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Self-assembled hydrogel fibers for sensing the multi-compartment intracellular milieu

Praveen Kumar Vemula1,2,3*, Jonathan E. Kohler4, Amy Blass4, Miguel Williams4, Chenjie Xu1,2,3, Lynna Chen1,2,3, Swapnil R. Jadhav5, George John5, David I. Soybel4† & Jeffrey M. Karp1,2,3

1Department of Biomedical Engineering, Center for Regenerative Therapeutics and Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115 USA, 2Harvard-MIT Division of Health Science and Technology, 65 Landsdowne Street, PRB 313 Cambridge, MA 02139, USA, 3Harvard Stem Cell Institute, 1350 Massachusetts Avenue, Cambridge, MA 02138 USA, 4Department of Surgery, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA, 5Department of Chemistry, City College of New York, 138th Street, Convent Avenue, New York, NY 10031, USA.

Targeted delivery of drugs and sensors into cells is an attractive technology with both medical and scientific applications. Existing delivery vehicles are generally limited by the complexity of their design, dependence on active transport, and inability to function within cellular compartments. Here, we developed self-assembled nanofibrous hydrogel fibers using a biologically inert, low-molecular-weight amphiphile. Self-assembled nanofibrous hydrogels offer unique physical/mechanical properties and can easily be loaded with a diverse range of payloads. Unlike commercially available E. coli membrane particles covalently bound to the pH reporting dye pHrodo, pHrodo encapsulated in self-assembled hydrogel-fibers internalizes into macrophages at both physiologic (37°C) and sub-physiologic (4°C) temperatures through an energy-independent, passive process. Unlike dye alone or pHrodo complexed to E. coli, pHrodo-SAFs report pH in both the cytoplasm and phagosomes, as well the nucleus. This new class of materials should be useful for next-generation sensing of the intracellular milieu.

The transportation of therapeutic or diagnostic agents and reporter molecules across the cell membrane into multiple intracellular compartments remains a significant challenge due to the selective permeability and biological complexity of eukaryotic cell membranes. Active transport via endocytosis, phagocytosis and pinocytosis are thought to be the major mechanisms mediating uptake of particles. However, controversy over specific mechanisms that mediate internalization still exist1,2. Specialized mammalian cells associated with the immune response readily engulf solid particles with diameters > 750 nm through phagocytosis3,4. In humans, professional phagocytes such as macrophages, monocytes, neutrophils, and dendritic cells display the most permissive internalization of large particles (>1 μm), while smaller particles are internalized via endocytosis by a wide range of cell types. Professional phagocytes engulf and digest bacteria and tissue debris, an essential element of their function in early and late immune responses, wound healing, and cellular recycling5-7. The bactericidal effect of phagosomes relies on acidification and oxygen radicals to create a hostile environment for microbes. However, some intracellular pathogens are known to evade the phagosome by perturbing phagosomal membrane maturation and modulating acidification8-10. Although multiple types of pH sensing probes have been developed, efficient delivery of such probes into specific intracellular compartments remains a significant challenge. Existing technologies for measuring phagosomal pH depend on active internalization of particles tagged with reporters that are amenable to chemical modification11-14, and are not capable of concomitant reporting of the nucleus and cytoplasm, or of multiplexed reporting of multiple intracellular parameters. These considerations emphasize the need for different kinds of reporter vehicles, capable of reporting activity across multiple cellular compartments and the cytoplasm.

Current delivery systems are reporter-specific and generally custom-made by covalently linking fluorescent reporters to particles (1–10 μm) such as latex beads or bacterial wall fragments11-14. For example, covalently attached pHrodo on bacterial-particles and dextran-particles have become a gold standard to track phagocytosis and endocytosis, respectively15,16. However, not all fluorescent dyes are amenable to covalent binding, and chemical modification can compromise or eliminate the reporting properties of the dye, limiting the potential
scope of this approach to a narrow range of molecules that retain their activity after binding. Alternative strategies are similarly limited. For example Modi et al recently reported the development of a DNA 1-switch to monitor endosomal pH using fluorescence resonance energy transfer (FRET). Although those nanosensors function adequately, they are limited by cost and scalability given that new designs are required for each condition to be sensed. Non-covalent delivery systems have traditionally been hindered by multiple constraints. Physical encapsulation of dyes in a polymer matrix often generates heterogeneity in the system, and the leaching of the sensor dyes causes instability, thus reducing the life time and reproducibility of the sensor. Thus, there is a significant unmet need to develop a platform delivery vehicle that could be loaded with a wide range of reporters without covalent modification and that could be readily internalized into multiple intracellular compartments including the cytoplasm and nucleus. Ideally, once internalized the vehicle/reporter construct should remain stable and rapidly, reversibly, and reproducibly respond to and report intracellular physiology in real-time.

To address these design criteria, we have explored the use of custom-designed self-assembled gel fibers (SAFs) that can physically encapsulate reporter molecules. Intracellular compartments may contain high concentrations of proteolytic enzymes and reactive oxygen species; thus SAFs should be relatively inert to enable long-term stability following internalization. In addition, SAFs should not exhibit buffering capacity that could alter the pH of the microenvironment and interfere with a pH sensing dye. Additionally, the ideal delivery vehicle would permit the encapsulation of multiple reporters to enable multiplexed readouts, such that multiple intraphagosomal functions could be tracked concurrently. To fulfill these criteria, we have designed and synthesized an amphiphilic derivative of the nonvolatile, odorless, color-stable, water-soluble, and essential (see Methods). Importantly, the presence of pHrodo altered neither the self-assembling ability of the amphiphile nor the morphology of the self-assembled hydrogel. Repeating a similar procedure in the presence of pHrodo dissolved in PBS (40 μg) produced a pHrodo-encapsulated self-assembled hydrogel. Subsequently, SAFs were isolated from the bulk hydrogel (see Methods). Importantly, the presence of pHrodo altered neither the self-assembling ability of the amphiphile nor the morphology of the fibers (Fig. 1a). This significant shift of 42 cm⁻¹ was lower than in a methanolic solution of Tris-12 (1629 cm⁻¹) was lower than in a methanolic solution of Tris-12 (1629 cm⁻¹) was lower than in a methanolic solution of Tris-12 (1629 cm⁻¹) was lower than in a methanolic solution of Tris-12 (1629 cm⁻¹) was lower than in a methanolic solution of Tris-12 (1629 cm⁻¹). This significant shift of 42 cm⁻¹ could be attributed to the strong hydrogen-bonding interactions of the carbonyl groups of amides in the self-assembled state, which clearly suggests that hydrogen-bonding enhances the self-assembly of these amphiphiles to form fibrous structures as depicted in Fig. 1b.

To confirm the location and function of SAFs, a pH-reporter fluorescent dye (pHrodo) was encapsulated into SAFs. pHrodo is a well-known fluorophore dye used for sensing changes in pH across the physiologic range (pH 4–8) in phagosomes and endosomes of mammalian cells through customized delivery vehicles. To design effective SAFs, we have synthesized a low-molecular-weight amphiphilic molecule that forms a fibrous hydrogel through self-assembly, and physically encapsulates dyes such as pHrodo without the need for covalent modification (Fig. 1a). To facilitate uptake through either endocytosis or phagocytosis, we have developed a method to isolate SAF particles from the bulk hydrogel with an appropriate size to maximize internalization.

**Results**

**Design and characterization of self-assembled hydrogels.** To generate a TRIS amphiphile that could assemble into a hydrogel, we conjugated dodecanoic acid to create Tris-12 (Fig. 1a), whereby the introduction of an aliphatic chain promotes self-assembly through van der Waals interactions (in addition to hydrogen bonding from Tris hydroxyl groups). In addition, this amphiphile was engineered to resist digestion by esterases and reactive oxygen species and exhibit minimal to no buffering ability through converting the TRIS primary amine, which is required for buffering capacity, to an amide. We also anticipated that the hydrogen-bonding interactions of the carbonyl groups of amides would aid in the assembly process. Importantly, unlike synthesis of many amphiphilic hydrogelators, the synthesis of the Tris-12 amphiphile was obtained through a single high-yielding step (see Methods). Furthermore, the crude products showed comparable self-assembling ability to the purified form of Tris-12, which may permit the development of these amphiphiles on an industrial scale for a wide range of applications which is often a roadblock in self-assembled hydrogels.

**Tris-12** exhibited excellent self-assembling ability in multiple solvents including aqueous (acidic and basic) and organic solvents (for detailed self-assembly characterization see Supplementary Table S1). Typically, 1–4% (wt/v) solid Tris-12 in phosphate buffered saline (PBS) was heated to 80°C until dissolved, and upon cooling to ambient temperature Tris-12 self-assembled into bulk hydrogels. Repeating a similar procedure in the presence of pHrodo dissolved in PBS (40 μg) produced a pHrodo-encapsulated self-assembled hydrogel. Subsequently, SAFs were isolated from the bulk hydrogel (see Methods). Importantly, the presence of pHrodo altered neither the self-assembling ability of the amphiphile nor the morphology of the fibers (Fig. 1c,d).

Through a series of ab initio calculations, X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FT-IR), the geometry of Tris-12 was optimized in the gaseous state, and lengths of single amphiphile molecules and possible lamellar assemblies were calculated. In XRD experiments, xerogel of Tris-12 showed long distance spacing of 3.1 nm, which is larger than the molecular length (1.86 nm from the optimized geometry calculations) and less than double the extended molecular length of Tris-12 (Fig. 1b, & see Supplementary Information). This is likely explained by interdigitated molecular packing as shown in Fig. 1b. In this model, hydrophilic hydroxyl groups are exposed to the outer solvent while hydrophobic chains are highly interdigitated, which is consistent with earlier reported polyhydroxyl based self-assembled amphiphiles. In addition, in FT-IR spectra of the Tris-12 amphiphile the carbonyl stretching value of the amide group in the self-assembled gel (1629 cm⁻¹) was lower than in a methanolic solution of Tris-12 (1672 cm⁻¹). This significant shift of 42 cm⁻¹ could be attributed to the strong hydrogen-bonding interactions of the carbonyl groups of amides in the self-assembled state, which clearly suggests that hydrogen-bonding enhances the self-assembly of these amphiphiles to form fibrous structures as depicted in Fig. 1b.

**Buffering capacity of self-assembled fibers.** To be an effective vehicle for reporter dyes, the carrier should not exhibit buffering capacity under a wide range of pH. A series of experiments were performed to confirm the absence of buffering capacity by the SAFs (without pHrodo). The pH of a concentrated solution of
SAFs was continuously monitored during the serial addition of small volumes of dilute acid (HCl) and base (NaOH). The SAFs solution exhibited a linear pH change over the pH range of 4 to 10.5, which suggests that fibers do not have buffering capacity (Supplementary Fig. S1). Thus, we envisioned that the encapsulated dye should accurately report the pH of the compartment without interference from the SAFs vehicle.

**Encapsulation of pHrodo dye in SAFs.** Encapsulation of pHrodo in SAFs was characterized via UV-vis absorption spectrophotometry (absorption and emission maxima for pHrodo are 530 and 590 nm, respectively (Fig. 2a and b)). As illustrated in Fig. 2a, Tris-12 SAFs do not have a discrete absorbance peak between 300 and 700 nm, allowing encapsulated pHrodo to be detected by absorption spectroscopy without interference from its vehicle. Physical encapsulation of pHrodo dye did not significantly alter its inherent spectral properties (absorption maximum shifted a marginal 5 nm, while emission spectra remained similar, Fig. 2a and b).

**Stability of encapsulated pHrodo in SAFs and its response to pH.** The stability of pHrodo encapsulated SAFs (pHrodo-SAFs) was investigated over a pH range from 2 to 8.5. To ensure pHrodo-SAFs are stable in a broad range of pH, and pHrodo remains confined within the fibers and does not leak the dye over time, pHrodo-SAFs were incubated in solutions of varying pH. To mimic the presence of proteolytic enzymes, fibers were also incubated with or without esterase enzyme (300 U/mL) at multiple pH conditions. Data in Fig. 2c indicates that pHrodo-SAFs are stable, and the dye remains within the self-assembled fibers over a wide range of pH. In the presence degradative enzymes over 3 days, >85% of the dye remained stable within the self-assembled fibers, and no further loss was observed beyond this time-point. As demonstrated in Fig. 2c, negligible release of the dye was observed at pH 4. In addition, pHrodo-SAFs were lyophilized and stored at room temperature. After regular intervals (1, 4, 8 and 10 weeks), re-dispersion of pHrodo-SAFs in PBS did not release the dye (confirmed with UV-vis spectra, data not shown).

The responsiveness of pHrodo was examined in its solubilized form, in its entrapped form in SAFs, and in its covalently bound form with commercially available pHrodo-conjugated E. coli cell wall particles (pHrodo-E. coli) at specific pH's ranging across the physiologic spectrum from 4–7.5. As shown in Fig. 2d, the fluorescence response of the entrapped dye closely mirrored that of the free-solubilized form of pHrodo and the pHrodo covalently conjugated to E. coli particles (Fig. 2d). These results suggest that encapsulation of pHrodo in SAFs did not compromise its ability to sense changes in pH.

**Uptake of pHrodo-SAFs by macrophages, and sensing of intracellular pH changes through pHrodo-SAFs.** Micron sized particles are known to internalize into macrophages through endocytosis and phagocytosis39,40; thus it was anticipated that micron-sized
pHrodo-SAFs (Fig. 1d) would be internalized by macrophages through phagocytosis. As shown in Supplementary Fig. S2, uptake of pHrodo-SAFs into acidified intracellular compartments at 37°C was demonstrated by discrete, punctate regions of high fluorescence within the cell suggesting that pHrodo-SAFs are localized in phagosomes. In addition to discrete punctate signals, and unlike E.coli-bound pHrodo, fluorescence was also observed throughout the cytoplasm, and was modulated by changing cytoplasmic pH, suggesting that pHrodo-SAFs also deliver pHrodo into the cytoplasm. Unlike phagosomal uptake, this effect was noted at both 37°C and 4°C, suggesting that cytoplasmic delivery occurs by an energy-independent process.

Localization of pHrodo-SAFs in intracellular compartments. Under physiologic conditions, pHrodo-SAFs reported pH in multiple intracellular compartments, with dominant concentration in acidified phagosomes and the cytoplasm (Fig. 3a,b). After clamping the intracellular pH to 5, to maximize pHrodo fluorescence, cells were fixed and labeled with DiO (cell membrane staining dye, ex/em 484/501 nm), V450 anti-mouse CD107a (lysosome staining, ex/em 406/450 nm) and TO-PRO®-3 (nucleus staining, ex/em 642/661 nm). Co-localized confocal scanning fluorescence images (Fig. 3a,b and Supplementary Fig. S3, S4, S5) suggest that cells loaded with pHrodo-SAFs (internalized at 37°C) localized pHrodo predominantly in the cytoplasm while a significant amount of dye was also observed in the phagosomes, with a minute quantity of pHrodo consistently localized in the nucleus. On the contrary, pHrodo-E.coli particles were confined to phagosomes. A modest amount of free dye was internalized (Fig. 3a,b), presumably via endocytosis of media. Transmission electron microscope experiments should be performed to visualize the location of the fibers.

In contrast, under hypothermic (4°C) conditions that inhibit active transport free dye and pHrodo-E.coli particles did not enter into cells, whereas large quantities of pHrodo-SAFs continued to internalize into the cytoplasm (Fig. 3c,d and Supplementary Fig. S5, S6). pHrodo-SAFs internalized by macrophages at 4°C co-localized to both the cytoplasm and phagosomes (Fig. 3c, d and Supplementary Figure S5, S6).

Function of internalized pHrodo-SAFs. To quantify the response of internalized dye to intracellular pH modulation in a population of cells, and to test the utility of pHrodo-SAFs in high-throughput, plate-reader based experiments, pHrodo-SAFs and pHrodo-E.coli particles were internalized into macrophages at 37°C (in independent experiments) and nigericin treatment was used to clamp the pH of the cytoplasm, nuclei and intracellular compartments to the pH of the extracellular solution35. Intracellular pH was subjected to pH cycling between 5 and 7 by exchange of nigericin solutions at each pH. A 1.5-fold increase in fluorescence was observed for internalized pHrodo-SAFs when the intracellular pH was clamped from native pH 7.4 to acidic pH 5 (Fig. 4a) suggesting that pHrodo-SAFs were present within the cytoplasm. On the contrary, upon clamping the pH to 5, a similar result was not observed in pHrodo-E.coli internalized-macrophages (Fig. 4a). These observations suggest that pHrodo-E.coli particles were confined into already acidic phagosomes but not present in the cytoplasm or nucleus, which is
in agreement with the previous reports^{11,36}. Returning the pH from 5 to 7 caused a drop in fluorescence that indicates that pHrodo physically encapsulated in SAFs, like pHrodo bound to E. coli particles, retain their ability to respond to changes in microenvironmental pH.

pHrodo-E.coli particles are known to internalize through an energy-dependent phagocytosis^{11,36}. On the contrary, uptake of pHrodo-SAFs into macrophages at 4 °C was comparable with the uptake at 37 °C (Fig. 4b and a, respectively), suggesting an energy-independent process. To further characterize the mechanism for uptake of pHrodo-SAFs and pHrodo-E.coli particles we inhibited endocytosis and actin-function with latrunculin A and cytochalasin D^{37} respectively, at both 37 °C and 25 °C. These inhibitors blocked the uptake of pHrodo-E.coli particles while internalization of pHrodo-SAFs was not affected (Fig. 4c, d). These results suggest that pHrodo-SAFs can enter cells in an energy-independent, non-endocytotic, passive manner. Importantly, internalization of pHrodo-SAFs did not affect the viability of macrophages compared to untreated control cells up to 48 hr (see Supplementary Fig. S7). To demonstrate that change in the observed fluorescence is due to pHrodo dye responding to the change in pH, DiO (a dye inert to pH change, ex/em 484/501 nm) was co-encapsulated with pHrodo in SAFs, and internalized into macrophages at 37 °C. Intracellular pH was subjected to pH cycling between 5 and 7 by exchange of nigericin solutions at each pH. Change in fluorescence emission at 590 nm (pHrodo) and 501 nm (DiO) were quantified (see Supplementary Fig. S8). As anticipated, pHrodo emission at 590 nm changed in response to a pH change, while DiO emission at 501 nm did not show any change. This suggests that the observed fluorescence change throughout this study is indeed due to the response of pHrodo dye to changes in micro-environmental pH, and shows the potential for multiplex readouts of SAFs encapsulating multiple dyes simultaneously.

Live cell monitoring of intracellular pH. Following uptake of pHrodo-SAFs by macrophages at 37 °C, intracellular pH was studied by quantification of fluorescence of individual cells using real-time fluorescence microscopy (ex/em 530/590 nm). In Fig. 5a, individual lines represent the signal from a single cell in a representative experiment. These results indicate that pHrodo-SAFs report the change in intracellular pH in an efficient, reproducible, and rapid manner in the range that pHrodo is known to sense (pH 4 to 7.5). The response to incremental changes in pH over time was measured (Fig. 5b, characteristic sigmoidal graph) showing that the ability to dynamically sense incremental changes in pH over multiple cycles (i.e. reversibility) was preserved.

To demonstrate that internalized pHrodo-SAFs are capable of reporting physiologic changes in intraphagosomal pH, fluorescence was measured during exposure to modulators of phagosomal pH (Fig. 5c). Bafilomycin A is a known selective inhibitor of the proton pump vacuolar ATPase (V-ATPase) that is responsible for the acidification of phagosomes^{38}. Thus, addition of bafilomycin-A increases phagosomal pH. Upon addition of bafilomycin-A (200 nM) to pHrodo-SAFs loaded cells, pHrodo fluorescence decreased by 35% within 30 mins (±4%, n = 4, P < 0.05, (Fig. 5c) indicating that...
pHrodo-SAFs internalized within the phagosomes specifically report changes in phagosomal pH. Real-time fluorescence microscopy of individual cells (pHrodo-SAFs internalized cells, Fig. 5d) reveals that upon addition of bafilomycin-A, there was a moderate decrease in fluorescence. Whereas cells subsequently treated with nigericin and clamped the pH to 5 followed by 7.5 triggered a sudden increase and decrease in fluorescence intensity, respectively (Fig. 5d). These results reveal that the majority of SAFs were localized within the cytoplasm.

To delineate the retention of SAFs in intracellular compartments, a kinetic study was performed by quantification of fluorescence intensity of pHrodo at different time points (0.1, 12, 24 and 48 hr) during post-fiber internalization. pHrodo-SAFs were internalized into RAW 264.7 macrophages, and at each time point intracellular pH was clamped to pH 5.2 and fluorescence intensity was measured. Results in Supplementary Fig. S9 suggest that although the total fluorescence decreased over time, a significant amount of pHrodo-SAFs remain inside cells at least for 48 hr. The decrease is likely due to exocytosis given that gels remain stable when incubated in the presence of degradative enzymes or over a wide range of pH (Fig. 5). The percentage of total fluorescence corresponding to pHrodo dye (ex:530/em:590 nm) was 100, 40, 31 and 22.5% at 0.1, 12, 24 and 48 hr, post fiber internalization, respectively (n = 9). While a 50–60% decrease in the fluorescence of dye loaded SAFs was observed during first 12 hr, likely due to SAF exocytosis, ~25% of SAFs remain inside the cells for at least 48 hr.

**Discussion**

There is a significant need to develop materials that target intracellular compartments for delivery of a wide range of payloads, from fluorescent reporters of physiologic states as described here to small-molecular drugs, antibodies, or gene-silencing RNA and DNA\(^4\). Covalent binding to micron-scale particles such as *E. coli* cell walls...
can reliably deliver a limited range of payloads to phagosomes. However, few approaches are available to target similarly large payloads to the cytoplasm and nucleus, or to deliver molecules that are not amenable to covalent binding. We anticipated that self-assembled fibers formed from amphiphilic small molecules with an overall neutral surface charge could efficiently cross the cell membrane into the cytoplasm, bypassing the process of endocytosis and delivering a pH-reporting dye in an energy-independent manner.

**Figure 5 | Live cell imaging of response of pHrodo-SAFs internalized within macrophages to cyclical modulation of pH and response to a proton pump inhibitor.** (a), Spectra signal from three individual macrophages shows response to pH cycling of pHrodo-SAFs internalized in murine macrophages upon alternate addition of acid and base (time of addition shown by black arrows). (b), A plot of emission of pHrodo at 590 nm as a function of time while exposed to incremental changes in pH. This reversible cycle of pH produced a mirror response. Each line in the spectra represents the signal from an independent macrophage. (c) and (d), Effect of bafilomycin (inhibitor for the vacuolar ATPase) on pH change in macrophage phagosomes. Clamping of pH by addition of bafilomycin and nigericin was followed via fluorescence emission of pHrodo dye (encapsulated within SAFs). Each line in (d) in the spectra represents the signal from an independent macrophage (n = 6).

In summary, SAFs offer the opportunity to deliver desired agents into the intracellular environment at lower temperatures or other metabolically inactive states. SAFs further permit encapsulation of dye without the necessity of covalent binding to a delivery vehicle, while not impacting the function of the payload. We have demonstrated their ability to report pH using a dye, pHrodo, otherwise deliverable only by covalent binding to bacterial particles and thus limited to the phagosome. pH changes across multiple cellular compartments are measurable in both in live cell experiments and in high-throughput plate reader based assays.

**Methods**

**Reagents and solutions.** Except where noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). pHrodo dye was purchased from Invitrogen (Carlsbad, CA). Calcium Ringers was prepared with the following composition: NaCl 145 mM, KH₂PO₄ 2.5 mM, MgSO₄ 1 mM, CaCl₂ 1 mM, D-Glucose 10 mM, HEPES 10 mM. High-K⁺ Ringers: NaCl 105 mM, KCl 40 mM, KH₂PO₄ 2.5 mM, MgSO₄ 1 mM, CaCl₂ 1 mM, D-Glucose 10 mM, HEPES 10 mM.

**Synthesis of Tris-12 amphiphile.** To a solution of 2-Amino-2-hydroxymethylpropane-1,3-diol (1.21 g, 10 mM) in anhydrous dimethyl sulfoxide (DMSO, 10 mL), 1 equivalent of methyl dodecanoate (1.24 g, 10 mM) and 2 equivalents of anhydrous K₂CO₃ (2.14 g, 15 mM) was added and stirred at room temperature. Following a 48 h
reaction that was quenched with ice cold water (40 mL), stirring continued for 30 minutes. While precipitate that was formed during quenching was filtered and washed with cold water (5 × 10 mL). Drying under vacuum produced Tri-12 amphiphile in its pure form (2.84 g, 94% yield).

Encapsulation of pHrodo in self-assembled fibrous particles. Tri-12 (8 mg) was placed in a scintillation glass vial with 20 µl of DMSO solution of pHrodo (40 µg) was added, vortexing to dissolve. A further addition 180 µl water (either PBS, or double distilled water which was passed through Chelex 100 resin), the vial was tightly closed with a screw-cap and heated to 70 °C for 2 minutes to dissolve the Tris-12. As the vial cooled to room temperature the fibers self-assembled within 30 minutes. After one hour, 0.5 mL of water was added to the vial and the gel was uniformly dispersed through mixing with a pipette. The gel was transferred to an epipendorf tube, which was subjected to centrifugation (15,000 rpm for 7 minutes). Following removal of the supernatant, fibers were rinsed with 1 mL of water and subjected to centrifugation. This process was repeated 7 times to completely remove non-encapsulated pHrodo. The final concentration of the dye in the fibers was established by dissolving the fibers in DMSO and measuring the absorbance against a standard curve of known dye concentrations. The concentration of pHrodo in the fibers was calculated to be 20 µg of pHrodo in 2 mg of fibers.

Scanning electron microscopy (SEM). To examine the morphology of the fibers, 20 µl of fibrous-dispersed water was placed on carbon-tape attached to an aluminum grid and dried overnight under ambient conditions, followed by coating with a thin-layer of Au (30 nm) before being observed with a Field emission-scanning electron microscope (FSEM, FEI, Hillsboro, Oregon) using 10 kV.

X-ray diffraction (XRD). XRD measurements were acquired using a Bruker AXS D-8 Discover with GADDS diffractometer using graded d-space elliptical side-by-side multilayer optics, monochromated Cu-Kα radiation (40 kV, 40 mA), and imaging plate.

UV-vis spectroscopy. UV-visible spectra of the amphiphiles was acquired with a Cary100 BIO spectrophotometer using a quartz cuvette of 1-cm path length.

Spectroscopy and in vitro fluorescence measurements. Absorbance of 50 µl of 10% Tri-12 fibers solution, with or without 20 µg/ml pHrodo, was measured in 1 nm increments using a 96-well microplate absorbance spectrometer (SpectraMax, 4, BioTek Inc, Winoski, VT). To measure fluorescence, the same plate reader and nanofiber solutions were used. 5 µl of nanofiber solution was added to wells containing 100 µl buffered Calcium Ringer’s solution titrated to pHs from 4 to 10 using HCl and NaOH. Fluorescence was measured using excitation and emission filters of 530/20 (nm) and 590 (nm), respectively. All experiments were performed in triplicate.

Cell culture. RAW 264.7 murine macrophages (ATCC) were maintained on plastic Petri dishes at 37°C and 5% CO2 in culture media consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin/streptomycin/fungizone, 10 mM HEPES buffer, 100 µM nonessential amino acids, and 2.5 x 10⁻³ M 2-mercaptoethanol (Invitrogen Corp., Carlsbad, CA). Cells were passaged every three days.

pH modulation. RAW 264.7 macrophages on glass coverslips in 3 mL of cell culture media were incubated with pHrodo-SAFs (0.4 µg of pHrodo dye, MW ~1000) for three hours. Selected individual cells that showed particle uptake were imaged using a Nikon TE-2000U inverted real-time fluorescence microscope (Nikon Inc, Melville, NY). Digital images were captured using a digital CCD camera (Hamamatsu ORCA-ER, Bridgewater, NJ). Pixel intensity was measured using image-processing software (Universal Imaging Corp., Downingtown, PA). Images were acquired every 30 seconds with an exposure time of 0.3 s. After equilibrating in a Calcium Ringer’s solution for five minutes, the solution was exchanged for alternating pH solutions of high-K⁺ solutions (Universal Imaging Corp., Downington, PA). Images were acquired every 30 s, with a 2-minute rinsing 2 times with Ringer’s buffer, cell membranes were stained with DIO (10 µM in Calcium Ringer’s buffer) for 20 min at 37°C. Then cells were washed with Ringer’s buffer twice and cell endosomes were stained with V450 Anti-mouse CD11b (0.4 µg, BD Horizon) in 0.5 mL Calcium Ringer’s buffer for 30 min at room temperature. Finally, after rinsing three times with Calcium Ringer’s buffer, nuclei were stained with 1 µM TO-PRO-3 dye in Calcium Ringer’s buffer for 15 minutes. After rinsing 3 times with Ringer’s buffer and with DI water, cover-slips were mounted on glass slides in ProLong Gold (Invitrogen) anti-fading medium.

Retention time of pHrodo-SAFs inside cells. RAW 264.7 macrophages were seeded into 96 well plates (Corning) at the density of 100,000 cells/well. One hour after seeding, cell media was re-placed with 100 µl Ca2⁺ Ringer’s buffer (pH ~ 7.2) containing 0.1 mg/ml pHrodo-SAFs. After a 3 hour incubation at 37°C, buffer was removed and cells were washed twice with PBS and plated in culture media at 37°C. From 6 to 24 hours after washing with PBS triche prior to treatment with K⁺ Ringer’s buffer (pH ~ 5.2). The fluorescent signal was measured with ex/em: 530 nm/590 nm by SpectraMax® microplate reader (Molecular Devices, Sunnyvale, CA).

Scanning electron microscopy (SEM). To examine the morphology of the fibers, 20 µl of fibrous-dispersed water was placed on carbon-tape attached to an aluminum grid and dried overnight under ambient conditions, followed by coating with a thin-layer of Au (30 nm) before being observed with a Field emission-scanning electron microscope (FSEM, FEI, Hillsboro, Oregon) using 10 kV.

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Spectroscopy and in vitro fluorescence measurements. Absorbance of 50 µl of 10% Tri-12 fibers solution, with or without 20 µg/ml pHrodo, was measured in 1 nm increments using a 96-well microplate absorbance spectrometer (SpectraMax, 4, BioTek Inc, Winoski, VT). To measure fluorescence, the same plate reader and nanofiber solutions were used. 5 µl of nanofiber solution was added to wells containing 100 µl buffered Calcium Ringer’s solution titrated to pHs from 4 to 10 using HCl and NaOH. Fluorescence was measured using excitation and emission filters of 530/20 (nm) and 590 (nm), respectively. All experiments were performed in triplicate.

Cell culture. RAW 264.7 murine macrophages (ATCC) were maintained on plastic Petri dishes at 37°C and 5% CO2 in culture media consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin/streptomycin/fungizone, 10 mM HEPES buffer, 100 µM nonessential amino acids, and 2.5 x 10⁻³ M 2-mercaptoethanol (Invitrogen Corp., Carlsbad, CA). Cells were passaged every three days.

pH modulation. RAW 264.7 macrophages on glass coverslips in 3 mL of cell culture media were incubated with pHrodo-SAFs (0.4 µg of pHrodo dye, MW ~1000) for three hours. Selected individual cells that showed particle uptake were imaged using a Nikon TE-2000U inverted real-time fluorescence microscope (Nikon Inc, Melville, NY). Digital images were captured using a digital CCD camera (Hamamatsu ORCA-ER, Bridgewater, NJ). Pixel intensity was measured using image-processing software (Universal Imaging Corp., Downingtown, PA). Images were acquired every 30 seconds with an exposure time of 0.3 s. After equilibrating in a Calcium Ringer’s solution for five minutes, the solution was exchanged for alternating pH solutions of high-K⁺ Ringer’s containing 10 µM nigericin at pH 5 and pH 7.4. For high-throughput measurements of physiologic pH changes, cells were plated on black fluorescence microplates (Corning Costar) at a density of 25 x 10⁴ cells/well. After allowing cells to settle for one hour at 37°C and 5% CO2, culture media was exchanged for Calcium Ringer’s solution containing 20 µl of concentrated fiber solution/mL. Cells were placed in a humidified room air incubator at 37°C for 3 hours, subsequently washed once with Calcium Ringer’s before addition of Calcium Ringer’s with or without the vascular H⁻/ATPase inhibitor bafloymycin-A (200 nM). Results were read on a microplate reader (SpectraMax, 4, BioTek, Winoski, VT) using excitation filters of 530 nm and emission filters of 590 nm.

Determination of cell viability. Cell viability was assessed by monitoring uptake and intracellular conversion of nonfluorescent calcine-AM to fluorescent calcine, using a modification of previously reported method6, pHrodo-SAFs were internalized into RAW264.7 cells, were subsequently plated on three different plates for 4, 24 and 48 hr time points. At each time point, wells were gently rinsed with Ringer’s buffer, and solutions were replaced with identical solutions containing calcine-AM (8 µM) for 40 min, after which fluorescence from each well (excitation (Ex) 485 nm, emission (Em.) 528 nm) was recorded.

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Author contributions
J.M.K., D.I.S., P.K.V. and J.E.K. conceptualized the idea, conceived experiments, analyzed the results and wrote the manuscript. A.B., M.W., C.X., L.C. and S.R.J. conducted experiments. G.J. helped to analyze data, wrote manuscript, and all authors reviewed the manuscript.

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