Published in final edited form as:

Biomaterials. 2011 December ; 32(35): 9383–9390. doi:10.1016/j.biomaterials.2011.08.064.

Real time monitoring of biomaterial-mediated inflammatory responses via macrophage-targeting NIR nanoprobes

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Abstract

Medical implant-mediated inflammatory responses, often involving high levels of macrophages, are typically determined by histological analyses. These methods however are time consuming and require many animals to monitor the kinetics of inflammatory reactions and to generate reproducible outcomes. Recent studies have shown that activated macrophages in inflamed tissue express high levels of folate receptor (FR). In this study, FR-targeting NIR nanoprobes were fabricated and then tested for their ability to detect and quantify the extent of biomaterial-mediated inflammatory responses *in vivo*. Indeed, FR-targeting nanoprobes preferentially accumulate on activated macrophage surfaces. When administered intravenously, we found that the FR-targeting nanoprobes distinctively gathered in the inflamed tissues and that a different extent of FR-targeting nanoprobe gathering could be found in tissues implanted with different types of biomaterials. Most importantly, we found that there was a good relationship between the extent of inflammatory reactions and the intensity of nanoprobe-associated NIR signal in tissue. Our results support that FR-targeting NIR nanoprobes can be used to monitor and quantify the extent of macrophage recruitment and the degree of an implants' biocompatibility in real time.

1. Introduction

Implantable medical devices are increasingly important in the practice of modern medicine. Prospect shifts in population demographics towards older age groups, coupled with continued medical progresses, will lead to increased utilization of medical devices in the years ahead. Unfortunately, shortly after implantation, most implants accumulate substantial numbers of phagocytes as a result of biomaterial-mediated inflammatory reactions. The interactions between phagocytes and biomaterials have been associated with chronic inflammation and fibrotic reactions surrounding many types of implants [1]. For example, severe inflammatory responses might cause the degradation and failure of many implantable devices and the dissolution of surrounding tissues [2,3]. Therefore, substantial research has been placed on the development of biomaterials with improved blood and tissue biocompatibility.

To assist the search for better biomaterials, many *in vitro* and *in vivo* models have been developed to test a material's biocompatibility. For example, several *in vitro* systems,

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including phagocyte/platelet adhesion, activation, and blood compatibility, have been used to assess the blood and cell compatibility of the biomaterials [4-8]. Although these *in vitro* models provide good indication and evaluation of a material's biocompatibility, *in vivo* animal testing is often necessary to provide critical evaluations of biomaterials' cell and tissue compatibility [9]. However, there are many limitations associated with *in vivo* testing. First, since immune responses are dynamic reactions, the evaluation of tissue and cell responses at different time points are needed to depict the overall reactions. Second, many animals are needed at each time point to overcome the large variations of cell/tissue responses between individual animals. Finally, histological and chemical evaluations of the implant-associated tissues are often required to analyze the tissue responses to biomaterial implants. Despite the assistance of automatic tissue sectioning and staining processes, histological analyses remain to be time-consuming and tedious. In addition, sequential histology analyses on the same tissue are often needed to establish quantitative results for the whole tissue [10]. Consequently, these technical challenges significantly delay the development of medical implants with improved tissue and cell compatibility. Therefore, there is a need for the development of a fast, accurate, and simple method to assess the extent of foreign body reactions *in vivo*.

Recent development of *in vivo* imaging systems has attracted great interest in different research disciplines. Several noninvasive imaging techniques, including computed tomography, magnetic resonance imaging, positron emission tomography and ultraonography [11-14], have been developed to visualize and quantify the inflammatory responses *in vivo*. These techniques rely solely on structural changes and, therefore, are not able to identify non-traumatic, acute and localized inflammatory reactions. Several fluorescence-labeled cells and particle-based sensors have been developed for a wide range of applications in biological research and clinical diagnosis [15-22]. Of late, NIR fluorescence has been widely used for *in vivo* imaging because of reduced absorption and scattering of photons by tissues, and also reduced interference from auto-fluorescence signals [23].

It is well established that biomaterial implants are surrounded by large numbers of monocytes/macrophages (MΦs) and the activities of recruited/activated MΦs play an important role on pathogenesis of foreign body reactions [1,3]. It is therefore generally believed that the quantification of recruited MΦs would serve as a good assessment for the extent of inflammatory responses to biomaterial implants. Lately, FITC-conjugated MΦ-antibody F4/80 was used to image macrophage recruitment at biomaterial implant sites [24]. Although the results are very interesting, the FITC-F4/80 probe can only provide qualitative, not quantitative, assessment of foreign body reactions for the following reasons. First, the FITC-F4/80 produces an emission wavelength close to the autofluorescence emitted by skin with limited *in vivo* sensitivities. Second, although MΦ-antibody F4/80 has good cell specificity, the high cost of the antibody limits its use as an imaging probe for systemic administration. Interestingly, several recent studies found that activated MΦs over-express folate receptor (FR), which has a high affinity to FR-targeting probes *in vitro* [25-28]. Although FR-targeting probes have been tested for their ability to detect inflammatory arthritis [29-33], the ability of FR-targeting probes to quantify the degree of inflammatory responses was not determined. Furthermore, it has not previously been investigated whether or not FR-targeting probes may be employed in real-time to quantify the degree of biomaterial-mediated inflammatory responses and also to assess the extent of implant biocompatibility *in vivo*.

To find the answer, the following study was aimed at fabricating FR-targeting nanoprobe and testing their use in identifying and quantifying the degree of biomaterial-mediated inflammatory responses *in vivo*. Specifically, NIR dye-trapped Poly(*N*-isopropyl

acrylamide-*co*-styrene) nanoparticles were synthesized and then conjugated with folate. The efficacy of FR-targeting nanoprobe in recognizing activated MΦs was studied *in vitro*. Using an *in vivo* imaging system, the effectiveness of FR-targeting nanoprobe in determining MΦ recruitment to the biomaterial implantation site was assessed. Finally, by comparing nanoprobe fluorescence intensities and histological evaluations, we explored the possibility of using FR-targeting nanoprobe to continuously monitor and quantify the extent of inflammatory responses to different biomaterial implants *in vivo*. This enhanced ability of FR-targeting nanoprobe may lead to future *in vivo* determination of a material's biocompatibility.

2. Materials and methods

Materials

N-isopropylacrylamide (NIPAM), styrene (St), divinylbenzene (DVB), Dodecyl Trimethyl Ammonium Bromide (DTAB), 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (V50), 1,1',3,3,3',3'-Hexamethylindotricarbocyanine (IR750), folate/folic acid, tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. *N*-(3-aminopropyl) methacrylamide (APMA) was procured from Polysciences (Warrington, PA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Thermo scientific (Rockford, IL). Goat anti-mouse folate/folic acid receptor polyclonal antibody (sc-16387) and a rat anti-mouse MAC-1 (sc-23937) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated rabbit anti-goat and goat anti-rat secondary antibodies were bought from Jackson ImmunoResearch Laboratories (West Grove, PA).

Methods

Preparation and characterization of folate receptor(FR)-targeting nanoprobe

—PNIPAM-*co*-St nanoparticles were synthesized by emulsion polymerization as described previously [34,35] with the supplement of 5 mole% APMA to create -NH₂ bearing nanoparticles. The average diameter and size distribution of PNIPAM-*co*-St nanoparticle were determined using Photon Correlation Spectroscopy (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). The morphology of the nanoparticles was also characterized using a JEM-1200EX transmission electron microscope (JEOL, Japan) as described earlier [36]. The trapping of IR750 dye into PNIPAM-*co*-St nanoparticles was then carried out according to published procedures [37]. The loading efficiency of IR750 dye was estimated according to the following equation: Loading efficiency = $(M_{\text{loaded-IR750}} / M_{\text{NP}}) \times 100\%$. where $M_{\text{loaded-IR750}}$ is mass of the entrapped dye obtained by the calibration curve of IR750 and M_{NP} is mass of the nanoparticles. Absorbance and fluorescence spectra of free and loaded IR750 were determined by UV-Vis spectrophotometer (Lambda 19 Spectrometer, PerkinElmer, MA) and fluorescence spectrometer (Shimadzu RF-5301PC, Japan), respectively. The photo stability of loaded IR750 vs free IR750 was evaluated by measuring the change in emission intensity before and after continuous exposure for 3.5 hours under an incandescent lamp (100 W, GE lighting). FR-targeting nanoprobe was fabricated by coupling folate into the IR750-loaded nanoparticles (folate: NP = 2:5 weight ratio) using an established EDC procedure [38,39]. Coupling efficiency of folate was estimated to be 0.3wt% (folate percentage of the nanoprobe) by using UV spectroscopic method as described in previous publications [40-42].

Cytotoxicity assay of FR-targeting nanoprobe—Cytotoxicity of the FR-targeting nanoprobe was evaluated using a standard MTS assay [43]. In brief, mouse 3T3 fibroblasts were seeded in a 96-well plate at the density of 1×10^4 cells/well in DMEM medium supplemented with 10% fetal bovine serum (FBS) + 1% antibiotics for 24 hours with 5%

CO₂ at 37°C. The culture medium in each well was then replaced with 200 µL of complete DMEM medium in the presence or absence of various concentrations of FR-targeting nanoprobe. After 24 hours incubation, the medium was then removed and adherent cells were washed three times with phosphate buffered saline (PBS, pH 7.4). Twenty µl of CellTiter 96® Aqueous One Solution Reagent (Promega, USA) and 100 µl of DMEM medium were added into each well and incubated for 4 hours. Finally, the absorbance of MTS reaction was measured at 490 nm using a SpectraMax 340 Spectrophotometric plate Reader (Molecular Devices, USA).

In vitro MΦ targeting of FR-targeting nanoprobe—Murine Raw 264.7 MΦs (ATCC, Manassas, VA.) were used as model cells to assess the ability of FR-targeting nanoprobe in finding activated MΦ. Raw 264.7 MΦ were cultured in folate free Hank's balanced salt solution (HBSS, Sigma Aldrich, St. Louis, MO) in the presence of 10% FBS and 1% antibiotic at 37°C with 5% CO₂ until 75% confluence. Culture media was deprived of folate to prevent down-regulation of the cell surface FR, which usually occurs when cells are cultured with elevated folate concentrations [44]. Prior to each experiment, cells were plated at 300,000 cells/500 µl/well in 24 well plates and then cultured overnight. To up-regulate cell surface FR expression, cultured Raw 264.7 MΦs were activated by the supplement of 1.0 µg/ml lipopolysaccharide (LPS) (from E-coli, Sigma, St. Louis, MO) for 4 hours as described earlier [45]. After LPS incubation, cells were washed twice with HBSS to remove non-adherent cells. Various treated nanoprobe were then incubated with adherent cells for different periods of time, followed by washing cells with PBS buffer three times to remove unlabeled nanoprobe. The extent of nanoprobe accumulation was then quantified by measuring fluorescence intensity at emission of 830nm (excitation at 760 nm) using a Tecan Infinite M 200 plate reader (San Jose, CA). In addition, to verify whether nanoprobe accumulation is mediated by conjugated folate, a nanoprobe cell adhesion study was carried out in the presence of various concentrations (2.3 -18 µM) of free folate as established earlier [44].

In vivo inflammation models—Female Balb/c mice (20-25 gram), purchased from Taconic Farms (Germantown, NY), were used in this investigation. The animal experiments were approved by the University of Texas at Arlington Animal Care and Use Committee (IACUC). To test the efficacy of FR-targeting nanoprobe in targeting distal inflammatory responses, subcutaneous inflammation was triggered by the subcutaneous injection of LPS as described earlier [46]. Briefly, LPS (100µg/50 µl saline/animal) or saline (50 µl, as a control) was injected subcutaneously in the back of mice. After injection for 24 hrs, mice were administered intravenously with FR-targeting nanoprobe or control nanoprobe (IR750-loaded nanoparticles) (0.1mg/mouse) and whole body images were taken subsequently at various time points. It should be noted that a 24 hours post-LPS administration time point was found to prompt the maximal inflammatory responses in our pilot study. To investigate foreign body reactions to particle implants, PLA particles (~8 µm in diameter) and PEG particles (~150 nm in diameter) were tested. These particles were synthesized using established procedures [47-49]. For *in vivo* testing, PLA and PEG particles (100 µl, 10% w/v) and saline (100 µl) were injected subcutaneously in various dorsal regions of the mouse. After particle implantation for 24 hours, mice were administered with FR-targeting nanoprobe (0.1mg per mouse) and then imaged.

In vivo animal imaging—Whole body images of mice were taken using KODAK *In vivo* FX Pro system with excitation wavelength of 760nm an emission wavelength of 830nm (f/stop, 2.5; no optical filter, 4×4 binning). After background correction, regions of interests (ROIs) were drawn over the implantation locations, and the mean fluorescence intensities

for all pixels in the fluorescent images were calculated using Carestream Molecular Imaging Software, Network Edition 4.5 (Carestream Health).

Immunohistochemical evaluation of biomaterial-mediated tissue responses—

To determine the extent of inflammatory responses to implants, at the end of the study, implants and surrounding tissues were isolated for immunohistochemical evaluation as described earlier [50,51]. Immunohistochemistry was performed to quantify the number of FR+ cells and CD11b+ cells using established procedures [30,50,51]. Briefly, to assess FR+ cells, some tissue sections were stained with a goat anti-mouse folate receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated rabbit anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories, PA). To evaluate inflammatory cells, some tissue sections were stained with inflammatory cell marker CD11b (rat anti-mouse MAC-1, Santa Cruz Biotechnology), monocyte/macrophage marker (MOMA-1, Santa Cruz Biotechnology) and peroxidase-conjugated goat anti-rat secondary antibodies (Jackson ImmunoResearch Laboratories, PA). All slides were treated with 3,3'-diaminobenzidine substrate system and counterstained with hematoxylin. Positive immunoreactions appeared as dark brown staining on a blue background. All histological imaging analyses were performed on a Leica microscope (Leica, Wetzlar, Germany) and processed using NIH ImageJ [50,51]. To visualize the distribution of FR-targeting nanoprobes in tissues, fresh tissue sections were imaged using Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY) with a cooled CCD camera as previously described [52,53].

Statistical analyses—All the results will be expressed as mean $\pm$ Standard error of the mean (SEM). One-way analysis of variance (ANOVA) and student t-test were performed to compare the difference between groups. A value of $p \leq 0.05$ was considered to be significant. Linear regression analyses was used to determine the relationship between fluorescent intensities and cell numbers in vitro, as well as assessing the correlation between fluorescent intensities and macrophage cell numbers in vivo. The coefficient of determination (R^2) was calculated providing a measure of correlation.

3.Results

Fabrication and characterization of FR-targeting nanoprobes

PNIPAM-*co*-St nanoparticles (NPs), synthesized by emulsion polymerization, had average diameter of ~ 100 nm at 25°C . The NPs were found to be highly monodispersed as shown in Fig 1A. TEM images of the PNIPAM-*co*-St NPs revealed homogeneous and spherical morphology although the diameter of particles reduced to ~ 40 nm due to dehydration during the process of sample preparation (Fig. 1B) [54]. IR750 is a hydrophobic dye with maximum absorbance at 731 nm and maximum emission at 758 nm (Fig. 1C-D). To label NPs, IR750 was physically encapsulated into the polystyrene-rich domains of the nanoparticles by the hydrophobic association. The IR750-NPs loading efficiency was calculated to be about 3.5% (w/w) according to the calibration curve: $y = 36.252 - 0.0061x$, $R^2 = 0.99$. As reported elsewhere [36], a red shift of ~ 24 nm and ~ 32 nm was observed for the absorbance and emission of the loaded IR750 (Fig. 1C-D), respectively. This may be ascribed to the enhanced interactions between segments of the polystyrene chain, leading to a decrease of transition energy of chromophores of IR750 [55].

Additionally, as the hydrophobic microenvironment of polystyrenes was impermeable to solvent (water) and oxidative agents, such as dissolved oxygen, the photostability of the physically-entrapped IR750 was substantially improved (Fig. 1D) [36,55]. After exposure to light for 3.5 hours, emission intensity was decreased by $\sim 30\%$ for free IR750 but only by

~3% for the loaded IR750 (Supp. Fig.1). FR-targeting nanoprobe were obtained by conjugating folate into IR750-loaded nanoparticles using carbodiimide coupling methods. The cytotoxicity of FR-targeting nanoprobe was determined using 3T3 fibroblasts and MTS assay (Fig.1E). We find that the FR-targeting nanoprobe trigger no statistically significant cytotoxicity over the studied concentration range (up to 0.2 mg/ml), indicating that the above-prepared nanoprobe possess adequate cell compatibility for further *in vivo* testing.

Effectiveness of FR-targeting nanoprobe on targeting activated MΦ *in vitro*

To investigate the interaction between FR-targeting nanoprobe and activated MΦ, LPS-treated rat Raw 264.7 MΦs were incubated with FR-targeting nanoprobe or control nanoprobe for either 15 or 30 minutes. The results show that a substantial amount (~9 fold compared with controls) of FR-targeting nanoprobe were associated with activated MΦ after 15 minutes of exposure and that the uptake of FR-targeting nanoprobe increased after 30 minutes of exposure (~10 fold compared with controls) (Fig. 2A). To further confirm the role of nanoprobe surface-bound folate in targeting activated MΦ, we performed a competition binding test in which free folate molecules were introduced to the medium before the addition of FR-targeting nanoprobe. As expected, fluorescence intensity is substantially decreased with increasing concentrations of free folate, suggesting that FR-targeting nanoprobe have high affinity for activated MΦ (Fig. 2B). Furthermore, by incubating FR-targeting nanoprobe with different numbers of activated MΦ, we find that there is a linear relationship between the activated MΦ numbers and fluorescence intensities (Fig. 2C). This data supports our hypothesis that FR-targeting nanoprobe may be used to quantify the number of activated MΦ *in vitro*.

Detection of activated MΦ *in vivo* using FR-targeting nanoprobe

To test whether FR-targeting nanoprobe can be used to detect activated MΦ accumulation in inflamed tissue *in vivo*, a LPS induced subcutaneous inflammation model was used. After 24 hours LPS challenge, FR-targeting nanoprobe or control nanoprobe were administered intravenously. Our results reveal that the fluorescence intensities at the LPS injection site, regardless FR-targeting nanoprobe or control nanoprobe, were substantially higher than saline controls (Fig 3A-B). We also determined the ability of nanoprobe to distinguish inflamed vs. control tissues by calculating their enhancement ratios (ratios of fluorescence intensity at LPS-site vs. saline-site). Indeed, the enhancement ratio for FR-targeting nanoprobe is higher than that for their controls 18 hours after nanoprobe injection. At 24 hours, our results show a substantially higher fluorescence enhancement ratio (~2.7 fold) in the FR-targeting nanoprobe group than that in its control group (Fig 3C). Histological evaluations also show that the LPS challenge resulted in tissue with ~6 times higher FR+ inflammatory cells than saline treatment (Fig. 3D-E). As expected, we also found that the LPS-challenge triggered ~6 times higher CD11b+ inflammatory cell recruitment than the saline control (Fig. 3F-G). These results strongly support our hypothesis that FR-targeting nanoprobe have the ability to noninvasively detect activated MΦ and may be used to monitor the biomaterial-mediated inflammatory processes in real time.

Quantification of foreign body reactions *in vivo* using FR-targeting nanoprobe

Subsequent studies were carried out to explore the possibility of using FR-targeting nanoprobe to assess inflammatory reactions to different biomaterial implants *in vivo*. PLA and PEG particles were chosen as model materials which have been shown to trigger strong and weak inflammatory responses, respectively [48,56]. After prior injection (24 hour incubation) with different types of particles or saline as injection control, the FR-targeting nanoprobe were introduced via tail vein injection and the animals were subjected to whole-body imaging at various time points (Fig. 4A). In general, fluorescent signals in both PLA and PEG sites increased over time, and the signal intensities in PLA implantation sites were

consistently higher than those in PEG sites (Fig. 4A). Furthermore, saline controls had very low or no fluorescent signals during the same time periods. The varying trends of fluorescence intensities were consistent and statistically significant between different test materials 6 hours after FR-targeting nanoprobe injection (Fig. 4B). To determine the distribution of the nanoprobe in the inflamed tissue, NIR microscopic images of sections of PLA implant sites were taken (Fig. 4C). The microscopic images show that abundant FR-targeting nanoprobes accumulated in tissue adjacent to the PLA implant and that substantially less nanoprobes are found in normal tissue.

Immunohistochemical analyses reveals that, after 24 hour implantation, the saline injection prompted negligible CD11b+ inflammatory cell recruitment, and that the PLA implants are consistently infiltrated by more CD11b+ inflammatory cells than the PEG implants (Fig. 4D). Similar trends were also found for monocyte/macrophages (MOMA+ cells) recruitment. Overall, it was found that the PLA implants recruited 3 times higher CD11b+ inflammatory cells and MOMA+ macrophages than PEG implants (Fig 4E). To determine the relationship between FR-targeting nanoprobe accumulation and implant-mediated macrophage responses, we correlate both the CD11b+ inflammatory cells and MOMA+ macrophage count with the fluorescence intensity. Our results show good near linear trends in both cases ($R^2=0.80-0.96$) (Fig 4F). These results support our assumption that FR-targeting nanoprobes can be employed as an effective imaging probe to detect and to assess the extent of biomaterial-mediated macrophage recruitment and inflammatory responses *in vivo*.

4. Discussion

In the present study, FR-targeting NIR nanoprobes based on PNIPAM-*co*-St nanoparticles were prepared to noninvasively evaluate inflammation by targeting activated MΦs. The uniform nanoparticle with an average diameter of ~100 nm makes it a suitable vehicle for *in vivo* imaging. Previous studies suggest that particles ranging from 10 nm to 200nm are optimal for prolonging blood circulating times [57]. Furthermore, the presence of PNIPAM segments on the nanoparticles may greatly reduce the binding of plasma proteins to the particles, thus enhancing particle bio-distribution [58-60]. Finally, it has been reported that PNIPAM and PNIPAM-based nanoparticles exhibit no or little cytotoxicity [48,54].

Our *in vitro* studies demonstrate that FR-targeting nanoprobes can be used to assess the number of recruited and activated MΦs *in vivo*. The success of this strategy is supported by many previous findings. Specifically, activated MΦs have up-regulated FR expression which has previously been used for targeting molecules [29,39]. Interestingly, similar to our findings, prolonged incubation time was found to enhance uptake of FR-targeting nanoprobes which may be ascribed to a folate-mediated endocytosis by MΦs [28]. It is possible that prolonged exposure to activated MΦs may lead to the endocytosis of initial adherent FR-targeting nanoprobes while allowing additional probes to be bound to FRs on the cell surface. Such internalization may increase the resident time of FR-targeting nanoprobes and increase the sensitivity of such nanoprobes for detection of activated MΦ at the implantation sites *in vivo*.

FR-targeting nanoprobes were found to preferentially accumulate in the LPS-induced inflamed tissue. These results support the hypothesis that such nanoprobes can be used to detect activated MΦs and to indirectly assess the extent of inflammatory responses in tissue. This assumption is supported by many previous observations. First, it is well established that the administration of LPS prompts a localized inflammatory responses with the upregulation of many inflammatory cytokines [61,62] and the accumulation of inflammatory cells, including activated MΦ [30,56,63]. Second, by targeting activated MΦ, several strategies

have been developed to deliver therapeutic and imaging agents [25,28,29,31,64]. Finally, FR-targeting imaging probes have been developed to noninvasively analyze inflammatory activity in atherosclerosis and arthritis in situ [29-31,44]. Interestingly, our study has also uncovered that, despite of a lack of folate, some control nanoprobe were found in the inflammatory tissue. The cause of non-specific accumulation has yet to be determined. However, it has been suggested that nanoparticle accumulation in tissue could be mediated by nonspecific phagocytosis or entrapment in the interstitial space due to the “enhanced permeability and retention” effect (EPR effect) within the inflammation tissues [63,65].

Using polymer particles as model implants, our studies have preliminarily explored the possibility of using FR-targeting nanoprobe to detect activated MΦs in the biomaterial implantation sites. Further analyses revealed that there is a good relationship between FR-targeting nanoprobe accumulation and CD11b+ inflammatory cell/macrophage recruitment in the implantation sites. These results demonstrate that the FR-targeting nanoprobe can be used not only to determine the inflammation location as reported earlier [25,30,31], but also to estimate the extent of biomaterial-mediated macrophage recruitment and associated inflammatory responses. We believe that FR-targeting nanoprobe can also be used to quantify the degree of inflammatory responses to other types of biomaterial implants. Since the FR-targeting nanoprobe are designed to target activated MΦs, representing the major subset of the inflammatory cells, we believe that FR-targeting nanoprobe would provide heightened sensitivity and reliability in the assessment of cellular responses to biomaterial implants, as opposed to other imaging probes currently testing foreign body reactions. Equally important, we anticipate that the FR-targeting nanoprobe-based *in vivo* fluorescence imaging can provide an alternative method for analyzing biomaterial biocompatibility in a rapid, non-invasive and realtime manner. This would greatly improve our understanding of the processes and factors governing foreign body responses to biomaterials. Furthermore, this fluorescence imaging technique may have a practical application in the evaluation and diagnosis of implant safety and performance.

5. Conclusions

FR-targeting NIR nanoprobe have been developed to target activated MΦs. These nanoprobe were found to have high affinity for activated MΦs *in vitro* and in the LPS-induced inflamed tissue *in vivo*. Further study has uncovered that these FR-targeting nanoprobe can be used to detect activated MΦs surrounding biomaterial implants and to assess the overall inflammatory reactions to the subcutaneous implants. These results support that the FR-targeting nanoprobe-based imaging model developed here can provide a rapid noninvasive technique for parallel evaluation of biomaterial tissue compatibility *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant EB007271. The authors would like to thank Drs. Wei Chen and Digant Dave for their facility support to carry out some of the experimental works.

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Fig. 1. (A) Size distribution of PNIPAM-*co*-St at 25 °C. (B) Transmittance electron microscope (TEM) images of P(NIPAM-*co*-St). (C) Absorbance spectra of free and loaded IR750. (D) Emission spectra of free and entrapped IR750. (E) Cytotoxicity study of FR-targeting nanoprobes using MTS assay.

Fig. 2.

In vitro study to assess the nanoprobe specificity to activated macrophages. (A) Influence of incubation time on the accumulation of FR-targeting nanoprobe and control nanoprobe onto activated macrophages. (B) Interference of different concentrations of free folate molecules on the binding of FR-targeting nanoprobe to activated macrophages; (C) Correlation between activated macrophage number and nanoprobe fluorescence intensity.

Fig.3.

In the LPS-induced inflammation model, the FR-targeting nanoprobes and their control nanoprobes were injected intravenously 24 hours after LPS subcutaneous administration. (A) The merged fluorescent signal with a white-light animal image for the FR-targeting nanoprobes and the control nanoprobes at different time points following nanoprobes administration. (B) The enhancement ratio of LPS-induced inflamed sites vs. saline control in the FR-targeting nanoprobes and the control nanoprobes at different time points. (C) The enhancement ratios of fluorescence intensities between FR-targeting nanoprobes and control nanoprobes in LPS-treated inflamed sites at 24 hours. (D) Representative FR+ cell staining and (E) FR+ cell number quantification in isolated tissue 24 hours following either LPS or saline treatment. (F) Representative CD11b+ cell staining and (G) CD11b+ cell number quantification of LPS- and saline-treated tissues isolated 24 hours after nanoprobes injection.

Fig.4.

In an implant-mediated inflammatory responses model, PLA particles, PEG particles and saline were implanted subcutaneously in the back of animals for 24 hours prior to FR-targeting nanoprobe intravenous administration. (A) The merged fluorescent signal with a white-light image captured at different time points following nanoprobe injection. (B) The fluorescence intensities in different implantation sites (PLA, PEG and saline) at different time points. After nanoprobe injection for 24 hours, the tissues surrounding implant sites were isolated for the following measurements. (C) NIR fluorescence (red) acquisition superimposed onto the correlative phase contrast microscopy image in the whole stitched image (20X) and enlarged view (40X). The tissue section was unstained and unfixed to preserve fluorescent signal. (D) Representative histological images and (E) quantification of CD11b+ inflammatory cells and MOMA+ macrophages in tissue surrounding PLA-, PEG- and saline-implanted tissue. (F) Correlation between CD11b+ cell number/macrophages number and fluorescence intensity at the implant sites.