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Near Infrared Fluorescence-Based Bacteriophage Particles for Ratiometric pH Imaging

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Abstract

Fluorogenic imaging agents emitting in the near infrared are becoming important research tools for disease interrogation *in vivo*. Often pathophysiological states such as cancer and cystic fibrosis are associated with disruptions in acid/base homeostasis. The development of optical sensors for pH imaging would facilitate the investigation of these diseased conditions. In this report, the design and synthesis of a ratiometric near infrared emitting probe for pH quantification is detailed. The pH-responsive probe is prepared by covalent attachment of pH-sensitive and pH-insensitive fluorophores to a bacteriophage particle scaffold. The pH responsive cyanine dye, HCyC-646, used to construct the probe has a fluorogenic pK_a of 6.2, which is optimized for visualization of acidic pH often associated with tumor hypoxia and other diseased states. Incorporation of pH insensitive reference dyes enables the ratiometric determination of pH independent of the probe concentration. With the pH-responsive construct measurement of intracellular pH and accurate determination of pH through optically diffuse biological tissue is demonstrated.

Introduction

The *in vivo* investigation of biological processes at a molecular level has received considerable attention for its potential application in early diagnosis and treatment of disease. Although the disruption of acid/base homeostasis is associated with a variety of diseases such as cancer, cystic fibrosis, and immune dysfunction(1–4), our understanding of the direct and indirect effects of these environments is limited. Due to their sensitivity and non-invasive nature, fluorescence-based imaging methodologies are well suited for interrogation of pH in biological systems. In order to obtain useful *in vivo* pH data, methods need to be employed in which the pH response of the probe is determined in a ratiometric fashion, allowing for measurement of local pH in a probe concentration independent manner. Even though small molecule(5–10) and nanoparticle(11–15) based systems have been employed for ratiometric pH determination in solution and *in vitro*, none are suitable for *in vivo* imaging applications. These probes typically use fluorescein(6,8–10,13–15) or other pH sensitive lumophores(5,7,11,12) with emission in the visible region. For efficient *in vivo* optical imaging, fluorophores that emit in the NIR are necessary due to the increased optical transparency and minimal tissue autofluorescence in this regime.(16) There are currently few reports of NIR pH responsive fluorophores in the literature.(17–21) These dyes have sub-optimal optical and solubility properties for imaging pH *in vivo*. Often the diseased states result in pH changes that are only slightly lower than that of physiological pH. For example, typical extracellular pH values reported for tumors are between 6.3 and 7.0.(3) Current NIR probes are not ideal for biological imaging because they are either

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irreversible(21) or have fluorogenic pK_a values that are too high(17,20) or too low(18) for effective imaging. Recently, we reported a new strategy for tuning the pK_a of pH responsive cyanine dyes to address these issues.(22)

Probes based on a nano-scaffold possess several advantages over small molecule pH sensors. Particle based approaches can easily incorporate a pH insensitive reporter dye allowing for the determination of H^+ concentration independent of the probe concentration. In addition, multiple copies of the reference and pH responsive dyes can be appended to a single particle, effectively increasing the brightness of the individual probe nanoparticle. Our research group has reported the development of targeted M13 bacteriophage particles functionalized with several hundred copies of NIR fluorophores for *in vitro* and *in vivo* receptor targeted imaging applications.(23,24) The M13 bacteriophage, is a flexible hetero-functional nano-platform (25) with a well investigated *in vivo* biodistribution profile.(26) Individual bacteriophage particles are approximately 880×6.6 nm in size and contain 2700 copies of the p8 coat protein with their amino termini exposed to the solvent. These amine groups are available for bioconjugation.(23,27) The preparation of nano-scale pH sensitive imaging agents based on these bacteriophage imaging platforms using optimized pH responsive and pH insensitive NIR fluorophores for potential *in vivo* imaging is detailed in this report.

Experimental Procedures

General Considerations

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI), TCI America (Portland, OR) or Acros Organics USA (Morris Plains, NJ) and were used as received. The starting indoles, 2,3,3-trimethylindolenine-5-sulfonic acid and 1-(ϵ -carboxypentyl)-2,3,3-trimethylindoleninium-5-sulfonate were prepared according to published procedures.(28) The succinimidyl esters of Cy 7 and Cy 5 were purchased from GE Lifesciences (Piscataway, NJ). All solvents, including anhydrous DMF, were purchased from Sigma-Aldrich. High performance liquid chromatography (HPLC) was performed on a Hitachi D-7000 instrument equipped with a L-7455 diode array detector. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker DPX-400 spectrometer at ambient temperature and referenced to tetramethylsilane (TMS) as an internal standard. Absorption spectra were collected on a Varian Cary 50-Bio UV/visible spectrophotometer (Palo Alto, CA). The extinction coefficient measurements for dye **2** were determined in 50 mM glycine buffer, pH 3.0, and were performed in triplicate using reverse phase C-18 and cation exchange chromatography purified dye. For determination of the extinction coefficient, of protonated **2**, fresh stock solutions of the dye were prepared for each trial by dissolution of 2–3 mg portions of the dyes, weighed on a Mettler AT201 analytical balance with an error of ± 0.01 mg, in 50 mM glycine buffer, pH 3.0, using a 10 mL volumetric flask. The extinction coefficient of the deprotonated form of **2** was determined in an analogous manner using aqueous NaOH, pH 10.0. Standard deviations for the extinction coefficient measurements were 5% or less. Fluorescence data were collected on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer (Edison, NJ) and were corrected for the detector sensitivity. Quantum yield measurements of the protonated form of **2** in 50 mM glycine, pH 3.0, were performed in triplicate following published procedures(29) and were collected using dilute samples (absorbance ≤ 0.10 OD) with Cy 5 ($\Phi = 0.27$)(28) in phosphate buffered saline as a standard. All samples for quantum yield determinations were excited at 610 nm. High-resolution electrospray ionization (ESI) mass spectra were collected on a Bruker Daltonics APEXII 3 T Fourier transform mass spectrometer in the Department of Chemistry Instrumentation Facility (DCIF) at the Massachusetts Institute of Technology. Low-resolution ESI mass spectra were obtained using a Waters/Micromass ZQ 4000 mass spectrometer. Fluorescence microscopy was performed on Zeiss Axiovert 100 TV microscope with a 40 $\times$ objective using filter sets from Omega Optical. The NIR reference dye, Cy 7 was imaged using

a 740 ± 12.5 nm band-pass filter for excitation and a 780 nm long pass filter for emission. The pH sensitive dye was imaged using a 610 ± 10 nm band-pass filter for excitation and a 670 ± 20 nm band-pass filter for emission.

Fluorescence reflectance imaging (FRI) data were acquired on a Maestro imaging system from CRi (Woburn, MA). The pH-responsive HCyC-646 (**2**), was imaged using a 590 to 645 nm band-pass excitation filter and the LCD emission filter tuned to 670 nm. The pH insensitive reference, Cy 7, was imaged using a 690 to 750 nm band-pass excitation filter and the LCD emission filter tuned to 775 nm. All Cy 7 images were exposed for 97 ms and all images using dye **2** were exposed for 68 ms.

HCyC-646 Methyl Ester, **1**

To a solution of 1-(ϵ -carboxypentyl)-2,3,3-trimethylindoleninium-5-sulfonate (884 mg, 2.5 mmol) was added chloromalondehyde (266 mg, 2.5 mmol) in 15 mL of MeOH. This solution was sealed in a thick-walled glass pressure reactor and heated at 70 °C for 30 min giving a yellow-green solution. After cooling, 2,3,3-trimethylindolenine-5-sulfonic acid (598 mg, 2.5 mmol) was added, the pressure reactor was re-sealed, and the solution was heated at 70 °C for an additional 5 h giving a dark blue solution. After cooling to room temperature, the solvent was removed by rotary evaporation. The crude blue solid was subjected to reverse-phase C-18 column chromatography (Isolute 70 g RPC18 cartridge) eluting with increasing concentrations of CH₃CN (0, 5, and 10%) in H₂O to afford **1** (163 mg, 9.6%). HPLC analysis of the final product indicates > 97% purity with dye **2** being the major contaminant. ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.1 (broad s, 1H), 8.39 (d, 1H, *J* = 13.8 Hz), 8.27 (broad d, 1H, *J* = 12.8 Hz), 7.85-7.84 (two overlapping singlets, 2H), 7.67-7.63 (two overlapping doublets, 2H), 7.40 (d, 1H, *J* = 8.3 Hz), 7.23 (d, 1H, *J* = 8.1 Hz), 6.37 (broad d, 1H, *J* = 13.8 Hz), 4.13 (t, 2H, *J* = 6.4 Hz), 3.55 (s, 3H), 2.30 (t, 2H, *J* = 7.3 Hz), 1.76-1.33 (m, 18H). MS-ESI [M+H]⁺: calcd for C₃₂H₃₈ClN₂O₈S₂⁺ 677.2, found 677.2.

HCyC-646, **2**

Intermediate **1** (102 mg, 0.15 mmol) was added to 5 mL of 1 M NaOH and heated at 80 °C for 1.5 h. After cooling, the solution was acidified by addition of 10% aqueous HCl and the solvent was removed by rotary evaporation. The blue solid was purified by reverse-phase C-18 column chromatography (Isolute 70 g RPC18 cartridge) eluting with increasing concentrations of CH₃CN (0, 10, and 15%) in H₂O. Following solvent removal, the product was subjected to cation exchange chromatography (Dowex 50WX8 hydrogen form resin) to give pure **2** (88 mg, 88%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.1 (broad s, 1H), 8.39 (d, 1H, *J* = 13.8 Hz), 8.28 (d, 1H, *J* = 12.7 Hz), 7.85 (two overlapping singlets, 2H), 7.67-7.63 (two overlapping doublets, 2H), 7.40 (d, 1H, *J* = 8.3 Hz), 7.23 (d, 1H, *J* = 8.1 Hz), 6.37 (broad d, 1H, *J* = 13.4 Hz), 6.24 (d, 1H, *J* = 13.4 Hz), 4.14 (t, 2H, *J* = 5.9 Hz), 2.20 (t, 2H, 7.2 Hz), 1.76-1.37 (m, 18H). HRMS-ESI [M+H]⁺: calcd for C₃₁H₃₆ClN₂O₈S₂⁺ 663.1596, found 663.1613.

HCyC-646 Su, **3**

To a solution of **2** (19.9 mg, 0.03 mmol) in 1 mL of anhydrous DMF was added 11.5 mg (0.045 mmol) of *N,N'*-disuccinimidyl carbonate (DSC) and triethylamine (31 μ L, 0.225 mmol). After stirring for 4 h, the product was precipitated by addition of *tert*-butyl methyl ether to give a blue solid. This material was further purified by preparative reverse phase HPLC chromatography using a gradient of 100% H₂O with 0.1% TFA to 60% CH₃CN in H₂O with 0.1% TFA to yield **3** (18.9 mg, 83%) as a blue solid. HPLC analysis of the product indicates the presence of approximately 5% dye **2** as an impurity. ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.1 (broad s, 1H), 8.38 (d, 1H, *J* = 13.8 Hz), 8.27 (broad d, 1H, *J* = 13.5 Hz), 7.85-7.84 (two overlapping singlets, 2H), 7.65-7.63 (two overlapping doublets, 2H), 7.39 (d, 1H, *J* = 8.3 Hz), 7.22 (d, 1H, *J* = 8.1 Hz), 6.37 (broad d, 1H, *J* = 13.7 Hz), 6.23 (d, 1H, *J* = 13.4 Hz), 4.14 (broad

t, 2H), 2.80 (s, 4H), 2.69 (t, 2H, J = 7.2 Hz), 1.77-1.37 (m, 18H). HRMS-ESI $[M-H]^-$: calcd for $C_{35}H_{37}ClN_3O_{10}S_2^-$ 758.1614, found 758.1592.

Bacteriophage Preparation and Labeling

Wild type M13 bacteriophage were amplified by infection in *Escherichia coli* and their titer was determined according to published procedures.(30) Following titer determination, the bacteriophage were labeled simultaneously with the succinimidyl esters of Cy 7 and HCyC-647 using a modified literature procedure.(31) Briefly, Cy 7 succinimidyl ester (40 μ L of a 5 μ g/ μ L stock solution in anhydrous DMSO) and HCyC-646 (20 μ L of a 5 μ g/ μ L stock solution in anhydrous DMSO) were added to 1×10^{13} plaque forming units (pfu) of wild type M13 bacteriophage in 2 mL of 100 μ M bicarbonate buffer, pH 8.3. The resulting solution was allowed to incubate for 1 h at room temperature in the dark. Following incubation, the labeled bacteriophage was precipitated by addition of 350 μ L of a PEG 8000/2.5 M NaCl solution (40% w/v PEG 8000) and allowed to stand on ice for 30 min. The bacteriophage was then pelleted by centrifugation at 10,000 G for 15 min. After removal of the supernatant, the pellet was resuspended in 833 μ L of DPBS buffer. This suspension was then subjected to two additional rounds of the PEG precipitation using 166 μ L of the PEG 8000/2.5 M NaCl solution. The final labeled bacteriophage was resuspended in 1000 μ L of DPBS. The concentration of the labeled bacteriophage (in pfu) was determined by comparing the absorbance values of 10-fold dilutions of the initial unlabeled bacteriophage (5×10^9 pfu/ μ L) to the labeled bacteriophage at 280 nm. The degree of labeling for both dyes was determined from a 10-fold dilution of the stock labeled bacteriophage in 100 μ M PBS, pH 8.0, using the extinction coefficients of Cy 7 ($\epsilon = 200,000 \text{ M}^{-1}\text{cm}^{-1}$ at 750 nm)(28) and deprotonated HCyC-646 ($\epsilon = 50,000 \text{ M}^{-1}\text{cm}^{-1}$ at 506 nm). Typical dye labeling using this procedure gives 400–500 copies of each dye per bacteriophage particle.

Sensor Calibration

The pH-dependent response of the sensor was determined based on the ratio of the fluorescence emission bands of the Cy 7 and HCyC-646 fluorophores on the bacteriophage particles in a series of 50 mM phosphate/citric acid buffer solutions from pH 4.9 to 9.3. For the in vitro calibrations, Cy 7 was excited at 715 nm and the fluorescence emission was integrated from 730 to 825 nm. Whereas HCyC-646 was excited at 610 nm and the fluorescence emission was integrated from 630 to 725 nm.

For the cell imaging experiments, in situ standard curves were generated on the same day as the imaging was performed using a procedure modified from the literature.(5) The ionophore nigericin was used for intracellular pH equilibration of all samples.(5) Briefly, RAW cells on 24 well plates were first incubated at 37 °C with the pH-responsive bacteriophage (at a final concentration of 1 μ M Cy 7 on the bacteriophage) in DPBS, pH 7.4 for 1 h. Following removal of the pH-sensitive bacteriophage-containing buffer, the cells were washed $2 \times 100 \mu$ M with the appropriate pH nigericin solutions. Nigericin solutions were prepared by adding 100 μ L of a 10 $\times$ nigericin stock solution (100 μ g/mL nigericin, 1.3 M KCl, and 10 mM $MgCl_2$ in distilled water) to 900 μ L of 50 mM phosphate/citric acid solutions with pH values between 5.0 and 7.5 in increments of 0.5 pH units. The cells were then incubated in the different pH nigericin solutions at 37 °C for 30 min to equilibrate the intracellular pH with the buffer pH and were then imaged by fluorescence microscopy. Images of at least three cells were used to acquire the fluorescence ratio between Cy 7 and HCyC-646 at each pH value for generation of the standard curve.

In the mouse imaging experiments, standard curves were generated by acquiring fluorescence images of the Cy 7 and HCyC-646 signals from 50 μ L volume phantom tubes containing the pH-responsive bacteriophage. For all phantoms, the bacteriophage was adjusted to a Cy 7

concentration of 1 μM . The bacteriophage probe was suspended in a series of 50 mM phosphate/citric acid buffer solutions with pH values between 5.0 and 7.0 in increments of 0.5 pH units. The phantoms were inserted subcutaneously into a mouse, which had been previously sacrificed, prior to imaging in order to correct for differences in light attenuation through tissue between the Cy 7 and HCyC-646 imaging channels. The fluorescence signals from the individual imaging channels were further corrected for background fluorescence by subtracting the average fluorescence intensity from a region of interest (ROI) adjacent to the bacteriophage-probe phantoms from the average signal intensity of ROIs covering the entire phantom. As with the *in vitro* and intracellular-based standard curves, the Cy 7/HCyC-646 ratios were used to generate a standard curve used for subsequent imaging experiments.

Results and Discussion

The water-soluble pH sensitive dye, HCyC-646 used in this report was prepared by the condensation of 1-(ϵ -carboxypentyl)-2,3,3-trimethylindoleninium-5-sulfonate and 2,3,3-trimethylindolenine-5-sulfonic acid with chloromalondehyde and then converted to its activated succinimidyl ester for bioconjugation (Scheme 1). In acidic environments, HCyC-646, exists primarily in its protonated emissive form with absorption at 646 nm and fluorescence emission at 670 nm (Scheme 2, and Figure S1 Supporting Information). The protonated dye has an extinction coefficient of $200,000 \text{ M}^{-1}\text{cm}^{-1}$ at 646 nm and a quantum yield of 8% in aqueous media. At neutral or basic pH, however, deprotonation of the indole nitrogen is accompanied by a large hypsochromic shift in the absorption maximum to 506 nm and loss of NIR fluorescence emission. With a pK_a of 6.2, HCyC-646 is ideally suited for potential imaging of biologically relevant acidic environments such as those found in tumors (32) and other disease states.(33,34) In addition, the long wavelength absorption and emission of HCyC-646 lie within the optimal window for *in vivo* imaging between approximately 650 and 900 nm.(16)

To prepare the ratiometric bacteriophage probe, the succinimidyl esters of the pH-sensitive dye and an additional pH-insensitive reference dye, Cy 7, were conjugated to the surface of the bacteriophage particles. In a typical labeling procedure approximately 400–500 copies of each dye could be attached to the surface of the bacteriophage particles. Once labeled, the bacteriophage respond to pH in a ratiometric manner with the HCyC-646 emission signal varying with pH and the Cy 7 signal remaining stable from pH 5 to 9 (Scheme 3 and Figure 1). This pH range matches well with the expected pH values in biological systems.

The labeled bacteriophage can be used for intracellular pH imaging. Incubation of the pH-responsive probe with RAW cells for one hour, results in internalization of the phage particles into acidic intracellular vesicles. Cells were imaged with filter sets optimized for HCyC-646 and Cy 7. To obtain pH map data, the ratios of the Cy 7 and HCyC-646 signals were taken and then correlated to an *in situ* standard curve. Using this approach, it is possible to visualize intracellular pH (Figure 2). The cellular pH readings were found to lie between 6.5 and approximately 5.0. These values agree well with reported pH ranges for acidic intracellular vesicles such as endosomes, phagosomes, and lysosomes.(35) These data are also in good agreement with intracellular pH readings from a recently published fluorescein-based ratiometric pH probe.(13) In addition to the ability of the pH-responsive bacteriophage to image intracellular pH, the probe can be used to image through optically diffuse biological tissue. To demonstrate the ability of the phage particles for imaging through tissue, pH adjusted phantoms containing a 50 μL solution of the probe adjusted to a concentration of 1 μM with respect to Cy 7 were inserted subcutaneously into mice and imaged by fluorescence reflectance imaging (Figure 3). As with the cell imaging experiments, the fluorescence signals were ratioed and mapped to a standard curve to generate the pH maps. There is excellent correlation between the ratiometric-based pH readings and the electrode measured pH values of the phantoms. For

example, the ratiometrically-measured pH of the phantom in Figure 3a of 6.1 compares well with the actual pH of 6.16. In Figure 3b, a ratiometrically measured pH of 6.5 matches well with the actual pH of 6.60. The ability to clearly visualize different pH values in this range should prove invaluable for *in vivo* imaging of pathophysiological processes associated with acidic pH. One current limitation of this system arises from differences in the tissue penetrating ability of light at the emission wavelengths of the two fluorophores (Table S1, Supporting Information). The shorter wavelength fluorescence emission at ~670 nm from HCyC-646 shows stronger tissue absorbance than the ~775 nm emission from Cy 7. To correct for the light penetrating abilities at the different imaging wavelengths fluorescence data for the standard curve were obtained under identical conditions to those in which the unknown pH phantoms were imaged.

In summary, we report the development of a new nano-scaffold for fluorescence-based pH imaging, both intracellular and through optically diffuse biological tissue. The pH sensitive cyanine dye, HCyC-646, used in this report has a pK_a of 6.2, which is optimized for visualization of pH in a biologically relevant range. Through coupling of several hundred copies pH sensitive HCyC-646 and Cy 7, a pH insensitive dye, to the surface of bacteriophage particles, a bright pH-responsive ratiometric imaging platform was obtained. With this platform, it is possible to make intracellular pH measurements as well as determine pH through optically diffuse tissue. Current research is focused on development of new nanoparticle scaffolds, for preparation of additional ratiometric pH-sensitive systems. We are also exploring strategies in which correcting for differences in light penetration through diffuse biological tissue at the different imaging wavelengths would not be necessary. One potential approach is through the use of fluorescence lifetime-based measurements using pH-sensitive and – insensitive dyes, which have identical absorption and emission wavelengths, but different fluorescence lifetimes. Once these hurdles are overcome, *in vivo* fluorescence-based imaging of pH in disease will be feasible. Such systems could prove invaluable for identification and diagnosis of tumors and other diseased states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kellum JA, Song M, Li J. Extracellular Acidosis and the Immune Response: Clinical and Physiologic Implications. *Crit. Care* 2004;8:331–336. [PubMed: 15469594]
2. Coakley RD, Grubb BR, Paradiso AM, Gatzky JT, Johnson LG, Kreda SM, O'Neal WK, Boucher RC. Abnormal Surface Liquid pH Regulation by Cultured Cystic Fibrosis Bronchial Epithelium. *Proc. Natl. Acad. Sci. USA* 2003;100:16083–16088. [PubMed: 14668433]
3. Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. pH Imaging. *IEEE Eng. Med. Biol. Mag* 2004;23:57–64. [PubMed: 15565800]
4. Gillies RJ, Schornack PA, Secomb TW, Raghunand N. Causes and Effects of Heterogenous Perfusion in Tumors. *Neoplasia* 1999;1:197–207. [PubMed: 10935474]

Supporting Information:

Spectroscopic characterization of pH-responsive dye, **2**, and signal attenuation of the fluorophores through tissue. This material is available free of charge via the Internet at <http://pubs.acs.org>.

5. Overly CC, Lee K-D, Berthiaume E, Hollenbeck PJ. Quantitative Measurement of Intracellular pH in the Endosomal-Lysosomal Pathway in Neurons by Using Ratiometric Imaging with Pyranine. *Proc. Natl. Acad. Sci. USA* 1995;92:3156–3160. [PubMed: 7724533]
6. Martin GR, Jain RK. Fluorescence Ratio Imaging Measurement of pH Gradients: Calibration and Application in Normal and Tumor Tissues. *Microvasc. Res* 1993;46:216–230. [PubMed: 8246820]
7. Bassnett S, Reinisch L, Beebe DC. Intracellular pH Measurement Using Single Excitation-Dual Emission Fluorescence Ratios. *Am. J. Physiol.-Cell Physiol* 1990;258:C171–C178.
8. Murphy RF, Powers S, Cantor CR. Endosome pH Measured in Single Cells by Dual Fluorescence Flow Cytometry: Rapid Acidification of Insulin to pH 6. *J. Cell Biol* 1984;98:1757–1762. [PubMed: 6144684]
9. Heiple JM, Taylor DL. Intracellular pH in Single Motile Cells. *J. Cell Biol* 1980;86:885–890. [PubMed: 7410485]
10. Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH Measurements in Ehrlich Ascites Tumor Cells Utilizing Spectroscopic Probes Generated in Situ. *Biochemistry* 1979;18:2210–2218. [PubMed: 36128]
11. Tomasulo M, Yildiz I, Raymo FM. pH-Sensitive Quantum Dots. *J. Phys. Chem. B* 2006;110:3853–3855. [PubMed: 16509664]
12. Kim S, Pudavar HE, Prasad PN. Dye-Concentrated Organically Modified Silical Nanoparticles as a Ratiometric Fluorescent pH Probe by One- and Two-Photon Excitation. *Chem. Commun* 2006:2071–2073.
13. Burns A, Sengupta P, Zedayko T, Baird B, Weisner U. Core/Shell Fluorescent Silica Nanoparticles for Chemical Sensing: Towards Single-Particle Laboratories. *Small* 2006;2:723–726. [PubMed: 17193111]
14. McNamara KP, Nguyen T, Dumitrascu G, Ji J, Rosenzweig N, Rosenzweig Z. Synthesis, Characterization, and Application of Fluorescence Sensing Lipobeads for Intracellular pH Measurements. *Anal. Chem* 2001;73:3240–3246. [PubMed: 11476221]
15. Ji J, Rosenzweig N, Griffin C, Rosenzweig Z. Synthesis and Application of Submicrometer Fluorescence Sensing Particles for Lysosomal pH Measurements in Murine Macrophages. *Anal. Chem* 2000;72:3497–3503. [PubMed: 10952534]
16. Weissleder R, Ntziachristos V. Shedding Light Onto Live Molecular Targets. *Nat. Med* 2003;9:123–128. [PubMed: 12514725]
17. Zhang Z, Achilefu S. Design, Synthesis and Evaluation of Near-Infrared Fluorescent pH Indicators in a Physiologically Relevant Range. *Chem. Commun* 2005:5887–5889.
18. Strekowski L, Mason JC, Lee H, Say M, Patonay G. Water-Soluble pH Sensitive 2,6-Bis(Substituted Ethylidene)-Cyclohexanone/Hydroxy Cyanine Dyes that Absorb in the Visible/Near-Infrared Regions. *J. Heterocyclic Chem* 2004;41:227–232.
19. Cooper ME, Gregory S, Adie E, Kalinka S. pH-Sensitive Cyanine Dyes for Biological Applications. *J. Fluoresc* 2002;12:425–429.
20. Snee PT, Somers RC, Nair G, Zimmer JP, Bawendi MG, Nocera DG. A Ratiometric CdSe/Zns Nanocrystal pH Sensor. *J. Am. Chem. Soc* 2006;128:13320–13321. [PubMed: 17031920]
21. Almutairi A, Guillaudeau SJ, Berezin MY, Achilefu S, Fréchet JMJ. Biodegradable pH-Sensing Dendritic Nanoprobes for Near-Infrared Fluorescence Lifetime and Intensity Imaging. *J. Am. Chem. Soc* 2008;130:444–445. [PubMed: 18088125]
22. Hilderbrand SA, Weissleder R. Optimized pH-Responsive Cyanine Fluorochromes for Detection of Acidic Environments. *Chem. Commun* 2007:2747–2749.
23. Hilderbrand SA, Kelly KA, Weissleder R, Tung C-H. Monofunctional Near-Infrared Fluorochromes for Imaging Applications. *Bioconjugate Chem* 2005;16:1275–1281.
24. Kelly KA, Waterman P, Weissleder R. In Vivo Imaging of Molecular Targeted Phage. *Neoplasia* 2006;8:1011–1018. [PubMed: 17217618]
25. Khalil AS, Ferrer JM, Brau RR, Kottmann ST, Noren CJ, Lang MJ, Belcher AM. Single M13 Bacteriophage Tethering and Stretching. *Proc. Natl. Acad. Sci. USA* 2007;104:4892–4897. [PubMed: 17360403]
26. Zou J, Dickerson MT, Owen NK, Landon LA, Deutscher SL. Biodistribution of Filamentous Phage Peptide Libraries in Mice. *Mol. Biol. Reports* 2004;31:121–129.

27. Newton JR, Miao Y, Deutscher SL, Quinn TP. Melanoma Imaging with Pretargeted Bivalent Bacteriophage. *J. Nucl. Med* 2007;48:429–436. [PubMed: 17332621]
28. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. Cyanine Dye Labeling Reagents: Sulfoindocyanine Succinimidyl Esters. *Bioconjugate Chem* 1993;4:105–111.
29. Demas JN, Crosby GA. The Measurement of Photoluminescence Quantum Yields. A Review. *J. Phys. Chem* 1971;75:991–1024.
30. Kelly KA, Allport JR, Tsourkas A, Shinde-Patil VR, Josephson L, Weissleder R. Detection of Vascular Adhesion Molecule-1 Expression using a Novel Multimodal Nanoparticle. *Circ. Res* 2005;96:327–326. [PubMed: 15653572]
31. Jaye DL, Geigerman CM, Fuller RE, Akyildiz A, Parkos CA. Direct Fluorochrome Labeling of Phage Display Library Clones for Studying Binding Specificities: Applications in Flow Cytometry and Fluorescence Microscopy. *J. Immunol. Methods* 2004;295:119–127. [PubMed: 15627617]
32. Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. pH Imaging. *IEEE Eng. Med. Biol. Mag* 2004;23:57–64. [PubMed: 15565800]
33. Ricciardolo FLM, Gaston B, Hunt J. Acid Stress in the Pathology of Asthma. *J. Allergy Clin. Immunol* 2004;113:610–619. [PubMed: 15100663]
34. Song Y, Salinas D, Nielson DW, Verkman AS. Hyperacidity of Secreted Fluid from Submucosal Glands in Early Cystic Fibrosis. *Am. J. Physiol.-Cell Physiol* 2006;290:741–749.
35. Vieira OV, Bothelo RJ, Grinstein S. Phagosome Maturation: Aging Gracefully. *Biochem. J* 2002;366:689–704. [PubMed: 12061891]

Figure 1.

Fluorescence response of the labelled bacteriophage in a series of different pH 50 mM phosphate/citric acid buffers. The emission from the conjugated HCyC-646 (squares) varies with pH, whereas the Cy 7 signal (triangles) is pH insensitive. Excitation for HCyC-646 was at 610 nm and the emission signal was integrated from 630 to 725 nm. Excitation for Cy 7 was at 715 nm and the emission signal was integrated from 730 to 825 nm. The probe was adjusted to a concentration of 1 μ M with respect to Cy 7.

Figure 2.

Intracellular ratiometric pH imaging in RAW cells with the pH-responsive bacteriophage particles. Prior to Imaging, the cells were incubated with the ratiometric imaging probe in PBS, pH 7.4 adjusted to a final probe concentration of 1 μ M with respect to Cy 7. The fluorescence images from the HCyC-646 and Cy 7 were ratioed and correlated to the standard curve to generate the pH map.

Figure 3.

Fluorescence ratio pH maps through optically diffuse tissue of 50 μ L phantoms containing the pH-responsive probe adjusted to a concentration of 1 μ M with respect to Cy 7 in 50 μ M phosphate/citric acid buffers. The left and right panels show the reconstructed pH ratio maps from the phantoms and the pH maps overlaid on the bright field images, respectively. The ratiometrically determined pH values of 6.1 (A) and 6.5 (B) match well with the corresponding pH electrode measurements of 6.16 and 6.60.

Scheme 1.
Synthesis of the pH-responsive cyanine derivative.

Scheme 2.
The pH-dependent equilibrium of HCyC-646.

Scheme 3.
Fluorophore labeled bacteriophage particles.