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Microscopic investigation of topically applied nanoparticles for molecular imaging of fresh tissue surfaces

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Abstract

Previous studies have shown that functionalized nanoparticles (NPs) topically applied on fresh tissues are able to rapidly target cell-surface protein biomarkers of cancer. Furthermore, studies have shown that a paired-agent approach, in which an untargeted NP is co-administered with a panel of targeted NPs, controls for nonspecific behavior of the NPs, enabling quantitative imaging of biomarker expression. However, given the complexities in nonspecific accumulation, diffusion, and chemical binding of targeted NPs in tissues, studies are needed to better understand these processes at the microscopic scale. Here, fresh tissues were stained with a paired-agent approach, frozen, and sectioned to image the depth-dependent accumulation of targeted and untargeted NPs. The ratio of targeted to untargeted NP concentrations – a parameter used to distinguish between tumor and benign tissues – was found to diminish with increasing NP diffusion depths due to nonspecific accumulation and poor washout. It was then hypothesized and experimentally demonstrated that larger NPs would exhibit less diffusion below tissue surfaces, enabling higher targeted to untargeted NP ratios. In summary, these methods and investigations have enabled the design of NP agents with improved sensitivity and contrast for rapid molecular imaging of fresh tissues.

Keywords

nanoparticle; molecular imaging; optical tags; SERS nanotags; SERS nanoantennas; nanomedicine; Raman spectroscopy; biomedical optical imaging

1. Introduction

Early detection and effective treatment of cancer are essential for minimizing morbidity and mortality. Existing cancer screening methods include imaging techniques (*e.g.* X-ray, CT, MRI, white-light endoscopy, and ultrasound) and cytological approaches (Pap smear or occult blood detection). These traditional detection methods, however, are often limited in terms of detection sensitivity and the ability to differentiate between benign and malignant lesions, which ultimately hinders their ability to detect cancers at the earliest stages. [1–4]

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For intraoperative guidance of tumor resection, a variety of imaging approaches have also been employed, including 2-D specimen X-ray and frozen-section pathology. However, the sensitivity and specificity of specimen X-ray is limited and frozen-section pathology suffers from sampling limitations and artifacts, especially for tissues with high lipid content (*e.g.* breast tissue) that do not freeze well. [5–7]

Molecular imaging approaches have the potential to identify tumors with a high degree of sensitivity and specificity. [8–10] However, studies have shown that there is significant molecular heterogeneity amongst different patients, and even within individual patients over time due to molecular changes associated with tumor progression. [11, 12] Therefore, multiplexed imaging of many biomarkers is necessary to detect tumors in diverse patient populations with high sensitivity.

Advances in nanotechnology have enabled multiplexed molecular profiling of tumors for early detection and intra-operative guidance of tumor resection. [13–16] A variety of nanoparticles (NPs) are now available for use as contrast agents in medical imaging; these NPs include quantum dots, carbon nanotubes, gold NPs, and nanowires. [17–21] Surface-enhanced Raman scattering (SERS) NPs have attracted particular interest as contrast agents for optical imaging studies owing to their brightness, low toxicity, photostability, as well as the unique and well-defined light spectrum emitted by each flavor of SERS NP when illuminated at a single common wavelength (*e.g.* 785 nm). [22–31] Since each flavor of SERS NP has a distinct spectral signature that identifies them from each other, many biomarkers can be targeted simultaneously with a panel of NP flavors (each targeted to a different biomarker) to acquire a more complete picture of the molecular phenotype of tissues. Note that these SERS NPs are often referred to as optical tags, SERS nanotags, or SERS nanoantennas. [32–35] However, to be consistent with the terminology used in our previous publications, we will continue to use the term SERS NPs here. Surface functionalization of these and other NPs with targeting moieties (*i.e.* antibodies, oligonucleotides, aptamers, peptides, and other small molecules) can impart the NPs with a high specific binding affinity to a variety of cell-surface cancer biomarkers for enhanced retention of the NPs at biomarker-positive cancer sites. [19, 36, 37]

Previously, we have developed a NP-based molecular imaging (NMI) strategy, with which we have demonstrated that rapid multiplexed molecular detection is possible under time-limited staining and detection conditions, as encountered in point-of-care clinical settings. [24–26, 38–41] In our initial studies, fresh tissue specimens were topically stained with a panel of SERS NPs (*in vivo* or *ex vivo*) and imaged after a brief rinse step to rapidly identify/quantify the presence of tumor-related biomarkers (Supplementary Figure 1). Unfortunately, uneven NP delivery, retention, and wash out at different tissue locations often results in misleading image contrast. [25, 26] For example, we have observed that increased diffusion and passive retention can often result in higher nonspecific accumulation of topically applied NPs in benign tissues in comparison to denser tumor tissues. [25] One method that has been utilized to mitigate these non-specific effects is to co-administer an untargeted (control) NP flavor simultaneously with the targeted NP(s) during imaging experiments. By calculating the ratio of the concentration of targeted NP(s) to the untargeted control NP, the effects of nonspecific accumulation may be normalized away. We have

shown that these NP ratios provide a linear and quantitative readout that correlates with biomarker expression levels. [25, 26, 39, 40]

Despite the strengths of such a ratiometric imaging approach, nonspecific accumulation and passive retention of NPs is often quite high, which can limit the sensitivity and accuracy of biomarker detection/quantification. In order to better understand and quantify the specific vs. nonspecific accumulation of our NPs, here we developed a method to investigate the diffusion and accumulation of targeted and untargeted NPs as a function of depth in fresh tissues at the microscopic scale. In short, we stained fresh tissues with a paired-agent approach using fluorophore-labeled NPs, and then snap froze the tissues at various time points to immobilize the NPs within the tissues. We then performed fluorescence microscopy of thin frozen sections of these tissues (cross-sectional views of the tissue) and quantified the penetration and binding of the targeted and untargeted NPs as a function of depth. The findings from these studies led us to hypothesize that the use of larger NPs could reduce the diffusion of the NPs, and therefore reduce the nonspecific accumulation of the NPs in tissue. We show that this allows for molecular imaging of fresh tissue surfaces with higher NP ratios (targeted vs. untargeted) compared with our original NPs, and that the staining can be achieved more rapidly than before (6-min topical application).

2. Materials and methods

2.1. SERS NPs and functionalization

SERS NPs were purchased from BD (Becton, Dickinson and Company). These NPs consist of a 60-nm diameter gold core, a layer of Raman reporters adsorbed onto the surface of the gold cores, and a 30-nm thick silica coating that encapsulates the gold cores, resulting in an overall diameter of ~120-nm. TEM images were obtained to confirm uniform size distribution of 120-nm NPs (Supplementary Figure 2). This size distribution is consistent with what has been measured in the past by others utilizing these same types of commercial SERS NPs. [42,43] The two “flavors” of NPs used here are identified as S420 and S440, and each of these emits a characteristic Raman spectrum attributable to chemical differences in the Raman reporter layer. The NPs, which contain reactive thiols at their surface, were first reacted with fluorescent maleimides and then conjugated to monoclonal antibodies (mAbs) via a bifunctional PEG linker (Thermo Scientific, PI22112). The NP flavor S420 was reacted with the fluorophore DyLight 650-4xPEG Maleimide (Thermo Scientific, Product No. 62294) and then conjugated with an anti-EGFR mAb (Thermo Scientific, MS-378-PABX) at 500 molar equivalents per NP, while the NP flavor S440 was reacted with fluorophore DyLight 550-2xPEG Maleimide (Thermo Scientific, Product No. 62292) and then conjugated with an IgG1 isotype control mAb (Thermo Scientific, MA110407) at 500 molar equivalents per NP. All reactions were performed using a reaction buffer consisting of 10 mM MOPS (Sigma-Aldrich, part No. M1254) buffer, pH 7.25. The NP conjugates were stored at 4°C and protected from light before use. UV-VIS spectrophotometry (Agilent 8453) was used to measure the concentration of the NP conjugates. Hereafter, the EGFR-targeted NPs and the IgG1 isotype control NPs will be referred to as “targeted NPs” and “untargeted NPs,” respectively.

Previous flow cytometry experiments demonstrated preferential binding of the EGFR-targeted NPs to A431 cells, which highly overexpress EGFR. Experiments have shown that the isotype NP is a highly accurate control for the nonspecific behavior of targeted NPs. This has been demonstrated using both flow cytometry and imaging of EGFR-negative cells and tissues, respectively, in which both the targeted and untargeted NPs have always exhibited identical levels of nonspecific accumulation. [24–26, 39]

2.2. Tumor xenografts

Nude mice (Taconic Farm Inc.) were used to develop tumor xenografts. Approximately 1×10^6 A431 cancer cells were suspended in matrigel (BD biosciences, 354234) in a 1:1 volume ratio to form a 100- μL mixture. At 7–9 weeks of age, nude mice were subcutaneously implanted with the cell mixture (1×10^6 cells per injection) subcutaneously on their flanks. After 2–4 weeks, when the tumors reached a size of 8–10 mm, the mice were euthanized by CO_2 inhalation and the implanted tumors were surgically removed. Benign tissue (control) was obtained from male Fischer 344 inbred rats (10 weeks, Harlan Laboratories, Inc.), which were euthanized via inhalation of CO_2 , followed by the surgical removal of $1 \times 1 \text{ cm}^2$ sections of normal muscle tissue. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC, No. 4345-01) at the University of Washington.

2.3. Staining, rinsing, and snap-freezing of fresh tissue specimens (micro-NMI)

Fresh tissue specimens were stained for up to 15 min with an equimolar mixture of the targeted NP and the untargeted NP (150 pM). Tissue staining was accomplished by placing the tissue surface of interest onto a drop of the staining mixture and gently dipping the specimen in and out of the solution ($\sim 50 \mu\text{L}$), which has been shown in previous publications to promote convection of the NP mixture at fresh tissue surfaces, allowing for accelerated binding of NPs to their respective biomarker targets. [40, 44] The tissues were then submerged briefly ($\sim 2 \text{ s}$) into 20 mL of phosphate buffered saline (PBS) – to rinse off unbound NPs at the surface of the tissue – and placed in a $10 \times 10 \times 5 \text{ mm}^3$ cryomold containing Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek USA, INC.). The cryomold was quickly frozen ($< 10 \text{ s}$) in 2-methylbutane (Sigma Aldrich) chilled in liquid nitrogen and cryosectioned into 10- μm -thick sections. These sections were then placed on a glass slide, fixed in formalin, and covered with a cover slip. This procedure is illustrated in Figure 1 and is hereafter referred to as “micro-NMI.”

2.4. Fluorescence microscopy

Fluorescence microscope images were acquired on a Leica DMI8 widefield microscope with a 20x Plan Apochromat 0.70 numerical aperture (NA) lens. The ET555 emission filter was used for imaging untargeted NPs (reacted with DyLight 550 fluorophores) and the ET645 emission filter was used for imaging targeted NPs (reacted with DyLight 650 fluorophores). A DFC9000 sCMOS camera was used for fluorescence detection. Images were collected using the Leica Application Suite and processed in MATLAB. The effective pixel size (Nyquist sampling) was 0.46 μm .

2.5. Image processing

The image processing method is shown in Figure 2 for a tissue section from a single A431 tumor xenograft stained for 15 min with an equimolar NP mixture. Bright field and fluorescence images are shown on the left. The average of all raw line profiles oriented perpendicular to the tissue surface is shown for the targeted NPs (top row) and untargeted NPs (bottom row). The fluorescence intensity was calibrated using fluorescence images of the staining mixture at a known concentration (here, the staining concentration was 150 pM). The tissue autofluorescence background was measured from the average intensity of the unlabeled tissue, and was subtracted from the NP profiles ("Background subtraction"). The maximum intensity of the NP intensity profiles was assumed to correspond to the tissue surface, which defines the zero depth on the x axes in Figure 2 ("Crop to surface"). Finally, the depth-integrated NP concentrations (area under the curve) were evaluated to verify that the micro-NMI results agreed with the signals obtained with the wide-area NMI technique ("Depth-integrated NP concentration"). Note that this assumes that the penetration depth of the NPs is small enough that the wide-area NMI technique collects the depth-integrated signal from all NPs at each point (pixel) on the tissue that is probed during raster-scanned spectral imaging (*i.e.* the effects of tissue scattering/absorption are negligible at such short tissue depths of $<20\ \mu\text{m}$). All image processing was carried out in MATLAB R2015a.

2.6. Silica NP thiolation and conjugation

To investigate the impact of NP diameter on tissue diffusion and molecular imaging contrast, silica NPs (SiNPs) with diameters of 200-nm and 300-nm were purchased from NanoComposix (SISN200-10M and SISN300-10M) and conjugated to fluorescent dyes and antibodies in a similar manner to our SERS NPs. SiNPs were used due to the lack of commercial SERS NPs available in different sizes. Note that since the SERS NPs are encapsulated in a silica shell, the behavior of these NPs (SERS NPs and SiNPs) are similar in terms of diffusion and chemical binding (both specific and nonspecific) when functionalized in a similar manner. To enable PEGylation and conjugations comparable to that of our SERS NPs, sulfhydryl groups were introduced on the SiNP surfaces by heating the SiNPs for 1 hour at 72°C in ethanol containing 1% (v/v) (3-Mercaptopropyl) trimethoxysilane (Sigma Aldrich) according to a protocol described previously. [45] The surface-modified SiNPs were thoroughly washed with ethanol and water to remove the surface modifier and were then redispersed in the reaction buffer (10 mM MOPS, pH 7.25). PEGylated SiNPs were then functionalized in the same manner as the SERS NPs –each NP size was subjected to two different sets of conjugation reactions: 1) DyLight 650-4xPEG Maleimide and an anti-EGFR mAb or 2) DyLight 550-2xPEG Maleimide and an IgG1 isotype control mAb. UV-VIS spectrophotometry (Agilent 8453) was then used to measure the concentration of the SiNP conjugates.

To ensure that the brightness of the fluorescence from each of the NP sizes was relatively well-matched, the NP concentrations used in the conjugation reactions were tuned such that total surface area of the NPs (total sum of the mixture) was identical to that of the 120-nm SERS NPs used in previous experiments (and reacted with the same concentration of fluorophores). To verify that this was true, we used a spectrofluorometer (Perkin Elmer, LS-50B) to show that the different NP sizes exhibited comparable levels of fluorescence for

aliquots with identical total NP surface areas (Supplementary Figure 4). Flow cytometry experiments were performed to verify that the binding of the targeted NPs to EGFR-positive cell lines was also comparable in magnitude for all NP sizes when staining cells with NP concentrations that were matched in terms of total NP surface area (Supplementary Figure 5).

3. Results

We first verified that the signals obtained with micro-NMI agree with our standard wide-area NMI approach for imaging fresh tissue surfaces (Figure 3). Fresh tumor xenografts and benign tissue specimens (*ex vivo*) were stained with an equimolar NP mixture (150 pM for each NP flavor) and rinsed briefly (~2 sec). Wide-area NMI images were obtained according to the protocol described in Supplementary Figure 1. Ratiometric images of EGFR-targeted vs. untargeted NP concentrations showed elevated ratios (1.60 ± 0.18) for A431 tumor xenografts (which overexpress EGFR) and a ratio of unity (1.08 ± 0.05 ; negligible differential binding of the targeted NP) for benign tissues (Figure 3A). An identical tissue-staining protocol was used with identical tissues, which were then snap frozen after various staining time points and processed with the micro-NMI technique illustrated in Figure 1 and 2. Figure 3B illustrates the concentrations of targeted and untargeted NPs as a function of depth in the red curve (C_{tar}) and blue curve (C_{untar}), respectively. Depth-integrated NP concentrations ($\int C_{tar} dz$ and $\int C_{untar} dz$) were calculated for the areas shaded in red and blue, respectively. As described in the Methods section (“*Image Processing*”), the depth-integrated concentrations of targeted and untargeted NPs obtained with micro-NMI are directly proportional to the signals that would be collected with conventional wide-area NMI of an identical tissue specimen stained with an identical protocol. This is attributable to the fact that the NPs only accumulate within the first 20 to 30 μm beneath the tissue surface (see Figure 2), and surface imaging with a 785-nm light source (or other visible light source) would excite and detect all of these superficially located NPs with minimal tissue attenuation: the transport mean free path of tissue at visible wavelengths is typically $1/\mu_s \sim 1$ mm, and is even longer at near-infrared wavelengths. In Figure 3C, we demonstrate that the ratio of the depth-integrated concentrations (targeted vs. untargeted) agree (within <5% error) with the ratio obtained from wide-area NMI for various staining times (3 to 15 min), for both the tumor xenografts and benign tissues. We observe that for tumor xenografts, ratios increase with staining time and plateau at an average ratio of 1.59 ± 0.24 , whereas for benign tissue the ratios remain near unity for all staining times (0.90–1.09).

Figure 4 shows the average of thousands of concentration curves (*i.e.* thousands of line profiles) of targeted and untargeted NPs as a function of depth from the tissue surface after 15 min of staining. The preferential binding of targeted NPs in tumor xenografts can be seen in Figure 4A, whereas a similar behavior for both the targeted and untargeted NPs is observed in the benign tissues (Figure 4B). As in previous studies, a higher level of diffusion is observed in the benign tissue, presumably owing to the greater porosity of benign tissue compared to the dense tumor xenografts. Figure 4C and 4D show the concentration ratios of targeted vs. untargeted NPs as a function of depth for tumor xenografts and benign tissues, respectively. Figure 4C shows an elevated ratio of nearly 2 at the tissue surface, which diminishes as NPs diffuse deeper into the tissue. This suggests that by limiting the diffusion

of NPs into tissue, it could be possible to obtain higher ratios in conventional wide-area NMI and increase the contrast between tumor and benign tissue for improved margin assessment. Note that the ratio of the targeted vs. untargeted NPs appears to dip below unity at greater tissue depths. We hypothesize that this could be attributable to a “binding-site barrier” effect that has been previously reported by others, and which will be discussed in the next section. [46–50]

From the results of our micro-NMI experiments, we reasoned that minimizing diffusion would allow for higher binding ratios. In order to achieve reduced diffusion, we chose to utilize larger NPs, which would physically limit the diffusion of NPs into tissue. This is based on the generalized Stokes-Einstein equation in which the diffusion coefficient of a spherical particle in porous media scales inversely with particle size. [51] Since larger SERS NPs are not commercially available, we used silica NPs (SiNPs) that are 200-nm and 300-nm in diameter that were then PEG-ylated and functionalized with EGFR or isotype control mAbs in order to compare their staining behavior with our standard 120-nm silica-coated SERS NPs. As described in the Methods section (“*Silica NP thiolation and conjugation*”), the SERS NPs are encapsulated in a silica shell and the behavior of these NPs (SERS NPs and SiNPs) are therefore similar in terms of diffusion and chemical binding (both specific and nonspecific) when functionalized in a similar manner. Also, as described in the same Methods section, conjugation reactions were performed to ensure that the fluorescence and mAb labeling densities were roughly equivalent for all of the NP sizes based on total NP surface areas. Data confirming that the NPs were conjugated evenly across all sizes, and that their biomarker-targeting properties were similar (flow cytometry), may be found in Supplementary Figure 4 and 5.

In order to improve their utility for time-constrained clinical applications (*e.g.* surgical guidance), we endeavored to develop NPs that could yield adequate tumor-to-benign tissue contrast in less than 10 min (staining plus imaging time). Therefore, for these final sets of experiments, tumor xenografts were stained for just 6 min. We tuned the staining concentration, C_0 , for each NP size to ensure that the total NP surface area (and binding avidity, see Supplementary Figure 5) was relatively well-matched between all sets of experiments ($C_0 = 150$ pM, 54 pM, and 24 pM, respectively, for the 120-nm NPs, 200-nm NPs, and 300-nm NPs). After staining, the specimens were rinsed briefly and snap-frozen according to the micro-NMI method illustrated in Figure 1 and 2. Figure 5 shows the average and standard deviation of approximately 1000 micro-NMI concentration curves as a function of depth for each NP size. The depth at which the concentration of the untargeted NP diminished to 10% of the initial staining (starting) concentration is indicated in grey in Figure 5A. Figure 5B shows the depth-integrated ratio (the ratio of the integrated area under the two curves) for each NP size.

4. Discussion and conclusion

This study explored the diffusion and chemical binding of NPs targeted to cell-surface proteins that were topically applied onto the surfaces of fresh tissues. We have previously described the development of a NP-based molecular imaging (NMI) system to enable rapid multiplexed molecular imaging of fresh excised tissues for intraoperative guidance of breast-

conserving surgery, which is the most common treatment for patients diagnosed with early-stage breast cancer. [24–26, 38, 39, 41, 52] With this technology, fresh surgical specimens are topically stained with a panel of NPs (potentially *in vivo* or *ex vivo*) and imaged after a brief rinse step to rapidly identify/quantify the presence of tumor-related biomarkers. Owing to uneven NP delivery/retention/rinse removal at different tissue locations, which often generates misleading image contrast, we utilize a control NP flavor that is co-administered with the targeted NP flavor(s) in an equimolar mixture during imaging experiments to normalize for nonspecific effects. While this NP ratio has been shown in previous studies to provide an accurate representation of biomarker expression levels, nonspecific accumulation and passive retention of NPs is often quite high, which reduces the sensitivity and accuracy of detection (*i.e.* the ratios are lower than we would like).

Here, we have investigated the topical application of NPs on fresh tissue specimens at the microscopic level. We have developed a method to investigate the diffusion and accumulation of NPs near the tissue surface, a method we refer to as micro-NMI. We performed well-controlled “proof-of-concept” experiments using tumor xenografts with known biomarker receptor expression levels and uniform, reproducible tissue properties. We stained and rinsed these fresh tissue specimens with a paired-agent approach identical to that of our standard wide-area NMI approach, in which the targeted and untargeted NPs were labeled with different fluorescent dyes to allow thin frozen tissue sections to be imaged with a fluorescence microscope. [24,25,38,39,41] An image processing method was developed for analyzing the NP distributions (Figure 2). We verified that the depth-integrated concentrations (area under the curve) agreed with wide-area NMI signals by comparing ratios of targeted vs. untargeted NPs obtained with both methods (Figure 3). Analysis of NP concentration ratios as a function of depth (Figure 4) led us to hypothesize that by limiting the diffusion of NPs into tissue, higher NP ratios could be obtained with NMI, thereby improving tumor-to-benign tissue contrast and detection sensitivity. We tested this hypothesis using 200-nm and 300-nm silica NPs functionalized with the same concentration of mAbs (per NP surface area) as our standard 120-nm NPs. Figure 5 shows that larger NPs diffuse less into tissue (Figure 5A), resulting in higher overall targeted vs. untargeted NP ratios (Figure 5B). Furthermore, we were able to achieve such high NP ratios in less time (6 min of staining) compared with our previous experiments that required 10 min or more of staining when utilizing our standard 120-nm NPs.

Previous studies on NP delivery have suggested that a binding-site barrier, in which NP binding can impede NP penetration in tissue, may be preventing our targeted NPs from penetrating as deeply into tissues as our untargeted (control) NPs, which penetrate passively (unobstructed by a binding-site barrier) into the tissue. [47, 48, 53–55] This theory is supported by our experimental data in Figure 4A and 4C, where the ratio of the targeted vs. untargeted NPs dip below unity at the furthest depths within the tumor tissue. This suggests that our targeted and untargeted NPs are not being delivered identically, resulting in ratios that are less than ideal. Further studies are needed to verify that our experimental results are in fact attributable to a binding-site barrier effect. However, as a preliminary step, we developed a mathematical model of the diffusion of NPs topically applied in fresh tissue. This model included association and dissociation rate parameters describing the binding and unbinding of targeted NPs to their biomarker targets, respectively, whereas the untargeted

NPs are described only by passive diffusion into tissue. An illustration of this simple model, with governing equations, is shown in Supplementary Figure 6. Model simulations (shown in Supplementary Figure 6C) predict that, for biomarker-positive tumor tissue, the concentration of targeted NPs is elevated near the surface (attributable to binding) and drops to zero concentration at a shallower depth than the untargeted NPs, whereas for benign tissue both NP flavors diffuse similarly into tissue. These model simulations agree with our experimental work, suggesting that the presence of a binding-site barrier may be impeding the delivery of targeted NPs in comparison to untargeted NPs. Our results shown in Figure 5 further indicate that the effects of this presumed “binding-site barrier” are mitigated with the use of larger NPs, in which both the targeted and untargeted NPs remain closer to the tissue surface. These results are consistent with the excellent staining performance of larger NPs witnessed by other investigators in the past. [56]

In summary, the results of this study are significant in showing that larger NPs may be used in NMI to improve the ratio of targeted vs. untargeted NPs after a rapid staining protocol on fresh tissues. We note that while studies shown here employed the use of xenografts, further studies are needed to validate that higher ratios can be achieved with larger NPs on human tissues. This could potentially allow for better differentiation of tumor and benign tissues for clinical applications such as early detection and intraoperative assessments of surgical margins. Future clinical studies are also needed, with large numbers of patient specimens, to validate that these improvements are consistent across clinical cohorts. Note that we have already shown, in a recent clinical study, that NMI with 5 multiplexed SERS NP flavors (to target 4 cancer biomarkers) enables the detection of breast carcinoma with high sensitivity and specificity. [57] However, as we add more biomarker targets into our multiplexed panel, higher detection SNR and sensitivity will be needed (as well as faster staining times). We have shown through this study that it is possible to optimize the specific vs. nonspecific accumulation of our targeted NPs, as well as staining speeds, based on an improved understanding of their behavior at the microscopic scale (micro-NMI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Biographies

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Jonathan T. C. Liu received degrees in Mechanical Engineering at Princeton (B.S.E., 1999) and Stanford (M.S., 2000 & Ph.D., 2005). He was a postdoctoral fellow in the Department of Electrical Engineering (Ginzton Labs) and the Molecular Imaging Program at Stanford (2005–2009), and was later appointed as an instructor within the Stanford University School of Medicine (2009–2010). From 2010 to 2014, Jonathan was an assistant professor in the Biomedical Engineering Department at SUNY Stony Brook. In 2014, he was appointed as an assistant professor and then an associate professor (in 2016) in the Mechanical Engineering Department at the University of Washington in Seattle. His molecular biophotonics laboratory develops optical strategies for improving the detection, diagnosis,

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Figure 1. Micro-NMI method

(1) One SERS NP flavor was reacted with DyLight 550 and conjugated to IgG1 isotype control antibodies (mAbs), while a different SERS NP flavor was reacted with DyLight 650 and conjugated to anti-EGFR mAbs. (2) Fresh tissues were stained with an equimolar mixture of EGFR-targeted and untargeted (isotype-control) NPs, followed by (3) a brief rinse in PBS (~2 sec) to wash away any excess NP solution from the surface of the tissues. (4) The tissues were then placed in a cryomold containing OCT (freezing media). (5) The cryomold was snap-frozen in 2-methylbutane chilled in liquid nitrogen (<10 sec) and (6) cryosectioned into 10- μm -thick sections. These sections were then placed on a glass slide, fixed in formalin, and coverslipped. (7) A Leica DMI8 widefield fluorescence microscope was used to obtain bright field images of the tissue sections, as well as fluorescence images of the untargeted NPs (conjugated to DyLight 550) and the targeted NPs (conjugated to DyLight 650). Scale bar represents 25 μm .

Figure 2. Image processing

Bright field and fluorescence images of a tissue section from a tumor xenograft stained for 15 min with an equimolar NP mixture are shown on the left. The average of all line profiles oriented perpendicular to the tissue surface is shown for the fluorescence images of targeted NPs (top row) and untargeted NPs (bottom row). The tissue autofluorescence background was measured as the average intensity in the unlabeled tissue, and was subtracted from the NP profiles ("Background subtraction"). The maximum intensity of the NP intensity profiles was assumed to correspond to the tissue surface, which defines the zero depth on the x axes in "Crop to surface." Finally, the depth-integrated NP concentrations (area under the curve) were evaluated to verify that the micro-NMI results ("Depth-integrated NP concentration") agree with the signals obtained with the wide-area NMI technique. See text for more details.

Figure 3. Conventional wide-area NMI is used to verify the accuracy of the depth-integrated ratios calculated from micro-NMI

The ratio of the depth-integrated concentrations of the targeted and untargeted NPs are calculated because they provide a reliable estimate of biomarker expression levels that are much less sensitive to nonspecific effects such as uneven illumination, variable staining concentrations, and heterogeneous tissue properties (*e.g.* diffusion constants). (A) A fresh tissue specimen is stained with an equimolar mixture of targeted and untargeted NPs, rinsed briefly (~2 sec) to remove excess NPs from the surface, and imaged with a customized wide-area NMI device that produces a raster-scanned image of the tissue surface. A ratiometric image of EGFR-targeted vs. untargeted NPs shows elevated ratios for the A431 tumor xenograft (which overexpresses EGFR) and a ratio of unity (no differential binding of the targeted NP) for the benign tissue. (B) Depth-integrated concentrations (area under the curve) of targeted and untargeted NPs (plotted as a function of depth from the tissue surface) from the micro-NMI experiments are shown for tumor xenografts (left) and for benign tissues (right). Concentrations of targeted and untargeted NPs as a function of depth are shown in the red curve (C_{tar}) and blue curve (C_{untar}), respectively. Depth-integrated NP concentrations are calculated for the regions shaded in red and blue as $\int C_{tar} dz$ and $\int C_{untar} dz$, respectively. (C) Ratios are plotted as a function of staining time for both NMI (black circles) and micro- NMI (grey triangles), showing good agreement (<5% error). Error bars show the standard deviation from N = 5 experiments for each staining time.

Figure 4.

The average of approximately 1000 curves showing the concentration of targeted (red) and untargeted (blue) NPs as a function of depth from the tissue surface after the tumor xenografts (A) or benign tissues (B) were stained for 15 min. Shaded regions represent the standard deviation for approximately 1000 curves collected from $N = 3$ specimens of each tissue type. The concentration ratios of targeted vs. untargeted NPs as a function of depth are shown in (C–D) for tumor xenografts and benign tissues, respectively. Shaded regions represent the propagation of error from the shaded regions in A–B, respectively.

Figure 5. Comparing micro-NMI for 3 different NP sizes

Three different NP sizes were functionalized with either EGFR or isotype control mAbs. Tumor xenografts were stained with an equimolar mixture of targeted and untargeted NPs for 6 min, using either the 120-nm, 200-nm, or 300-nm diameter NPs. After staining, the specimens were rinsed briefly and snap-frozen for examination with the micro-NMI method. (A) The average of approximately 1000 concentration curves are shown for the targeted NPs (red line) and untargeted NPs (blue line) as a function of depth for each NP size and are normalized to the initial staining concentration. The initial staining concentrations are different for each NP size to account for differences in the total surface area of the NPs (see text for details). Shaded regions represent the standard deviation for approximately 1000 curves collected from $N = 5$ specimens for each NP size. The grey dashed lines and grey text indicate the depth at which the concentration of the untargeted NP diminishes to 10% of the initial staining (starting) concentration. (B) The depth-integrated ratios (targeted vs. untargeted NPs) for each NP size. Error bars are based on $N = 5$ experiments for each NP size.