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Synthesis of the Cyanine 7 labeled neutrophil-specific agents for noninvasive near infrared fluorescence imaging

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ABSTRACT

A neutrophil-binding peptide, cinnamoyl-F(D)LF(D)LF (cFLFLF), was labeled with the near infrared (NIR) fluorophore, Cyanine 7 (Cy7). This construct was modified with a polyethylene glycol (PEG, M_W 3.4 kDa) moiety in order to increase its solubility and bioavailability to circulating neutrophils. A preliminary non-invasive fluorescence imaging of a mouse model of ear inflammation with the fluorescent probe is presented.

Neutrophils are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. In a number of inflammatory disorders, neutrophils recruited to the site of injury and their subsequent release of reactive oxygen species (ROS) and proteolytic enzymes are responsible for the tissue damage associated with such disease conditions. Therefore, imaging technologies that could enable specific visualization of neutrophil activation and infiltration noninvasively are highly desirable. Noninvasive imaging of acute inflammation has been achieved by using nuclear imaging modalities with either ex vivo labeled leukocytes[1,2] or in vivo imaging probes directly targeting formyl peptide receptor (FPR) of neutrophils.[3–5] The strength of nuclear imaging is low tissue attenuation and quantitability of its nuclear signal. However, its wide application in research setting is impeded by the cost of the relevant instruments and the limited shelf life of the nuclear probes.

During last decade, the real time in vivo fluorescence imaging of small animals has gone through tremendous technological progress because of its merits of operational simplicity and low cost. As a result, considerable effort has been directed toward the development of new NIR fluorescent probes targeting in vivo molecular events and biochemical processes. However, to date, there has been no report on neutrophil-specific NIR fluorescence imaging agent.

In the current study, we synthesized and evaluated a series of fluorescent neutrophil-binding ligands: cFLFLF-PEG$_n$-Cy7 (n = 0, 12, and 76). The hexapeptide, cFLFLF, exhibits high affinity to FPR,[6] but its high hydrophobicity impedes the application in live animals. We addressed the issue by modifying the hydrophobic peptide with PEG moieties to enhance its bioavailability. The spectrum ($E_{excitation}/E_{emission}$: 745 nm/800 nm) of Cy7 provides better photon signal penetration through animal tissues because of the minimum tissue absorbance in the NIR region (700–900 nm).[7]

Peptides cFLFLFK-CONH$_2$ **4** and cFLFLF-PEG$_{12}$-K-CONH$_2$ **5** were synthesized on solid phase using conventional Fmoc chemistry starting from Fmoc-PAL-PEG-PS resin (0.18 mmol/g, Applied Biosystems, Foster city, CA). The procedure followed our previously published paper,[4] in which PEG$_{12}$ moiety (*N*-Fmoc-amino-dPEG™$_{12}$ acid) (Quanta Biodesign Ltd, Powell, OH) was inserted into the peptide sequence as an amino acid residue, while PEG$_{76}$ moiety (*tert*-Boc-PEG-NHS-3.4 kDa, Laysan Bio Inc., Arab, AL) was conjugated to the ώ-NH$_2$ of the lysine residue of **4** in aqueous solution to form peptide cFLFLFK-PEG$_{76}$-NH$_2$ **6**. The yield of the crude peptides was in the range of 80–85%. Cy™7 Mono NHS ester (GE healthcare, Piscataway, NJ) was conjugated to the ώ-NH$_2$ of the lysine residue of **4** and **5** or the terminal –NH$_2$ of the PEGylated **6** in an 1:1 mixture of acetonitril and sodium borate buffer (0.1 N, pH 8.5) at 4 °C for 2 h, followed by incubation at room temperature for another 1 h in dark. The yield of Cy7 conjugations was in the range of 59–97% (Scheme 1). All of the intermediates and the final products were purified by semi-preparative reverse phase high

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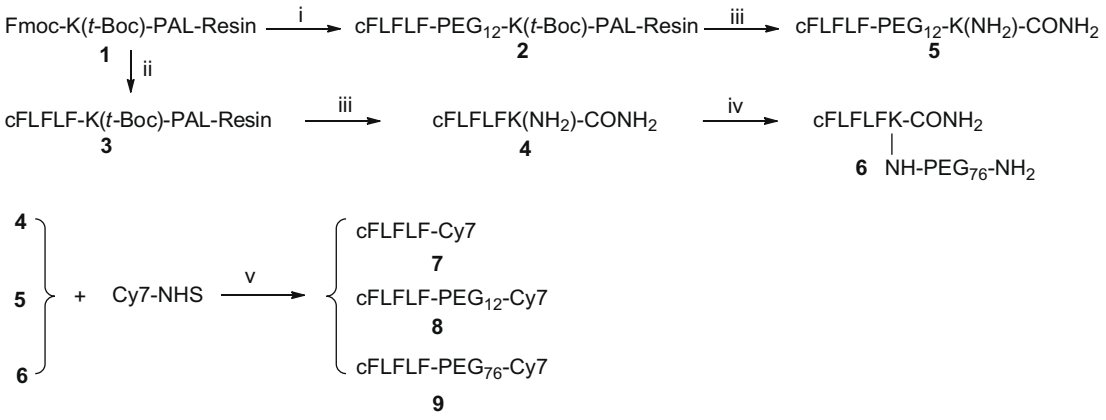

Scheme 1. Reagents: (i), (ii) Standard solid phase Fmoc chemistry; (iii) TFA(95%); (iv) 1—*tert*-Boc-NH-$PEG_{3.4kDa}$-NHS; 2—TFA; (v) CH_3CN/sodium borate buffer, pH 8.5, 4 °C to room temperature.

performance liquid chromatography (RP-HPLC).[8] The HPLC retention times of cFLFLF-Cy7 **7**, cFLFLF-PEG_{12}-Cy7 **8**, and cFLFLF-PEG_{76}-Cy7 **9** were 19.2 min, 16.9 min, and 16.7 min, respectively. The chemical purities of Cy7 conjugates were >95% and these pure samples were used for further experiments. Final Cy7 conjugated products were characterized by MALDI-TOF mass spectroscopy (W.M. Keck Biomedical mass spectrometry laboratory at University of Virginia) as follow: (1) cFLFLF-Cy7 **7**: calculated $[M]^+$: 1607.8; found $[M+H]^+$: 1608.2; (2) cFLFLF-PEG_{12}-Cy7 **8**: calculated $[M]^+$: 2207.1; found $[M+H]^+$: 2208.2; (3) cFLFLF-PEG_{76}-Cy7 **9**: average calculated $[M]^+$: 5007; found $[M+H]^+$: major peaks distributed from 4929 to 5150 with center peak at 5000.

Fluorescence spectra were recorded on a Fluorolog-3 spectrofluorometer (JOBIN YVON/HORIBA, Edison, NJ), with slit width of 5 nm and integration time of 0.2 s. Stock solutions (20 μM) of Cy7 and its peptide conjugates **7**, **8**, and **9** were prepared by dissolving solid sample in 97% aqueous solution with 3% DMSO. Fluorescence spectra were measured by adding 3 mL aliquots of the stock solutions into a quartz cuvette. The fluorescence intensities were normalized and plotted versus emission wavelengths. It turned out that the conjugation of peptides (with or without PEG) and Cy7 did not significantly alter the fluorescence emission spectra of Cy7 (Fig. 1). The excitation wavelengths used in all experiment were 745 nm. The fluorescence emission maximum

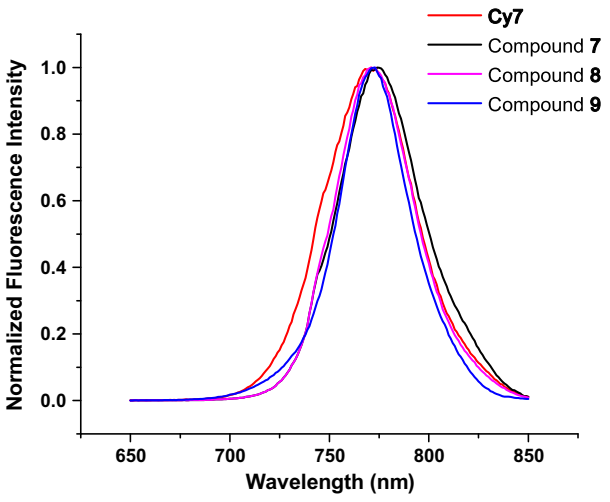

Figure 1. Normalized fluorescence spectra of Cy7 conjugated peptides. Excitation wavelengths were 745 nm for all experiments.

of Cy7 was measured to be 771 nm (vs 775 nm in PBS buffer, pH 7.45). The peptide conjugates showed 2–3 nm red shift in their fluorescence emission maximums versus that of Cy7.

The hydrophilicity of compounds **7**, **8**, and **9** was determined by measuring partition coefficient constant in *n*-octanol and water. In brief, the purified compound (5 nmol) was dissolved in an equal volume (0.6 mL/0.6 mL) mixture of *n*-octanol and deionized water, resulting in a final concentration of 4.15 μM that is in the range of linear relationship between fluorescence probe concentration and fluorescence intensity. After vigorous vibration for 10 min, the mixture was centrifuged at 10,000 rpm for 5 min. Aliquots (in triplets) of 100 μL were aspirated out carefully from each layer and transferred into separate wells of a fluorescence 96-well plate. The plate was placed under the CCD camera of Xenogen IVIS Spectrum (Caliper life science, Hopkinton, MA) and the fluorescence was measured with a filter set of E_x/E_m = 745/800 nm (auto exposure, medium binning, F/Stop = 2). Fluorescence intensity of each well was measured by drawing ROI on the well boundaries and recorded as average radiance ($p/s/cm^2/sr$) using software LiveImage 3.1. Partition coefficient parameter log *P* was calculated using the equation: log([fluorescence intensity in octanol]/[fluorescence intensity in water]). Fluorescence intensities to calculate log *P* were corrected by a factor of solvent effect. The factor was calculated based on different fluorescence intensities of the same concentration of probes in *n*-octanol and water. The average and standard deviation of log *P* values were calculated. It turned out that compound **7** showed most positive log *P* (1.35) and compound **8** demonstrated moderate improvement toward water solubility (log *P*, 0.49). Compound **9** exhibited the best hydrophilicity as log *P* of −1.13 which was comparable to our previously reported PET imaging probe cFLFLF-PEG_{76}-^{64}Cu (log *P*, −1.21).[5] Although Cy7 itself is quite water soluble with log *P* of −1.621 ± 0.001, incorporation of this dye is not enough to compensate the hydrophobicity of the cFLFLF moiety. PEGylation with a larger PEG_{76} moiety indeed improves the bioavailability of the FRP-targeting peptide sequence. As a result, compound **9** was further evaluated as an in vivo imaging probe with a mouse model of ear inflammation.

The mouse model of acute inflammation was induced by topical application of phorbol myristate acetate (PMA) [5 μg in 20 μL of DMSO and acetone (1:9, v/v)] (Sigma–Aldrich, St. Louis, MO) onto the left earlobes of the FVB mice, male, 3 months old, purchased from National Cancer Institute at Frederick (Frederick, MD). It prompted acute ear dermatitis, manifested by local swelling, erythema and infiltration of neutrophils.[9–11] The right ears of the same mice served as control and received only the vehicle [20 μL of DMSO and acetone (1:9, v/v)]. In vivo fluorescence imaging was

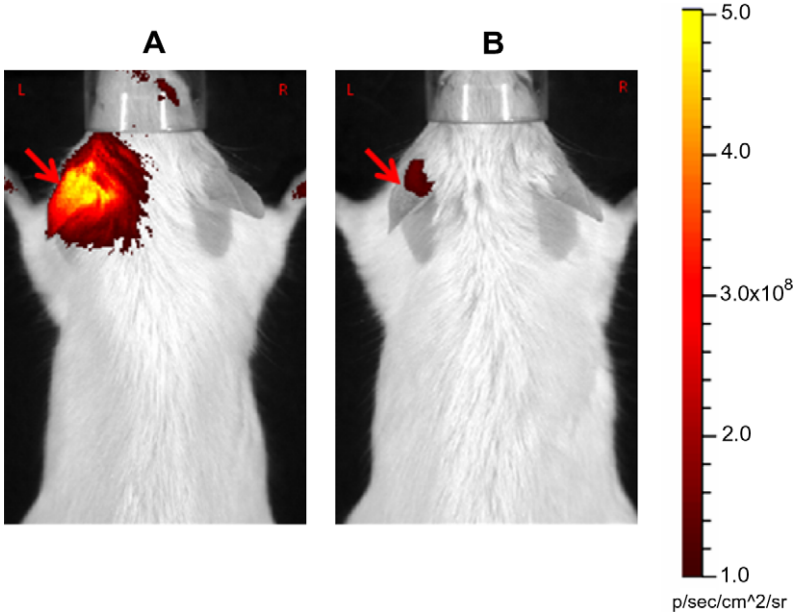

Figure 2. Representative fluorescence image at 3 h postinjection of cFLFLF-PEG$_{76}$-Cy7 **9** (2 nmol): (A) normal group; (B) blocking group pre-injected with 100 nmol (50 equiv) of non-fluorescence peptide **6** 1 h before injection of probe **9**.

performed at 24 h after PMA application. To test the in vivo FPR-specificity of probe **9**, a group of PMA treated mice was designed for blocking experiment. For the blocking mice, excess of the non-fluorescent peptide **6** was administered 1 h before the probe **9** injection.

All in vivo studies were carried out in compliance with University of Virginia Animal Study Committee's requirements for the care and use of laboratory animals in research. In vivo fluorescence imaging was performed on Xenogen IVIS Spectrum with excitation filter of 745 nm and emission filter of 800 nm. Images were acquired and analyzed on LiveImage 3.1 software (Caliper Life Science, Hopkinton, MA). Mice were anesthetized with isoflurane (2%) during probe injection and imaging. After 24 h of PMA application, mice (n = 5) were injected with 2 nmol of probe **9** via tail vein (iv). For the blocking group, mice were injected with 100 nmol (50 equiv) of peptide **6** at 1 h before probe injection. In vivo fluorescence imaging was performed at various time points postinjection (pi). All in vivo NIR fluorescence images were acquired using auto exposure (F/Stop = 2). Measurement ROIs were obtained from both PMA-challenged ears (left) and control ears (right). Background ROIs were recorded as the fluorescence emission from surrounding area of mice. Fluorescence intensities were normalized by subtracting the background ROIs from the measurement ROIs. A representative image is presented in Figure 2. The fluorescence intensity of PMA-challenged ear was significantly higher than control ear, as well as the PMA-challenged ear of blocking group. The ratio of the fluorescence intensity of the infected ear relative to the control ear (L/R) reached the peak value of 3.4 at 3 h pi while in blocking group it demonstrated a significant lower ratio of 1.9 ($P < 0.005$, compared to normal group). It proved the high FPR-specificity of probe **9** in vivo.

In conclusion, we have successfully synthesized cFLFLF-PEG$_{76}$-Cy7 **9** as a potential NIR fluorescence imaging probe for in vivo detection of neutrophilic activation and accumulation. The specificity of the probe was approved by an in vivo blocking experiment. In our mouse model of the PMA induced ear inflammation, probe cFLFLF-PEG$_{76}$-Cy7 **9** showed significantly higher accumulation in the infected ears than in the control ones. Further in vivo evaluation of this fluorescence imaging agent is ongoing in our laboratory, with the goal to correlate the live images with the details from the tissue microscopy data.

Acknowledgments

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8. HPLC was performed with a Varian system with ABI Spetroflow 783 UV detector using an Apollo C18 reversed-phase column (5 μm, 250 × 10 mm). The mobile phase changed from 60% solvent A (0.1% trifluoroacetic acid in water) and 40% solvent B (0.1% trifluoroacetic acid in 80% aqueous acetonitrile) to 100% solvent B during 30 min at a flow rate 3 mL/min. UV detector wavelengths were 215 nm for all the experiments.
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