Investigation of tumor cell targeting of a dendrimer nanoparticle using a double-clad optical fiber probe

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Abstract. Fluorescence quantification in tissues using conventional techniques can be difficult due to the absorption and scattering of light in tissues. Our previous studies have shown that a single-mode optical fiber (SMF)–based, two-photon optical fiber fluorescence (TPOFF) probe could be effective as a minimally invasive, real-time technique for quantifying fluorescence in solid tumors. We report improved results with this technique using a solid, double-clad optical fiber (DCF). The DCF can maintain a high excitation rate by propagating ultrashort laser pulses down an inner single-mode core, while demonstrating improved collection efficiency by using a high–numerical aperture multimode outer core confined with a second clad. We have compared the TPOFF detection efficiency of the DCF versus the SMF with standard solutions of the generation 5 poly(amidoamine) dendrimer (G5) nanoparticles G5-6TAMRA (G5-6T) and G5-6TAMRA-folic acid (G5-6T-FA). The DCF probe showed three- to five-fold increases in the detection efficiency of these conjugates, in comparison to the SMF. We also demonstrate the applicability of the DCF to quantify the targeted uptake of G5-6T-FA in mouse tumors expressing the FA receptor. These results indicate that the TPOFF technique using the DCF probe is an appropriate tool to quantify low nanomolar concentrations of targeted fluorescent probes from deep tissue. [DOI: 10.1117/1.2870105]

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1 Introduction

Noninvasive tissue fluorescence analysis by conventional spectroscopic techniques presents significant challenges because of the absorption and scattering of light in tissues.[1] Visualization of fluorescent probes in tissues, such as the distribution of a fluorescent drug, has been achieved by whole-body imaging.[2] However, this approach requires large amounts of a fluorescent material, and it is difficult to obtain quantitative information at low fluorophore concentrations. Our group has demonstrated a minimally invasive technique, using a two-photon optical fiber fluorescence (TPOFF)–based detection system for the quantification of tissue fluorescence both *in vitro* and in live animals.[3,4] As compared to single-photon excitation,[5] two-photon excitation has the advantage of having greater penetration into tissue samples.[6,7] Moreover, analytes with multiple emission wavelengths can be simultaneously quantified using a single-excitation laser beam due to the broad two-photon excitation cross-section spectra. As the two-photon excitation and emission wavelengths are widely

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separated and can be easily filtered, the sensitivity of detection is enhanced, especially for multiwavelength analytes. In our previous studies, we have used a single-mode fiber (SMF) to deliver femtosecond laser pulses into cell and tissue samples and to collect the emitted fluorescence back through the same fiber. This allowed the detection and quantification of targeted fluorescent antibodies and nanoparticles.[3,4,8]

One of the limitations of a SMF is that its small numerical aperture (NA, ~0.1) results in inefficient signal collection. To quantify low levels of localized fluorescence such as those that occur with tissue-specific targeting of a nanoparticle, a more sensitive *in situ* fluorescence quantification technique is required. Although the application of a multimode fiber (MMF) would enable efficient fluorescence collection through its large NA, the sensitivity for two-photon fluorescence is compromised because of its inefficient excitation and lower resolution. To solve these "trade-off" problems between the SMF and the MMF, we have recently reported the development of a double-clad photonic crystal fiber (DCPCF) that allows SMF-like high resolution excitation through its center core while collecting the emitted light efficiently through both the inner core and an outer core that has a large NA (~0.8).[9] We demonstrated the use of a DCPCF as a scanning microscope for visualizing fluorescent images of individual cells, using a gradient index lens attached at the end of the fiber. However, the use of a DCPCF for *in situ* fluorescence quantification is limited because of the entry of fluids into its outer core airspace by capillary action. In this paper, we demonstrate the application of a solid double-clad optical fiber (DCF) that, because it does not need air holes for the optical confinement in the outer core, avoids the entry of biological fluids into the fiber. Although the DCF has a slightly lower NA (~0.46) and collection efficiency versus the DCPCF, it provides a significant improvement over conventional single-clad SMFs. Our studies show for the first time that the improved sensitivity of a DCF as compared to that of a single-clad fiber allows sensitive quantification of targeted nanoparticles in tumor cells.

2 Materials and Methods

2.1 *Materials*

The generation 5 (G5) poly(amidoamine) (PAMAM) dendrimer was synthesized and characterized at the Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan. Methanol (MeOH, high performance liquid chromatography [HPLC] grade), acetic anhydride (99%), triethylamine (99.5%), dimethyl sulfoxide (DMSO, 99.9%), dimethylformamide (DMF, 99.8%), l-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC, 98%), citric acid (99.5%), sodium azide (99.99%), D_2O, NaCl, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Aldrich Co. (Milwaukee, Wisconsin) and used as received. The Spectra/Por dialysis membrane (MWCO 3500) and Millipor Centricon ultrafiltration membrane (YM-10) were from Fisher Scientific (Hanover Park, Illinois). The 6-carboxytetramethyl rhodamine, succinimidyl ester (6-TAMRA-SE, 6T-SE, properties: molecular=528, soluble in DMF and DMSO, absorption =546, emission=576 measured in MeOH, $\epsilon=9.5\times10^4$) was purchased from Invitrogen (Carlsbad, California). The KB cells were obtained from ATCC (Rockville, Maryland). Female, severe combined immunodeficiency (SCID) mice were purchased from Charles River Laboratories (Wilmington, Massachusetts). The SMF and DCF were from Newport (F-SBA; Irvine, California) and Thorlab (P-10/123DC, Newton, NewJersey). Roswell Park Memorial Institute (RPMI) cell culture medium, trypsin–ethylenediamine tetraacetic acid, penicillin/streptomycin, and Dulbecco's phosphate buffered saline (PBS, pH 7.4) were from Gibco/BRL (Gaithersburg, Maryland). Folate-deficient diet was from TestDiet (Richmond, Indiana). Folic acid (FA, 98%) and all other reagents were from Sigma (St. Louis, Missouri).

2.2 *Methods*

2.2.1 *Synthesis of G5-6T-FA*

We have previously established the G5-PAMAM (G5) dendrimer nanoparticle as a suitable platform for conjugating multiple molecules for specific targeting of drugs and imaging agents into cells.[10] The G5 dendrimer has several attractive properties as a molecular delivery platform, including uniformity, biocompatibility, defined branched chain structure, and the capability for chemically coupling multiple molecular entities to its surface amino groups. The three-dimensional architecture of the PAMAM dendrimers resembles globular proteins, and the G5 has the size of hemoglobin (~5 nm). PAMAM dendrimers are commercially available and are synthesized from an ethylene diamine (EDA) initiator core, with exhaustive Michael addition of methyl acrylate followed by condensation (amidation) reactions of the resulting ester with large excesses of EDA.[10] PAMAM dendrimers have recently been shown to be highly suitable for drug delivery, detection of biochemical functions such as apoptosis, and for tumor imaging.[10]

For the FA receptor-specific targeting *in vitro* and *in vivo*, we synthesized the targeted and nontargeted conjugates G5-6TAMRA-folic acid (G5-6T-FA; compound 7, Fig. 1) and G5-6TAMRA (G5-6T; compound 4, Fig. 1), respectively, using procedures similar to those we have described previously.[11] After the synthesis of each intermediary conjugate shown (Fig. 1), the product obtained was purified by intensive dialysis or by repeated ultrafiltration, using a 10-kDa membrane filter and using PBS followed by water. The purified compound was lyophilized before undergoing the subsequent reaction step.

G5 carrier. The G5 dendrimer platform used in this study had a number average molecular weight of 26 530 g/mol, as determined by gel permeation chromatography (GPC) equipped with multi angle laser light scattering and refractive index detectors. The average number of primary amino groups was 110, as determined by potentiometric titration.

G5 with 70 amino groups acetylated [G5-Ac(70)]. Initially the primary amino groups on the G5 were partially acetylated. Acetylation of the primary amino groups was used to neutralize the positively charged primary amines on the dendrimer surface from further reaction or intermolecular interaction within biological systems. The quantity 1.51859 g (5.724×10^{-3} mol) of G5 in 100 mL of absolute MeOH was reacted with 378.1 μL (4.007×10^{-3} mol) of acetic anhydride in the presence of 698.1 μL (5.009×10^{-3} mol, 25% molar excess) of triethylamine at room temperature and stirred overnight.

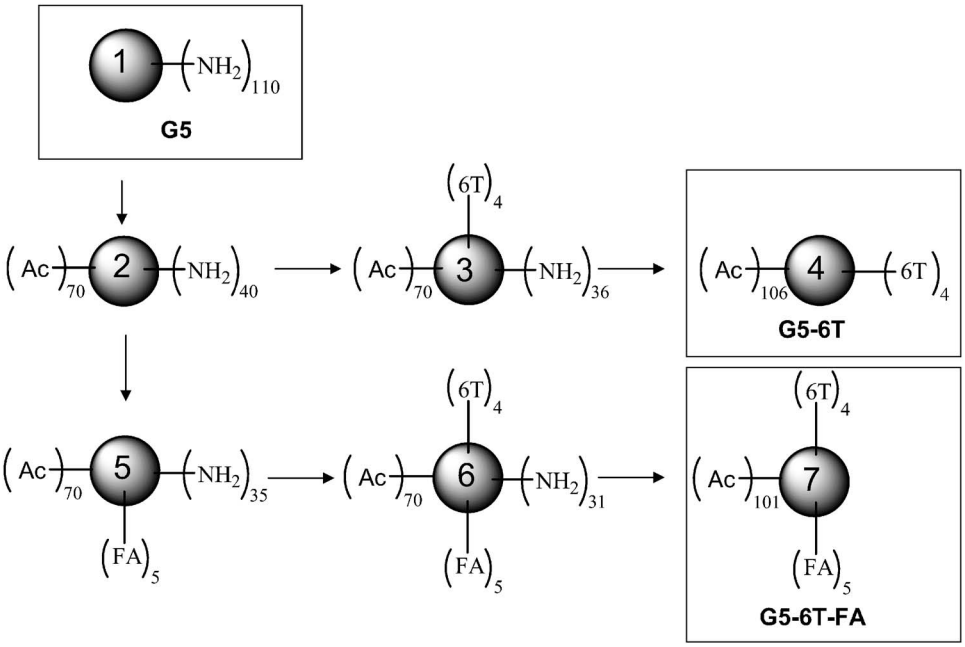

Fig. 1 Synthetic scheme for production of a generation 5 PAMAM dendrimer (G5)–based nanoparticle containing folic acid (FA) as the targeting molecule and 6TAMRA as the sensing dye for targeting FA receptor-expressing KB cells and tumors.

The yield of the purified product was 1.51208 g (89.64%), with an average of 70 acetyl groups as determined by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy.[12] Leaving 40 surface amines allows for the efficient attachment of multiple functionalities, such as receptor ligands, fluorescent dyes, and drugs.[11]

G5 with 70 acetyl groups and 4 covalently conjugated 6TAMRA [G5-Ac(70)-6T(4)]. To 46.4 mg (1.574×10^{-6} mol) of G5-Ac(70) in 17 mL of deionized (DI) water and 1.7 mL of 1 M $NaHCO_3$ solution was added dropwise 2.8 mL of 5-mg (9.478×10^{-6} mol) 6-TAMRA in DMSO solution at a reduced temperature. This was then stirred for 24 h. The yield of the purified product was 40 mg (68%).

Fully acetylated G5-6TAMRA [G5-Ac(fully)-6T(4)]["G5-6T," control conjugate for biological testing]. As a standard procedure after conjugation of multiple functionalities, any free amine groups remaining are neutralized by acetylation to obtain a fully neutralized final product. A mixture of 12.24 mg (3.876×10^{-7} mol) of G5-Ac(70)-6T(4) dendrimer conjugate, 1.65 μL (1.755×10^{-5} mol) of acetic anhydride, and 3.04 μL (2.18×10^{-5} mol) of triethylamine in 0.5 mL of absolute MeOH was allowed to react at room temperature by stirring overnight. The purified product obtained (G5-6T) had a molecular weight of 33 090 g/mol, as determined by GPC.

G5 containing five covalently conjugated folic acid molecules [G5-Ac(70)-FA(5)]. Conjugation of FA to the partially acetylated dendrimer was carried out via condensation between the γ-carboxyl group of FA and the primary amino groups of the dendrimer. Because of the higher reactivity of the γ-carboxyl group of FA as compared to its α-carboxyl, under the conditions used, conjugation takes place primarily through the γ-carboxyl. Leaving the α-carboxyl of FA free is a prerequisite for its full affinity toward the FA receptor. The FA was initially activated by reacting 65.37 mg of FA (1.48×10^{-4} mol) with a 14-fold excess of EDC (399.24 mg, 2.08×10^{-3} mol) in a solvent mixture of 48 mL of DMF and 16 mL of DMSO at room temperature. After 3 h, the FA-active ester solution obtained was added dropwise to an aqueous solution of the partially acetylated product G5-Ac(70) (0.79055 g, 2.683×10^{-5} mol) in 180 mL of water. The reaction was allowed to proceed at room temperature with intensive stirring for three days. The yield of the purified product obtained was 0.8543 g (99.8%), with five FA per dendrimer as determined by ^{1}H-NMR analysis.[11] In the ^{1}H-NMR of the purified G5-6T-FA, the absence of any sharp peaks and the observed broadening of the aromatic proton peaks were indicative of the lack of presence of any free FA and the presence of covalently conjugated FA. Additional characterization by gel permeation column chromatography (equipped with a UV detector) and HPLC analysis also failed to show any presence of free FA.

G5-Ac(70)-FA(5)-6T(4). To 100.07 mg (3.147×10^{-6} mol) of G5-Ac(70)-FA(5) in 35 mL DI water and 3.5 mL of 1 M $NaHCO_3$ solution was added dropwise 4.6 mL of 10 mg (1.896×10^{-5} mol) 6-TAMRA in a DMSO solution at reduced temperature, and it was stirred for 24 h. The yield of the purified product was 0.09612 g (90.1%).

G5-Ac(fully)-FA(5)-6T(4) ["G5-6T-FA," targeted conjugate for biological testing]. A combination of 21.74 mg (6.41×10^{-7} mol) of G5-Ac(70)-FA(5)-6T(4) dendrimer conjugate, 2.34 μL (2.484×10^{-5} mol) of acetic anhydride and 4.33 μL (3.105×10^{-5} mol) of triethylamine in 0.5 mL of absolute MeOH was allowed to react at room temperature overnight. GPC analysis of the product obtained (G5-6T-FA) gave a molecular weight of 35 200 g/mol. On the basis of the integration values of the methyl protons in the acetamide groups and the aromatic protons by ^{1}H-NMR analysis, the

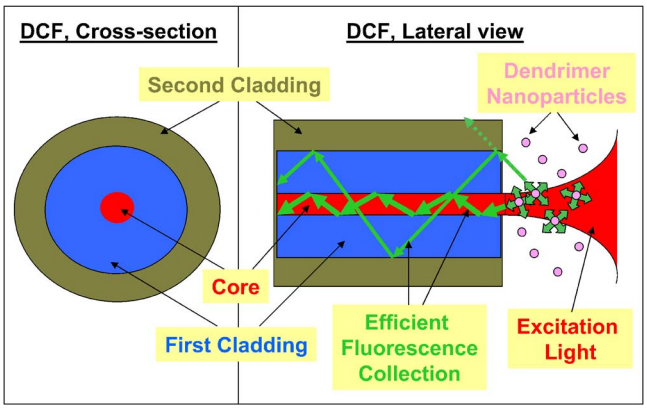

Fig. 2 Schematic drawing of the DCF having a 10-μm inner core, 123-μm first cladding, and 178-μm second cladding diameters. Unlike the SMF we have described previously, this newly developed DCF allows collection of emission fluorescence not only through the fiber core, but also through the inner cladding, resulting in increased efficiency.

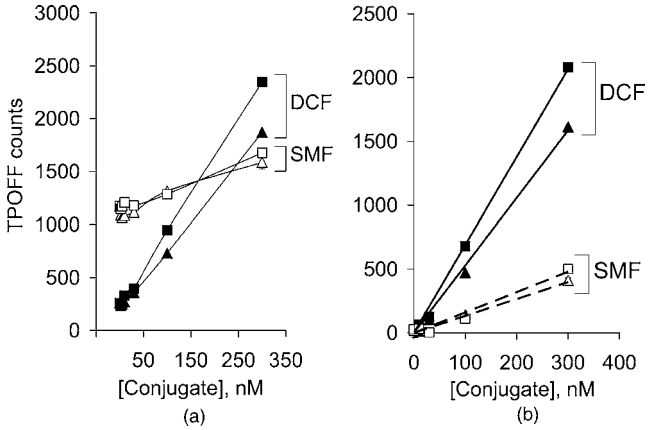

Fig. 3 (a) Comparison of TPOFF measurement of standard solutions of G5-6T (triangle symbols) and G5-6T-FA (square symbols) using SMF and DCF probes. The TPOFF photocounts of different concentrations of the standard solutions are measured by inserting the fiber into the solution. The data shown are the mean ± SE of four to five such counts taken for each concentration of the standard. (b) Data corrected for the detection background.

numbers of attached FA and 6TAMRA molecules were calculated to be 5 and 4, respectively.

2.2.2 *The TPOFF system—instrumentation*

The TPOFF fluorescence detection system used was similar to that previously described.[4] The SMF used in this study had a mode field diameter of 4.1 μM with a NA of 0.16. The DCF had a 10-μm diameter inner core with a NA of 0.07 and a 123-μm cladding with a NA of 0.46 (Fig. 2). Femtosecond laser pulses generated from a Ti:sapphire oscillator (Mira 900, Coherent, Inc., Santa Clara, California) with a pulse duration of 50 fs and center wavelength of 800 nm were used as the excitation source. Fibers were directly inserted into dye solutions, cell pellets, or into tumor samples through a 30-gauge needle. The fluorescence emission was collected through the same fiber and then separated from the excitation beam by a dichroic mirror. The fluorescence was then sent through a short-pass filter and counted using a photon-counting photomultiplier tube.

Cell culture and in vitro studies. The KB cell line, which expresses high levels of FA receptor on its membrane, was used for the receptor-specific targeting of the G5-6T-FA conjugate. The KB cells were cultured as described previously.[13] Cells were maintained in folate-free RPMI medium containing 10% serum to provide extracellular FA similar to that found in human serum. For *in vitro* studies, cells plated in 75-cc flasks were trypsinized, rinsed, and resuspended in phosphate buffered saline containing 0.1% bovine serum albumin (PBSB). The cells in suspension were treated with different concentrations of the conjugates for 1 h at 37°C, rinsed, and centrifuged to collect the cell pellets. The optical fiber was then inserted into different regions of the cell pellet and TPOFF counts were collected as described previously.[14]

Animal model and in vivo studies. For *in vivo* studies, KB cell tumors were developed in seven-week-old SCID mice as described before.[15] The mice were housed in a specific pathogen-free animal facility at the University of Michigan Medical Center in accordance with the regulations of the University's Committee on the Use and Care of Animals as well as with federal guidelines, including the principles of laboratory animal care. The animals were fed *ad libitum* with Laboratory Autoclavable Rodent Diet 5010 (PMI Nutrition-International, St. Louis, Missouri). Two weeks before tumor cell injection, the food was changed to a folate-deficient diet. The KB cell tumors were developed by subcutaneous inoculation of 1 000 000 cells each in the left and right flank areas. The cells were trypsinized, rinsed, and injected as a 0.1-ml suspension using sterile PBS. The tumors were allowed to reach 0.7 to 0.8 cm in diameter before analysis. We administered 15 nmols of the conjugates in 0.1 mL of PBS, or PBS alone as a control, through the tail vein. At the end of the study, 15 hours later, the mice were anesthetized with isoflurane and the tumors were excised and frozen. The optical fibers were inserted into the tumor through a 30-guage needle, with the tip of the fiber placed at the proximal end of the needle hole. The needle with the fiber was sonicated in a bath sonicator between tumor sample measurements. The fluorescence of multiple internal regions of the tumors was quantified using the two-fiber probes under identical conditions.

The statistical significance of differences among the groups was analyzed by the Student-Newman-Keuls test, with significance calculated at $p < 0.05$.

3 Results and Discussion

The fluorescence emissions of the standard solutions of G5-6T-FA and G5-6T were initially quantified using DCF and SMF probes (Fig. 3). The DCF fiber showed lower background fluorescence when measured in PBS buffer and a five-fold higher conjugate fluorescence as compared to the SMF. The background fluorescence obtained in PBS was 1175 ± 42 and 258 ± 4.6, respectively, when the SMF and DCF probes were used. This detection background was mainly from the autofluorescence of the fiber core. The fact that the SMF has a higher background was attributed to the impurity of the ma-

terial used for the SMF core. The low detection background of the DCF probe ensures the accurate measurements of samples with an extremely low concentration of fluorophores. Other independent measurements using the two fibers similar to what is presented in Fig. 3 have shown that the standard TPOFF counts were linear at least up to 1-μM concentrations of the conjugates. This is consistent with the linearity in TPOFF counts up to 100-μM concentrations of the "G5-fluorescein" conjugates we have previously demonstrated using the SMF.[14]

The background-subtracted counts [mean $\pm$standard error (SE)] obtained for 5, 10, 30, 100, and 300 nM of G5-6T-FA conjugate were, respectively, -13 ± 6, 65 ± 11, 127 ± 6, 679 ± 32, and 2079 ± 11 using the DCF, and -6 ± 5, 32 ± 19, 3 ± 2, 110 ± 8, and 501 ± 24 for the SMF [Fig. 3(b)]. This shows that the lower detection limit for the conjugate is $\sim$10 nM for the DCF and $>$100 nM for the SMF. The increased sensitivity of the DCF versus the SMF is due to the unique fiber structure, which allows the laser beam to propagate in single mode through the inner core for a high excitation rate while having an efficient collection of fluorescence through its outer core (first clad), and is also due to the lower background fluorescence of the former.

The DCF fiber was then utilized to quantify the *in vitro* targeting of the dendrimer conjugates in FA receptor-expressing KB cells. In untreated control cell pellets, the mean background fluorescence taken by the SMF and the DCF were 1313 ± 52 and 2089 ± 165 counts per second, respectively. This observation is in contrast to the increased background fluorescence of the SMF in PBSB. The increased DCF counts observed in the control cell pellet could be because of the autofluorescence of endogenous fluorophores. Several cellular components such as collagen, tryptophan, and porphyrins are known to be fluorescent, and low levels of these agents are apparently being detected by the more sensitive DCF fiber.

As shown in Fig. 4(a), the fluorescence of the cell pellet was significantly higher for the DCF fiber at all concentrations of the G5-6T-FA conjugate tested, consistent with results from the standard solutions. The increased sensitivity of the DCF fiber is further evident from the marked difference in the slopes of the two curves [Fig. 4(a)] at lower doses of the conjugate. Low levels of nonspecific binding of the control conjugate G5-6T were detected only by the DCF fiber [Fig. 4(b)]. The binding curves observed by the DCF fiber are indicative of the FA receptor-specific interaction we have previously reported.[13] The receptor-specific binding of the G5-6T-FA was further confirmed by the reversal of the binding of 300 nM of G5-6T-FA by preincubating cells with a 50-fold excess of free FA. The DCF-TPOFF counts in cells incubated with 300 nM of G5-6T-FA in the absence and presence of 15 μM of free FA were $15\,411 \pm 247$ and 2903 ± 546, respectively. The standard error for the measurement of different regions of the cell pellets varied from 2% to 20% for the different concentrations of the conjugate tested. As the TPOFF measurement quantifies the fluorescence of only a few microns surrounding the fiber, this variablity could result from the differences in the fluorescence intensities for the 4 to 5 cell regions tested in cell pellets at each concentration. A broad population distribution for binding of the conjugate has been observed by flow cytometry by measurement of the individual fluorescence of 10 000 cells.[3]

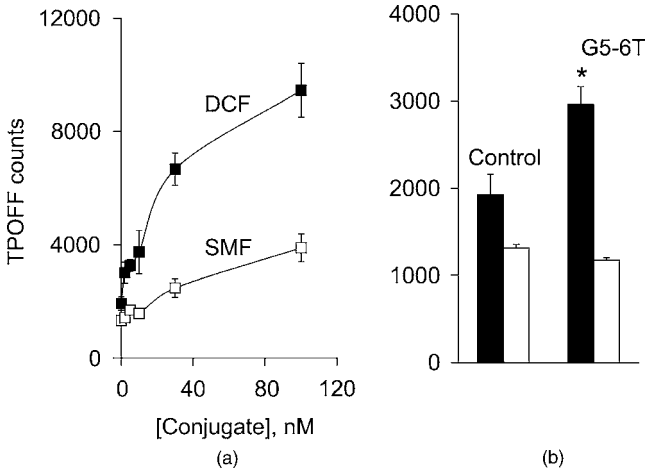

Fig. 4 (a) Comparison of the targeting of G5-6T-FA in KB cells determined by SMF- and DCF-TPOFF measurements. The KB cells in suspension were incubated with different concentrations of the conjugate for 1 h; the cells were rinsed; and the TPOFF counts were taken in the cell pellet. $p < 0.05$ for all DCF data points versus SMF, except for the zero concentration. (b) Comparison of the association of the control conjugate G5-6T (100 nM) on KB cells determined by DCF (filled bars) or SMF (open bars). $*p < 0.05$ versus the corresponding SMF counts. All data shown are the mean ± SE of four to five counts taken in different regions of each cell pellet.

The DCF probe was then used to confirm our previous findings[3,4] that the G5-6T-FA conjugate could target FA receptor-expressing tumors *in vivo* in mice. As shown in Fig. 5, there were approximately three-fold greater background-subtracted counts for the targeted conjugate as detected by the DCF probe in comparison to the counts obtained by the SMF

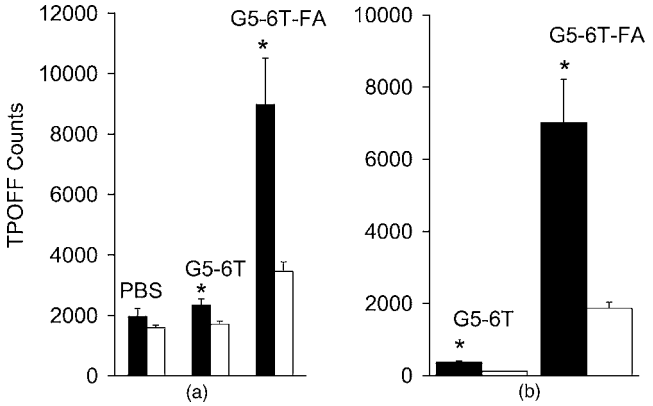

Fig. 5 (a) *In vivo* tumor targeting of G5-6T-FA, determined by TPOFF. The KB cell tumors were developed in SCID mice and were intravenously injected with 15 nmols each of the targeted conjugate G5-6T-FA, the control conjugate G5-6T, or their vehicle PBS. After 15 h, the tumors were isolated and the TPOFF counts were taken in different internal regions of the tumor using the DCF (filled bars) or SMF (open bars) fiber probe. The data shown are mean ± SE of counts taken in four to five different internal regions of four tumors for each condition. (b) TPOFF counts corrected for the background fluorescence of the PBS tumor, measured by DCF (filled bars) or SMF (open bars) fiber probe. $*p < 0.05$ versus the corresponding SMF counts.

probe. There was a three- to four-fold increase in the uptake of the targeted conjugate (G5-6T-FA) in comparison to the control conjugate G5-6T, which again is consistent with our previous findings.[3,4] Using the slope of the standard curve generated for the G5-6T-FA (Fig. 3), the concentration of the targeted conjugate in the tumor was 1016 ± 174 nM and 1251 ± 117 nM measured by the DCF and SMF, respectively. In keeping with these results, our previous studies using the SMF have shown the accumulation of 1 to 5 μM of G5-6T-FA in KB tumors.[3,4]

4 Conclusion

The results in this study demonstrate that the DCF-TPOFF has at least a three-fold improved tissue detection limit compared to the similar-in-diameter SMF used under similar conditions. Although the tissue background fluorescence due to endogenous fluorescent cell components is increased because of the improved sensitivity of the DCF probe, selection of appropriate fluorophores with emission wavelengths separated from endogenous emissions (e.g., red-shifted fluorophores such as Deep Red) for analysis is expected to further increase the resolution and sensitivity of the DCF. On the other hand, an understanding of the ratio of the sensitive endogenous fluorescence to that of the exogenously administered component may facilitate the more accurate determination of time-dependent changes with variables such as the power or coupling of the laser over time. The core of the DCF fiber used in this study is twice as large as that of SMF, causing lower peak intensity at the excitation end of the DCF fiber, and the usage of fibers with a comparable core size would further enhance the sensitivity of the DCF probe. The improved sensitivity of the DCF versus the SMF and the application of a small 30-guage needle make this a superior optical fiber probe technique in quantifying fluorescent signals in deep tissues. Nanomolar quantities of multiple fluorophores with different emission wavelengths or lifetimes could be discerned and quantified using this technique. We envision that the TPOFF probe will serve as a minimally invasive diagnostic tool for screening tumor markers, such as the epidermal growth factor–receptor 2 (HER2) in women with breast cancer, when used in conjunction with a fluorescent marker such as the fluorescently tagged antibody Herceptin. Currently, efforts are also underway to modify the DCPCF to prevent it from interacting with fluids while retaining its superior optical characteristics.

Acknowledgments

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