gold–Silica Nanoparticles for Raman Molecular Imaging of the Epidermal Growth Factor Receptor

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Abstract

The affibody functionalization of fluorescent surface-enhanced Raman scattering gold–silica nanoparticles as multimodal contrast agents for molecular imaging specific to epidermal growth factor receptor (EGFR) is reported. This nanoparticle bioconjugate reports EGFR-positive A431 tumors with a signal nearly 35-fold higher than EGFR-negative MDA-435S tumors. The low-level EGFR expression in adjacent healthy tissue is 7-fold lower than in the positive tumors. Validation via competitive inhibition reduces the signal by a factor of six, and independent measurement of EGFR via flow cytometry correlates at $R^2 = 0.92$.

1. Introduction

Colon cancer is the second leading cause of cancer death in US adults with 143 000 new cases and approximately 50 000 mortalities expected in 2010.^[1,2] Initially presenting as a benign adenomatous polyp, it is highly curable in stages I and II via surgical excision of the lesion. Even upon advancing into a dysplastic adenoma, colonic resection and adjuvant therapy result in a 73% cure rate.^[1] The prognosis for stage IV disease is quite poor. Thus, the disease management strategy involves monitoring the bowel for oncogenesis via occult blood testing and colonoscopy.^[3] The former is rapid, noninvasive, and offers positive predictive values between 0.87 and 6.13, but has sensitivity below 25% and lacks insight into the physiology of fecal blood.^[4,5] Colonoscopy is established, routine, and reduces mortality by 30%, yet still suffers from two major limitations:^[6,7] lack of molecular insight into the nature of the observed anatomy and difficulty in detecting lesions and metastatic foci that do not protrude from the colon wall, i.e., flat lesions.^[6,8,9] By relying solely on white light anatomic imaging of the colon, patient outcomes suffer because of limited endoscopic diagnostic ability. To wed the benefits of colonoscopy with understanding of colon cancer biology, we and others have proposed molecular imaging of the colon to increase early detection and potentially improve patient outcomes.^[10–13]

A number of proteomic, genetic, and nucleic acid biomarkers exist for the early detection and diagnosis of colon cancer, including the circulating proteins interleukin-6 and carcinoembryonic antigen, mutations to *K-RAS*, and even bulk fecal DNA.^[14,15] For imaging applications, one of the most attractive targets is the epidermal growth factor receptor (EGFR), a cell surface biomarker up-regulated in 80% of colon cancer cases.^[16,17] The EGFR marker is also up-regulated (10-fold) in human and murine aberrant crypt foci

(ACF), the apoptosis-resistant colonic crypts that correlate to a risk of adenoma or carcinoma.^[18,19] Thus, imaging EGFR during colonoscopy may be useful for the identification of ACF and other lesions not obvious during routine examinations.^[18,20,21]

Reports of EGFR molecular imaging include radioisotope and optical techniques.^[22,23] Although chromoendoscopy and small molecular fluorophores in tandem with targeting ligands have potential in this field, their low sensitivity and quick photobleaching as well as the high autofluorescence of colon tissue and difficulty in multiplexing beyond EGFR limit their utility.^[24] Quantum dots (QDs) offer an intensity and photostability superior to molecular dyes, and have remarkable application in cell staining, tracking, and characterization.^[25–28] Unfortunately, lingering concerns regarding their toxicity hamper further deployment of QDs in vivo.^[29] An exciting alternative to these fluorescent systems is the use of surface enhanced Raman scattering (SERS) nanoparticles (NPs).^[11,30–34] In SERS, the characteristic, inelastic (Raman) scatter of incoming photons by a small molecule reporter is increased 10^6 – 10^{14} -fold by the surface plasmon of a noble metal support (e.g., gold).^[35] While SERS has long attracted attention as a label-free detection modality in vitro, it has only recently been deployed by our group^[33,36] and others^[32,34,37,38] for molecular imaging experiments.

Although there are many reported formulations of SERS nanoparticles, this work focuses on gold core–silica clad NPs, which offer the stability and intensity critical for molecular imaging.^[39,40] Briefly, the core acts as the enhancing material for a small molecule reporter sealed on the surface by the silica coat. The silica is further utilized for the coordination of targeting ligands and is an inert material for use in vivo. Previously, the signaling capabilities of SERS NPs were examined by our lab both in vivo and ex vivo. Detection limits <10 pm were achieved with the capacity to multiplex and spectrally deconvolute five simultaneous SERS signatures.^[33,36] The present work extends these efforts to correlate the SERS signal to the presence of a molecular phenotype, e.g., EGFR. There are many EGFR-targeting protein scaffolds (IgG, knottins, scFvs, and affibodies), each with relative advantages and limitations.^[41] For this study, the affibody molecule was chosen due to its affinity in the nm range, low molecular weight/size (7 kDa), high stability, and ease of conjugation via its terminal cysteine. Previous affibody applications include in vivo imaging and therapy.^[42–45]

The following work describes the construction of multimodal affibody-coated Raman NPs for the molecular imaging of EGFR. First, a fluorescence modality (Alexa Fluor 647) is added to the NP to allow for high-throughput cell screens. Validation of the NP in a cell culture and its deployment to tumor samples from mouse models of human disease demonstrate the feasibility of EGFR molecular imaging via SERS NPs. The results indicate that the NPs quantitatively monitor EGFR and bring molecular imaging of colon cancer closer to fruition.

2. Results and Discussion

2.1. Native Nanoparticles

To develop a bioconjugation protocol, the NPs were first studied in their native form, without passivation and affibody functionalization. The size, zeta potential (ζ), and sedimentation time of native NPs was measured as a function of pH and ionic strength. The NPs were 139.4 ± 8.5 nm with a core of 64.3 ± 7.1 nm as determined by transmission electron microscopy (TEM, Supporting Information (SI), Figure S.1). Analysis of the NPs at 27 pm in 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 7.3 via dynamic light scattering (DLS) indicated the hydrodynamic radius to be 155 ± 34 nm with a polydispersity index of 0.089. A larger value from DLS than from TEM is typical and

reflects the hydrodynamic radius.^[46] The 27 pm concentration was selected for further experiments as it yielded optimized nonspecific binding (SI, Figure S.5D). This size regime is ideally suited to topical applications to the colon as it prevents uptake into the blood pool and maintains high concentrations in the bowel.

To study the impact ionic strength has on colloidal stability, ζ and sedimentation rate were measured for NP samples at 27 pm diluted in distilled water with NaCl added at eight different concentrations increasing from 0 to 500 mM (SI, Figure S.2A). As a descriptor of a nanoparticle's attraction for solvent, ζ is useful in monitoring NP aggregation and nonspecific binding.^[47] Values stronger than ± 10 mV indicate enhanced colloidal stability. The NP's ζ values are more negative than -15 mV, well above this threshold, suggesting higher colloidal stability through 77 mM NaCl. While the ionic strength of plasma is maintained at approximately 150 mM, a bowel prepared for colonoscopy has reduced salt concentrations.^[48] As ζ becomes neutral, the amount of time needed for the particles to settle decreases (SI, Figure S.2A). Near 200 mM NaCl, both ζ and sedimentation time show no appreciable change upon further increases in ionic strength and are stable at approximately -7 mV and 5 h, respectively.

Next, ζ was measured as a function of pH, important because the synthetic steps involved in functionalizing the NPs involve both basic and acidic conditions. Phosphate buffered saline (PBS; 10 mM) at five pH points between pH 4 and 11 was used to dilute the NP stock to 27 pm. While ζ became less negative at high acidity (pH = 4.25), the NPs remained very stable across the pH = 6–8 range typical of bioconjugation as indicated by $\zeta = -37$ mV (SI, Figure S.2B).

The silica shell was capped with 3-mercaptopropyl-trimethoxysilane (MPTMS), offering terminal thiol groups as reactive sites (Figure 1A). The 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) assay measured the number of thiols present on the NPs. Quantifying this amount is important for stoichiometric control of affibody loading. An average of $27\,300 \pm 3\,200$ thiols were measured per NP, or $1.2\text{--}1.5$ nm² per thiol via standardization versus MPTMS dissolved in 70% ethanol/water.

2.2. PEG

Strategies to reduce or eliminate nonspecific binding and to reduce aggregation in nanoparticle systems include changing the surface charge and coating. One of the most common coatings employed is polyethylene glycol (PEG).^[49] Specifically, PEG with a methoxy terminus and molecular weights between 1 and 10 kDa have a long-standing role in preventing nonspecific absorption of biological species to surfaces or uptake by the reticuloendothelial system.^[49,50] In addition, a large number of preparatives and whole bowel irrigation systems (e.g., GoLYTELY) utilize PEG (methoxy terminated; 3400 MW) prior to colonoscopy.^[51]

As a model for future PEGylation activities and to determine potential loading levels, NPs were coated with a PEG polymer with a distal fluorescein tag, maleimide reactive site (for thiol coupling via MPTMS), and MW = 3.4 kDa (Mal-PEG-FI). Starting PEG:NP ratios from 10^3 to 10^6 were incubated for 2 h, centrifuged, and the absorbance of the supernatant (excess, unreacted fluorescent PEG) compared to the initial stock solution. The difference in fluorescein absorbance at 490 nm before and after incubation with NPs was monitored and plotted in SI, Figure S.3A. The results for ten different concentrations are sigmoidal, with NP saturation estimated at 50 000 PEG per NP (1.5 nm² per PEG) by Langmuir analysis (see SI) and 15 000–20 000 ($3.8\text{--}5.0$ nm² per PEG) by direct fluorescent measurement (SI, Figure S.3B).^[52] The latter estimate better corresponds to the thiol estimates for DTNB and the value obtained by subtraction may be adversely influenced by PEG nonspecific binding.

To determine the effect various amounts of immobilized PEG have on NP stability and passivation, the ζ and size of each batch of these Mal-PEG-FINPs are plotted in SI, Figure S.4. Also studied were NPs functionalized with Mal-PEG2K-methoxy (PEG-MeO) and a short ($n = 3$ monomers) chain bis-maleimide PEG, BM(PEG). Although less common, short chain PEGs also induce stealth characteristics and prevent aggregation and may be superior for application that require a narrow PEG corona.^[53–55] Lower amounts ($<75\,000:1$ starting ratio) of PEG produce the most stable particles, with the small addition to hydrodynamic radius for PEG-MeO and Mal-PEG-Fl. In contrast, short length PEGs do not alter the ζ or size of the NPs at any of the concentrations studied (SI, Figure S.4). This BM(PEG) ligand also offers the potential both to passivate via PEG and to link affibody to the NPs (Figure 1). Because of this compact size and favorable effect on ζ , as well as the ease with which targeting ligands are next functionalized, the BM(PEG) ligand was selected for future synthesis. After synthesis with BM(PEG) only, NP size increased to 193.9 nm with a polydispersity index (PDI) of 0.141. The 19 nm increase (19 nm on each side for total 38 nm increase) due to PEG follows from the size of the ethylene glycol repeats and two maleimide groups. The PEG monomer is very hydrophilic, strongly influencing the NPs' hydrodynamic radius, and causes DLS increases larger than the length of PEG chain. The increase in PDI could also be due to aggregation and/or unintentional removal of smaller NPs during purification. Alternatively, for experiments linking a protein containing free amines, e.g. avidin, streptavidin, or IgG, an analogous *N*-hydroxysuccinimide (NHS) ester, Mal-PEG-NHS ester (SM(PEG)) could be used at the same concentrations as BM(PEG) (see SI).

2.3. Affibody

Two different EGFR-binding affibodies were evaluated in this study. The first was a commercially available dimer with a molecular weight of 13.9 kDa, an optical density at 280 nm of 2.47 at 1.0 mg mL^{-1} , and a molar extinction coefficient of $34\,200\text{ m}^{-1}\text{ cm}^{-1}$.^[56] The second was an acetylated (Ac), cysteine (Cys)-terminated three-helix affibody monomer (Ac-Cys-Z(EGFR:1907)) via solid phase peptide synthesis previously reported by our group.^[44,45] A Her-2 specific affibody (ZHER2:342) was also used as a control. Her-2 (also Her-2/Neu or EerB-2) is the human epidermal growth factor receptor 2.^[57] This peptide had a 1.0 mg mL^{-1} optical density (OD) = 2.74 at 280 nm. Both contained an N-terminal cysteine for sulphydryl chemistry, amenable to labeling and NP binding.

To first confirm that the affibodies bound EGFR before conjugation to NPs, flow cytometry (FC) was performed with affibodies labeled with Alexa 680 via the N-terminus cysteine and purified by size exclusion chromatography.^[58] The fluorophore-to-affibody ratios in the final products were 1.3 and 0.86 for the homemade and commercial conjugates, respectively. We used both conjugates to label the A431 cell line, which is an EGFR-overexpressing cell and RAMOS (EGFR-negative cells) and analyzed via FC. Mean fluorescent signal intensity (MFI) from the A431 line was 10.5 times higher than that from RAMOS via the commercial affibody and 39.7 times higher via the affibody monomer.

2.4. Integrated NP

The NPs were next conjugated with affibodies after integration of a fluorescent layer to provide a concurrent imaging system for high throughput cell assays via FC (Figure 1A). Although the Raman modality is the focus of this text, the current generation of Raman microscopy and cytometry prevents large ($n = 10\,000$) cell analysis procedures and thus FC was employed for rigorous confirmation of targeting. The fluorescent label was optimized independently of the affibody and titration of increasing amounts of maleimide-fluorophore, starting fluorophore-to-NP ratios of 10^4 , 10^5 , and 5.0×10^5 gave NPs with covalently labeled fluorophore at ratios of 160, 1050, and 1610, respectively. For intense signal, the 5.0×10^5 starting molar ratio of NP:fluorophore was selected for all preparations.

Next, to model the amount of protein that could be immobilized onto the surface via BM(PEG) and to deploy for other applications, we created both IgG and avidin-coated NPs (Table 1 and SI, Figure S.5). Other work bound the peptides RGD, RAD, and VRPMLQ to NPs via BM(PEG) and SM(PEG).^[13] These studies also measured the amount of protein bound per particle. Since the affibody is equipped with only the N-terminus cysteine reactive site, quantifying the amount bound to particle via a reporter molecule is difficult. While it is possible to estimate the amount covalently bound by the difference in absorbance at 280 nm of the sample before and after reaction, this technique is generally inaccurate.

The final product was diluted in normal saline to 0.1 nm for characterization. Its hydrodynamic radius was 214.0 nm with a PDI of 0.254 and $\zeta = -14.5$ with a sedimentation time of 7.25 h. A TEM image of the conjugation (SI, Figure S.1B) shows the core-shell structure intact. The affibody:NP ratio was estimated at 400–450 based on an IgG model (see SI, Figure S.6C), although indirect measurements based on ΔA_{280} indicated loading above 10 000 affibody:NP. This increase in PDI results from different affibody:NP ratios and may also be due to aggregation, which could potentially result in signal variation. A nearly monodisperse NP sample generally has a PDI < 0.1. While the PDI is larger and may cause larger variations in signal, the use of NPs with PDI values up to 0.3 is common.^[59]

A similar protocol (SI, Figure S.6A) was used for other EGFR-binding ligands and the results compiled in Table 1. The ligand loading levels were determined via fluorescence labeling of the ligands and were controlled easily via starting stoichiometry (SI, Figure S.6C,D). Interestingly, the particle size and ζ are similar at approximately 220 nm and -20 mV across the various types of ligands including affibody, avidin, and goat anti-mouse IgG (secondary IgG; 2° IgG). An important exception is anti-EGFR IgG. This ligand caused severe, macroscopic aggregation of the NPs during both cysteine (BM(PEG)) and amine-based (SM(PEG)) coupling. Reaction time and buffer, ligand ratios, and commercial vendor were varied to resolve the situation, with no success. The avidin and 2° IgG particles were effective at labeling cells when used with a primary IgG (SI, Figure S.6B,C). For direct EGFR imaging however, the affibody NPs were chosen for simplicity as they need no further reagents, e.g., biotinylated anti-EGFR IgG, for labeling.

Next, we determined the ability of the hybrid affibody-dimer nanoparticle to label cells. The A431 and MDA-435S (low EGFR) cells were stained with increasing concentrations of NPs from 13 to 40 pm with 27 pm producing the best signal versus nonspecific binding to control cells (SI, Figure S.5D). Additional wells were stained with the native NPs as well as those with PEG linker to determine this contribution to nonspecific binding. One well of the A431 cells were incubated 60 min prior with $5 \mu\text{L}$ of 1 mg mL^{-1} free affibody as a competitive inhibition control. The MFI of the fluorophore on NP surface in FC is reported in Figure 2. The MFI by FC of the A431 cell line was 599.2 and of the MDA-435S cell line was 39.0: a 15-fold increase in signal. When competitively inhibited, the MFI of A431 decreased to 138.1, a 4-fold reduction. An additional control incubated A431 cells with NPs coated with Her-2 affibody (ZHER2:342) identically to the EGFR affibody. This cell population had a MFI of 151.1. While A431 cells do express Her-2, it is at a much lower level than EGFR.^[60] The labeling capacity of affibody dimer versus monomer NPs was also studied. At isomolar coating densities, the commercial dimer affibody NPs produced 1.5-fold more signal and were used for the remainder of the study.

After FC, we examined the cells with Raman microscopy, removed by threshold pixels below 20% of the maximum, and overlaid the result with the white-light photomicrograph, resulting in Figure 3. To quantify these results further, Raman maps were converted to 8-bit grayscale images via ImageJ (NIH, Bethesda, MD) and analyzed for total area, area above threshold, mean pixel intensity, and integrated density. The mean intensity was then

corrected by the cell count in each field of view. Values of 0.04 ± 0.04 , $2.74 \times 10^{-3} \pm 3.88 \times 10^{-3}$, 0.10 ± 0.05 , 0.80 ± 0.16 , and 0.06 ± 0.05 a.u. were measured for the PEGylated NPs (Figure 3D), Her-2 targeted NPs (Figure 3E), competitive inhibition control (Figure 3F), targeted NPs (Figure 3G), and MDA-435S cells (Figure 3H), respectively. No signal was measured in cells incubated with native NPs (Figure 3C) or normal saline (Figure 3A). Cells incubated with affibody free of NPs gave a signal of 0.005 (Figure 3B). Cells incubated with targeted NPs (Figure 3E) are 8.0 ($p < 0.0023$ by analysis of variance (ANOVA)), 13.3 ($p < 0.015$), and 292.0 a.u ($p < 0.013$) times higher than the inhibited cells and MDA-435S, and Her-2 controls respectively. The low signal of the native NPs and PEG linker control suggest that nonspecific binding in these experiments derives mostly from ligand nonspecificity and PEGylation aberrations rather than bioconjugation inefficiencies and poor nanoparticle behavior.

A fundamental feature of molecular imaging is measurability of the biological target. To determine whether our NP system is capable of discriminating between different amounts of EGFR, five unique cells lines (A431, LoVo, U87MG, MDA-435S, and HT29) were analyzed for EGFR expression levels concurrently and independently with both FC and Raman imaging. Unlike in Figure 2, the FC signal here arises from a primary/fluorescent secondary antibody and not the fluorescent NP. Figure 4 plots the Raman intensity as detailed above for Figure 3 versus the MFI from FC. The Raman signal correlates with the levels of EGFR as determined by an independent assay at $R^2 = 0.92$.

2.5. Tumor Labeling

After validating the NPs in cell culture, we next deployed them to label tumor models mimicking those present on the colon wall. The labeling protocol models a bowel preparation protocol that involves a barium contrast agent, but with molecularly targeted material.^[48] Two sets of controls were performed—one varied the NP type versus constant tissue and the second varied the sample type in presence of consistent NP type. In the first example, all sample types were the EGFR positive A431 type. These were stained with both the EGFR-affibody NPs as well as untargeted (blank) NPs. As a further validation of EGFR imaging, tissue was stained with affibody NPs after the tissue was pre-incubated with free EGFR affibody for competitive inhibition.

In a typical analysis, 3–5 washed tumors were loaded on a quartz slide and raster scanned across the Raman microscope stage. Approximate areas were $2.0 \text{ cm} \times 5.0 \text{ cm}$, with 1.0 mm resolution for 1000 data points consisting of a Raman spectrum, and total acquisition time below 30 min. Spectra for three different sample types are presented in Figure 5A. Many different approaches to interpreting such spectra exist, including peak intensity, peak to baseline, principal component analysis, and dynamic least squares.^[36,61] The least squares approach was selected due to its long-standing role in Raman quantification and mixture deconvolution. Additional details on this approach have been reported elsewhere.^[33,36,61,62] The output of this method ranges between zero (no match) and unity (complete match), with the resulting number multiplied by 10^6 . The result of these maps and white-light photographs of the tumors are presented in Figure 5B. An initial analysis used the average of the five most intense pixels for one tumor set. This approach is useful because it does not attenuate the signal. Furthermore, differences in tumor size make averaging the total intensity difficult, and thus selecting the most intense pixel set out of each tumor image was utilized. The use of five pixels was explored below. The average of the most intense pixels for three replicate tumors for targeted NPs was 1072.6 ± 817.3 , untargeted NPs were 120.3 ± 99.1 and competitive inhibition tumors were 191.3 ± 138.2 . Tumor controls using normal saline or $50 \mu\text{g mL}^{-1}$ EGFR affibody had an undetectable signal. Thus, targeted NPs produced a signal 8.9 times more intense the untargeted NPs and 5.6 times higher than the

competitively inhibited samples, suggesting that the NPs specifically label the EGFR receptors in biological specimens.

The second set of controls all employed EGFR-targeted NPs, but varied the sample type. Samples included the EGFR over-expressing A431 tumors, low EGFR expressing MDA-435S tumors, and epidermis adjacent to the tumor location. This skin specimen helped model the ability of the NP probes to discriminate between tumor and the adjacent tissue present in the colon. While there are clearly large physiological and morphological differences between murine epidermis and normal bowel, both express low levels of EGFR.^[56,63] These samples ($n = 5$) were analyzed as above and the results plotted in Figure 5C. Signal from A431 tumors were 34.7-fold higher versus negative tumors and are significant at $p < 0.005$ by ANOVA. Versus skin, the intensity difference decreases to 7.1-fold, but is still significantly less than A431 tumors at $p < 0.009$ by ANOVA. When only the three most intense pixels were used, the significance changed to $p = 0.027$ for A431 versus epidermis and $p = 0.019$ for A431 versus MDA-435S tumors. When the seven most intense pixels were used, these values change to $p = 0.003$ and $p = 0.001$, respectively, suggesting that five is an acceptable number of pixels to use for future analysis.

3. Discussion

This work is the first use of Raman imaging via the intense and photostable silica nanoparticles and demonstrates important progress in molecular imaging, including the critical extensive validation of signal presented. However, some error sources can contribute to variability in SERS imaging. First, there is inherent variation in the Raman signal. Although the NP synthetic protocol is optimized, there does exist some particle-to-particle variation in size and intensity (SI, Figure S.1).^[39] As this approach to Raman imaging employs an ensemble method, this contribution is relatively low. There is also tumor heterogeneity in the intensity of EGFR expression in tumor tissue,^[64] that is, different areas of the tumor express different biomarkers at different levels. This is likely a more substantial contribution to variance. Finally, there are inconsistencies with the optics. Fluctuations with the laser and CCD variation, as well as the inability to maintain a stable focal plane across nonplanar tumors could cause unreal signal modulations. Nonspecific binding arises from both NP sources and ligand sources. While nonspecific binding of the targeted particles is low, Figure 2 and 3 do suggest that the affibody and PEGylation are the primary sources of poor specificity and not the NP surface chemistry.

Analysis in vivo was not investigated, as it is unnecessary for endoscopic measurements with the Raman colonoscope under development in our lab. Bowel preparation with the NP enema will also account for tumor heterogeneity by saturating all areas of the colon with contrast agent. Although the cell culture work and tumor xenografts described here are helpful models in determining the labeling efficiency of EGFR affibody NPs, a more useful sample would be tumors from within the bowel. Such a model is underway. Importantly, no substantial recalibration of the NPs will be needed when transitioning between murine and human EGFR, as the affibody studied here is reactive with both species.^[65] Raman-guided colonoscopy in the clinic would utilize either a topical (enema) application or oral ingestion of the contrast agent. Work in preparation for publication elsewhere suggests that the former is effective at specifically staining only the colon with no leakage to circulation or toxicity concerns.

Previously, scFv fragments were used to label EGFR in vivo with nonsilica-coated Raman NPs.^[34] The affibody ligand offers improved affinity (nM) over the scFv ligand, and the silica-capped NPs possess long-term (years) stability important for clinical applications. Other work uses the SERS signal of the ligand directly or a gold-polymer hybrid NP, but

illustrate no validation beyond cell culture.^[66,67] Molecular imaging for colonoscopy is being explored by other groups using positron emission tomography (PET) and other imaging modalities.^[68] Virtual colonoscopy with computed tomography (CT) or PET-CT is an interesting alternative, but still involves a high radiation dose.^[6,69,70] Capsule-assisted endoscopy is also intriguing, but is incapable of detecting flat lesions or glean molecular imaging content.^[71]

4. Conclusion

To the best of our knowledge, this is the first example of molecular imaging with core-shell Raman NPs and the first to deploy the affibody recognition element with Raman imaging. The small size, high affinity, and long-term stability of the affibody ligand allows the preparation of contrast agents not possible with traditional IgG. Future work will involve small animal models of colon cancer, smaller NPs for in vivo work, and CD44v6 for targeting.^[12] The Raman design offers seamless integration with existing colonoscopy instrumentation and is easily expandable to other endoscopic areas including the mouth, esophagus, stomach, bladder, and even intraoperative applications. The integration of specifically targeted NPs increases further the capacity for such imaging and is the next step toward Raman-assisted colonoscopy. Other molecular targets can be similarly imaged simply by changing the affibody or other ligand on the NP. Further, because of the facile spectral deconvolution of Raman mixtures, the multiplexing measurement of different molecular targets should be straightforward.

5. Experimental Section

Reagents

Gold core-silica shell SERS nanoparticles were purchased from Oxonica (Mountain View, CA). All NPs used in this study employed trans-1,2-bis(4-pyridyl)ethylene (BPE) as the Raman reporter.^[36] Phosphate buffered saline, 2-(*N*-morpholino) ethanesulfonic acid (MES), 3-mercaptopropyl-trimethoxysilane (MPTMS), (tris(2-carboxyethyl) phosphine (TCEP), and sodium chloride and were purchased from Sigma-Aldrich. From Pierce (Rockford, IL), we purchased 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), 1,11-bis-maleimido-triethyleneglyco (BM(PEG)), and succinimidyl-[(*N*-maleimido-propionamido)-tetraethyleneglycol] ester (SM(PEG)). Methoxy PEG of molecular weight 3.4 K and a fluorescein-capped version of the same were purchased from Rapp Polymere (Tubingen, German). Thiol reactive, maleimide-Alexa Fluor 647 and 680 were purchased from Invitrogen (Eugene, OR) and the affibody dimer was produced by Affibody, Inc., (Stockholm, Sweden).

Bioconjugation

Activation of the NP surface used 10 mM MES buffer of pH 6.8 for binding of maleimide-fluorophore to the surface and pH 7.3 for heterobifunctional linkers. For affibody binding, the peptide (at 15 000 molar equivalents per NP) was first reacted for 30 min with 5 mM TCEP to reduce any cysteine disulfides and expose a free sulfhydryl. This was then added to fluorescent NPs coated immediately prior with BM(PEG) (at 15 000 molar equivalents per NP) and washed to remove excess cross linker. The affibody-NP mixture was reacted for 2 h and purified via centrifugation and washing or dialysis.

Equipment

Size and zeta potential were obtained via a dynamic light scattering (DLS) on a Zetasizer-90 instrument from Malvern Instruments (Worcestershire, UK). The ζ measurements were made using the Smoluchowski model (parameters detailed in the Supporting

Information).^[47] Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Transmission electron microscopy (TEM) images were acquired on a Tecnai G2 F20 X-TWIN instrument from FEI (Hillsborough, OR). To measure SERS signals, we used a customized Raman microscope (InVia, Renishaw, Gloucestershire, UK). Since previous reports,^[33,36] this microscope has had additional customization including the integration of a 785 nm point source laser, piezo-controlled stage for micrometer-resolved spatial mapping, and a 1 inch CCD detector (where 1 inch $\approx$ 2.54 cm) for a spectral resolution of 1.07 cm^{-1} . For cellular studies, an infinity-corrected 20 $\times$ objective (NA = 0.4) was used. Mapping scans with dimensions of approximately 110 $\mu\text{m} \times 150 \mu\text{m}$ were collected in under 4 min and repeated up to five times on different fields-of-view at 3.7 μm resolution. Each spectrum was analyzed by least squares analysis by Wire 2.0 Software (Renishaw).

Cell Culture

A431, HT29, U87MG, and MDA-435S cells were grown in DMEM and LoVo cells used F-12K media all supplemented with 10% fetal bovine serum. For NP labeling, cells plated at 250 000 cells per well in a 12-well polystyrene plate and allowed to proceed overnight to 80% confluence. Staining volume was 200 μL of 1% BSA in PBS with varying amounts of NPs for 60 min on ice. Control cells were labeled with 1 μL of 1 mg mL^{-1} primary antibody to EGFR followed by 1 μL of 2 mg mL^{-1} of a secondary antibody labeled with Alexa Fluor 488. After incubation, cells were washed twice with 1% BSA/PBS to remove nonspecifically bound NPs, trypsinized at 37 $^{\circ}\text{C}$, and subjected to FC. After FC, the cells were centrifuged to a pellet and a 5 μL aliquot loaded onto a quartz microscope slide underneath the Raman microscope.

Animal Models

Both A431 and MDA-435S 5×10^6 cells were inoculated into nu/nu mice ($n = 5$) in 50% Matrigel (BD Biosciences) per tumor site and allowed to grow to diameters of 5–10 mm followed by explantation. All animal work was conducted in accordance with the Administrative Panel on Laboratory Animal Care at Stanford University. If not imaged immediately the tumors were stored in 1% BSA in PBS. Alternatively, the tumors were preserved with 4% formalin. In either case, the tumors were immersed in a polystyrene-well plate loaded with 300 μL of 27 pm NPs and incubated for 1 h on ice. After incubation, the tumors were washed three times with 1% BSA/PBS and examined with Raman microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Preparation of NPs: a gold core with Raman-active molecules is coated with a silica shell for stability and SERS intensity. The NPs are first coated with a fluorophore (star) for high throughput cell-based assays (A) and remaining thiols react with a bis-maleimido short ($n = 3$) PEG (coil) cross-linker (B). After removing excess PEG, affibodies specific to EGFR or Her2 are bound to the NP surface (C). RT denotes room temperature reactions.

Figure 2.

Validation of SERS NPs with Cultured Cells: EGFR positive (A431) and negative (MDA-435S) cell lines were labeled with fluorescent SERS NPs coated with Alexa Fluor 647 and EGFR affibody, and analyzed via FC. Controls include an isotype affibody (Her-2) and competitive inhibition (block) of A431.

Figure 3.

Raman Intensity Maps: white-light images with Raman intensity maps superimposed indicate differences between various samples types. The dashed line indicates the area within the white-light image subjected to Raman mapping. Here, EGFR positive A431 cells were incubated with normal saline (A), EGFR affibody unbound to NPs (B), native SERS NPs (C), PEGylated NPs (D), and NPs with an isotype control affibody (E). Both (F) and (G) show A431 cells incubated with NPs targeted to EGFR via an affibody, although (F) was previously incubated with free affibody ($25 \mu\text{g mL}^{-1}$) as a competitive inhibition control. Sample (H) is stained EGFR-negative MDA-435S cells with the EGFR-targeted NPs. Scale bar = $20 \mu\text{m}$.

Figure 4.

Independent Verification of EGFR Imaging by SERS NPs: five different cell lines with unique EGFR expression levels were analyzed by both FC (A) and Raman imaging (B), similar to Figure 3. The intensity of the two measurement modalities correlates above $R^2 = 0.90$ suggesting the Raman molecular imaging may be used to quantitatively examine EGFR expression levels.

Figure 5.

Validation of SERS NPs with explanted xenograft tumors: A) Representative spectra for A431 tumors labeled ex vivo with three different NP types: targeted (dots; via anti-EGFR affibody), targeted with competitive inhibition (solid), and untargeted (dashes; blank). Specific signals arise from the targeted NPs. B) Collections of spectra create Raman maps from whole tumors and signal quantitated in (C). Error bars represent the standard error and * indicates significance at $p < 0.01$.

Table 1

Characterization of SERS NPs with Various EGFR Ligands. Different strategies for labelling EGFR-positive cells with SERS NPs include avidin/biotin, primary antibodies, secondary antibodies, and antibodies. A bis-maleimide PEG cross-linked thiol-containing ligand and a maleimide-NHS heterobifunctional linker was deployed for amine applications. The amount of ligand per NP was determined via fluorescence measurements of purified NPs. Signal-to-background ratio (SBR) for avidin and IgG is defined as FC signal with 1 ° or biotinylated IgG and the NP versus fluorescent signal from NP alone without the EGFR-targeting ligand. For the antibody NPs, SBR is the ratio of the positive signal versus competitive inhibition or versus a negative cell line (*). n.d. = not determined.

Ligand	Linker	Ligand:NP	Zeta (ζ), [mV]	Diameter (DLS), [nm]	SBR	Stability
Avidin	SM(PEG) ₄	10:250	-16.8 ± 4.6	269 ± 146	7.7	Months
2 ° IgG	SM(PEG) ₄	290:925	-19.6 ± 4.5	230 ± 13	4.5	Months
2 ° IgG	BM(PEG) ₃	150:400	-19.6 ± 5.2	209 ± 58	13.5	Months
1 ° IgG	SM(PEG) ₄	n.d.	-20.0 ± 7.0	6500	n.d.	None
1 ° IgG	BM(PEG) ₃	n.d.	n.d.	5000	n.d.	None
Affibody	BM(PEG) ₃	410:925	-14.5 ± 5.0	214 ± 20	4.3 (15.4*)	Months