Magnetic resonance imaging (MRI) is a powerful non-invasive imaging technique that has greatly impacted basic biological research as well clinical diagnosis of cancer and other diseases. Conventional MR contrast agents are $T_1$ (e.g. Gd-DTPA) or $T_2$-based (e.g. iron oxide), which cause significant longitudinal or transverse relaxation of protons, respectively. Despite their success in many biological applications, one potential limitation is the lack of multichromatic features that allows for simultaneous detection of multiple signals. Recently, $^{19}$F has received significant attention in MR imaging and spectroscopy studies. Compared to $^1$H-MRI, $^{19}$F-MRI has little biological background owing to the low levels of endogenous fluorine in the body. Moreover, $^{19}$F has 100% natural abundance and its gyromagnetic ratio (40.06 MHz/T) is second only to $^1$H, which makes it more sensitive for detection over other nuclei.

Herein, we report on the development of “multi-colored” pH-activatable $^{19}$F-MRI nanoprobes with tunable pH transitions. Recently, extensive efforts have been dedicated to the development of stimuli-responsive nanoprobes. Various nanosystems that respond to pH, enzymatic expression, redox reaction, temperature, and light have been reported. Among these stimuli, the pH stands out as an important physiological parameter that plays a critical role in both the intracellular ($pH_i$) and extracellular ($pH_e$) milieu. For example, dysregulated pH was described as another hallmark of cancer, where a “reverse” pH gradient across the cell membrane is observed in cancer cells compared to normal cells. A variety of different types of MRI agents have been reported for measuring pH values, but all have a rather broad pH response which may limit the accuracy of the pH measurement, particularly when the pH perturbation in the pathological tissue is small. Moreover, it is often necessary to administer another pH-insensitive agent to correct for the contribution of agent concentration to obtain pH-sensitive signals, which makes the procedure complicated and difficult to perform.

Herein we describe the development of pH-sensitive $^{19}$F-MRI nanoprobes with a binary (ON/OFF) response to a specific, narrow pH transition (0.25 pH unit). We theorize that a collection of such nanoprobes where each pH transition is encoded with a specific $^{19}$F signature will allow for a simple readout of environmental pH value through an “activation barcode”. To demonstrate this proof of concept, we synthesized three $^{19}$F-MRI nanoprobes with different pH transitions and $^{19}$F-reporters (Scheme 1). Through these nanoprobes, we show in phantom studies the feasibility of using either $^{19}$F NMR spectroscopy or imaging to discriminate the pH difference.

Scheme 1. a) pH-activatable ON/OFF $^{19}$F-MRI nanoprobes from ionizable diblock copolymers. At $pH > pK_a$, the hydrophobic segments self-assemble into a micelle core leading to $^{19}$F signal suppression as a result of restricted polymer chain motion. Upon pH activation ($pH < pK_a$), micelle disassembly leads to dissociated unimers and a strong $^{19}$F signal. b) Structural formula of three representative diblock copolymers containing different pH responsive segments and $^{19}$F reporter moieties, their $pK_a$ values and $^{19}$F chemical shifts (in ppm, relative to trifluoroacetic acid, or TFA), respectively, are shown in parenthesis.
ences in the microenvironment (i.e. pH = 7.4, 6.5, 5.5, and 4.5).

The challenge in designing a set of multi-colored pH-activatable $^{19}$F-nanoprobes is two-fold: first is the availability of reporter molecules that can be distinguished by MRS/I. For this purpose, $^{19}$F is highly advantageous over $^1$H probes as many $^{19}$F reporter molecules have diverse chemical shifts and narrow peak widths that can be easily differentiated. The second is to devise an activation mechanism in which the signal intensities of these $^{19}$F reporter molecules are highly responsive to the pH changes in the environment. In this regard, we adopted a strategy of using changes in spin–spin relaxations between the micelle and unimer states to turn ON/OFF $^{19}$F signals in response to the pH value.$^{[3e,i]}$ $^{19}$F relaxations between the micelle and unimer states to turn OFF19F signals in response to the pH value.$^{[3e,i]}$ $^{19}$F relaxations between the micelle and unimer states to turn OFF19F signals in response to the pH value.$^{[3e,i]}$

We hypothesize that at pH $> pK_a$ of amphiphilic copolymers consisting of a hydrophilic poly(ethylene oxide) (PEO) segment and tertiary amine/ammonium segment (Scheme 1b). We hypothesize that at pH $> pK_a$, hydrophobic micelle assembly results in highly restricted chain motions and short spin–spin relaxation times ($T_2$) to effectively broaden and eliminate the $^{19}$F signals; at pH $< pK_a$, protonation of ammonium groups will result in micelle disassembly, conformational flexibility in the dissociated polymer chains (unimers), and reappearance of the previous $^{19}$F signal.

For initial development, we synthesized the poly(ethylene oxide)-b-poly-[diisopropylamino] ethyl methacrylate-trifluoroethyl methacrylate] (PEO-b-P(DPA-r-TFE)) copolymer using atom transfer radical polymerization method.$^{[14]}$ To investigate the optimal composition, we synthesized a series of PEO-b-P(DPA-r-TFE) copolymers with increasing molar ratios (5 to 75 mol%) of the TFE component (Table S1–S2, Figure S1 in the Supporting information). A higher TFE content should lead to more intense $^{19}$F signals, whereas too much TFE may override the pH response from the copolymer without TFE.$^{[5c]}$ An increase in TFE content ($\approx 20$ mol%) was observed in the formation of micelles at pH 7.4 (above its $pK_a$ of 6.1) and complete micelle dissociation at pH 5.0 (Figure S3a). The micelle–unimer transition was further corroborated by $^1$H NMR spectroscopy (Figure S3b) and dynamic light scattering, which showed the hydrodynamic diameters were changed from 40 to 6 nm at pH 7.4 and 5.0, respectively (Figure S3c).

![Image](image_url)

**Figure 1.** a) $^{19}$F spectra of 2 mg mL$^{-1}$ PEO-b-P(DPA$_{48}$-r-TFE$_{12}$) micelles in deuterated acetate buffers at different pH values. TFA was used as an external reference with its chemical shift set as 0. b) Normalized $^{19}$F signal intensity as a function of pH value. Data was obtained from (a).
poly[dibutylamino methacrylate]-nanoprobes. 19F spectroscopy was then performed for each experiment, in which four solutions at pH 7.4, 6.5, 5.5, and 4.5 were first prepared containing the same mixture of the three other is OFF (Figure 3a).

The OFF state). Such a barcode design allows for the direct readout of the microenvironment pH value within two a single color to each nanoprobe for the ON state (black for the microenvironment. In addition to TFE (2.3 ppm), we introduced two additional 19F reporter molecules (Scheme 1b, DFB and BTBF, δF = −33.2 and 13.3 ppm, respectively). These reporter molecules were incorporated into two new copolymers with different pH sensitivities, poly(ethylene oxide)-b-poly[2-(pentamethylene imino) methacrylate-r-2-(methacryloyloxy) ethyl 3,5-bis(trifluoromethyl) benzoate] (PEO-b-P(DBA-r-BTFB)) and poly(ethylene oxide)-b-poly[2-(dibutylamino) methacrylate-r-2-(methacryloyloxy) ethyl 3,5-difluorobenzoate] (PEO-b-P(DCA-r-DFB); Table S3). pH titration experiments demonstrated similar ultra-pH responsive properties of the two new copolymers (Figure S4). The pKa values of the PEO-b-P(C6A-r-BTFB) and PEO-b-P(DBA-r-DFB) copolymers were 7.0 and 5.0, respectively, in addition to PEO-b-P(DPA-r-TFE) (pKa = 6.1). Based on these pKa values, we defined a three-digit barcode where each digit corresponds to one nanoprobe (with pH 4.5 did not affect the signal contrast significantly, demonstrating successful 19F detection in biologically relevant media (Figure S5).

In addition to 19F spectroscopy, we also used 19F MRI to spatially resolve the nanoprobe activation map. A phantom sample was prepared in which four smaller tubes (each containing the same nanoprobe mixture in solutions at pH 7.4, 6.5, 5.5, and 4.5) were placed in a bigger tube with water only. T1-weighted 1H MRI images show similar signal intensity from all the tubes and the surrounding water (Figure 3c). For 19F MR imaging, we selectively activated each 19F reporter at its chemical shift to examine the nanoprobe activation. Based on results from each 19F channel, we were able to obtain the barcode information for the different regions of interest (Figure 3c). Potentially, by combining the 19F spectroscopy and imaging capabilities, we can generate a pH map where each voxel can be encoded with an activation barcode to indicate its environmental pH value with spatial discrimination.

In summary, we report the feasibility of a series of multichromatic pH-activatable 19F nanoprobes encoded with different 19F reporters at specific pH transitions. Compared to small molecular pH sensors (typically 2 pH unit for a 10-fold signal change across pKa), the pH response of these nanoprobes is extremely sharp (ΔpHON/OFF ≈ 0.25 pH unit) and can be used as binary indicators for a specific pH transition. The current three nanoprobe collection provides the proof of concept and allows for a qualitative measurement of environmental pH values. This nanoplatform can potentially overcome the instrument complexity and short T1 limitation of the 13C-based hyperpolarization probes. Moreover, compared to chemical exchange saturation transfer (CEST) or 1H agents with which small pH-dependent chemical shifts are quantified, the chemical shifts of 19F reporters are widely separated and easily differentiated for binary readout and data processing. Development of additional nanoprobes with more refined pH transitions will be useful to narrow the pH transitions and improve the precision of the pH measurement. In addition, use of hybrid nanoparticles to include all 19F-encoded polymers in one system could further unify pharmacokinetics and biodistribution during in vivo studies. Through a barcode map from 19F-imaging spectroscopy, it is conceivable to generate a pH map in three dimensions. Along with these exciting potentials, one main challenge in subsequent preclinical translation of these nanoprobes is the relatively low detection sensitivity of 19F-MRS/I. Optimization of MR scan time, pulse sequence or coil design should further improve the current detection limit (0.16 mgmL−1 19F). Image resolution can also be compromised to achieve higher detection sensitivity. Upon successful demonstration, the 19F nanoprobes will add to the existing arsenal of pH sensors to measure tissue pH values, an important physiological param-

SNR_ON/ON is 27-fold based on the 19F images, demonstrating that the 19F reporters on the polymers are highly responsive to the pH changes in the environment. In comparison, the SNR_ON/ON ratio from the 1H images was only 1.2.

Finally, we investigated the “barcode” concept using a mixture of 19F-MRI nanoprobes with different pH transitions and 19F reporter molecules to distinguish pH values in the microenvironment. In addition to TFE (δF = 2.3 ppm), we introduced two additional 19F reporter molecules (Scheme 1b, DFB and BTBF, δF = −33.2 and 13.3 ppm, respectively). These reporter molecules were incorporated into two new copolymers with different pH sensitivities, poly(ethylene oxide)-b-poly[2-(pentamethylene imino) methacrylate-r-2-(methacryloyloxy) ethyl 3,5-bis(trifluoromethyl) benzoate] (PEO-b-P(C6A-r-BTFB)) and poly(ethylene oxide)-b-poly[2-(dibutylamino) methacrylate-r-2-(methacryloyloxy) ethyl 3,5-difluorobenzoate] (PEO-b-P(DCA-r-DFB); Table S3). pH titration experiments demonstrated similar ultra-pH responsive properties of the two new copolymers (Figure S4). The pKa values of the PEO-b-P(C6A-r-BTFB) and PEO-b-P(DBA-r-DFB) copolymers were 7.0 and 5.0, respectively, in addition to PEO-b-P(DPA-r-TFE) (pKa = 6.1). Based on these pKa values, we defined a three-digit barcode where each digit corresponds to one nanoprobe (with pH 4.5 did not affect the signal contrast significantly, demonstrating successful 19F detection in biologically relevant media (Figure S5).

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