



Highly Sensitive Fluorescent pH Microsensors Based on the Ratiometric Dye Pyranine Immobilized on Silica Microparticles

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Abstract: Pyranine (HPTS) is a remarkably interesting pH-sensitive dye that has been used for plenty of applications. Its high quantum yield and extremely sensitive ratiometric fluorescence against pH change makes it a very favorable for pH-sensing applications and the development of pH nano-/microsensors. However, its strong negative charge and lack of easily modifiable functional groups makes it difficult to use with charged substrates such as silica. This study reports a methodology for noncovalent HPTS immobilization on silica microparticles that considers the retention of pH sensitivity as well as the long-term stability of the pH microsensors. The study emphasizes the importance of surface charge for governing the sensitivity of the immobilized HPTS dye molecules on silica microparticles. The importance of the immobilization methodology, which preserves the sensitivity and stability of the microsensors, is also assessed.


pH sensors based on small molecular dyes have been used in conjugation with various nano/microparticles to enhance their properties.^[1] As a substrate, fluorescent nano/microsensors based on silica have gained special popularity due to the improvements that they impart on the properties of encapsulated dye, such as photostability,^[2] good cytocompatibility^[3] and flexibility for easy functional modification.^[4] To incorporate sensing dye molecules into silica matrix, common ways involve


covalent linking, matrix entrapment- or charge-based immobilization.^[5] Covalent linking requires presence of certain functional groups on the dye molecule,^[1f] which is not possible many of the times. In addition, covalent bond formation can also cause unwanted change in the fluorescence properties of the dye and its sensitivity. Charge-based immobilization of positively charged dye molecules is possible due to their interaction with negatively charged silica, however many of the dye molecules are negatively charged and cannot be tethered using covalent bonding. One such particularly useful pH sensitive dye molecule is 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS), which is pyrene based and offers an extremely sensitive ratiometric pH-sensitive fluorescence emission. Compared to HPTS the other fluorophores like seminaaphtharhodafuor (SNARF), 2,7-bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein (BCEF), and Fluorescein-5-isothiocyanate (FITC) are frequently used in development of various kinds of pH sensors. Fluorescein-based indicators are quite common pH-sensitive dyes however they are prone to photoquenching and their fluorescence maxima and pK_a can change significantly after chemical substitution. SNARF-1 is a ratiometric dye (pK_a : 7.5) and is superior to fluorescein due to its higher stability and inertness towards other perturbations. Its bioconjugable forms make it one of the widely used pH probes. However, its fluorescence quantum yield is only around 10% that is a significant limitation. Comparatively, HPTS is one of the most inexpensive pH-sensitive ratiometric dye but compared to naphthoxanthene-based dyes like SNARF-1 with cell permeant ester, it has no membrane permeant form. Thus, it is difficult to link HPTS with other molecules or substrates and compromise in pH sensitivity has been observed due to chemical conjugation. HPTS shows an extremely high quantum yield (> 75%) and is overly sensitive to near-neutral pH values (pK_a : 7.2). In addition to being cell impermeant and nontoxic, pH values of as low as pH 4.4 can be easily measured using HPTS. A large Stokes shift is one of the most desired features of any sensor dye and HPTS is one such dye where the Stokes shift is more than 100 nm. Its high polarity makes it highly water soluble.^[6] Despite all its advantages, HPTS is still not fully exploited in the development of nanosensors, where the reason lies in difficulty to tether or immobilize it. Its high negative charge makes it difficult to be used along with negatively charged substrates such as silica and to covalently attach it using its sulphonic acid groups to other substrates which requires harsh chemical treatment and could also potentially compromise with the pH sensitivity. In the past, attempts have been made to use cationic

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polyelectrolytes to negate the overall negative charge of the silica to make its surface positive enough to attract negatively charged dye molecules, however, a significant change in their fluorescence properties was observed decreasing the overall applicability.^[7]

The current HPTS immobilization methodologies on planar as well as optical fibers involve dissolving the HPTS molecules in polymeric matrix like poly(vinyl alcohol),^[8] poly(2-hydroxyethyl methacrylate)^[9] or polyurethanes^[10] and then coat the intended planar surface or fiber with the HPTS containing matrix. Covalent immobilization of the HPTS in polydimethylsiloxane (PDMS)-based amphiphilic co-networks has also been reported however the pH sensitivity is significantly lower than free HPTS molecules.^[11]

In general, covalent modification methods have obvious advantages like stronger binding of the molecule for enhanced stability, however on many instances it can seriously perturb the intrinsic fluorescence properties of the molecule of interest. For example, reports on HPTS molecule covalently linked to polymeric matrix such PDMS-poly(2-hydroxyethylacrylate) has shown to result in 58-fold change in fluorescence emission ratio for corresponding pH change from pH 5 to 9.^[11] The free HPTS molecule on the other hand is much more sensitive and can show ~600 fold change in fluorescence emission ratio during transition from pH 4 to 8 (Figure 1A and Table 1).

Noncovalent modifications interfere less with the electronic state of the molecule and are thus less likely to influence its sensitivity. However, there could be problems related to leaching of the molecule over time. In our work, we used a more efficient noncovalent immobilization technique to hold the HPTS molecules on silica surface while maintaining appreciable pH sensitivity at the same time. Our proposed

approach could provide a better solution where HPTS and similar molecules could be coated without incorporating additional polymers such as hydrogels. In addition, the sensitivity of the molecule could also be optimized easily by manipulating the charges.

We have investigated different methodologies to immobilize HPTS on silica microparticles (SMPs) and developed a conjugation technique which keeps the HPTS bound to the silica particles with significant retention of its pH sensitivity. Here, the effect of silica particle surface charge was found to be linked with retention of HPTS molecules in addition to its involvement in governing the pH sensitivity. In the end, an overly sensitive HPTS-based pH sensor was developed that can act as a platform in the development of other HPTS-based pH sensors. Besides, the design methodology can be used for immobilization of other similar negatively charged molecules on silica-based substrates.

pH sensing with accuracy becomes especially important when minute fluctuations are to be observed in close vicinity of a cell or tumor structure.^[12] In biological systems, pH variation is associated with a functional change in protein and other biomolecular entities.^[13] These changes reflect at the systemic and organismic level as an outcome of a disease or malfunction.^[14] Due to the importance of pH, its accurate sensing under different conditions is very crucial to understand the underlying activities taking place in an area of interest. Among various techniques of pH sensing, fluorescence-based pH sensing offers several advantages, like noninvasiveness, sensitivity and ability to monitor it at very small scales in a biological system. Therefore, continuous efforts have been made in creating different kinds of fluorescence-based sensors with better biocompatibility, stability, sensitivity. For this purpose, small molecule-based pH sensors like fluorescein, SNARF and HPTS have been used frequently in their molecular form, however, confining or linking them to nano/microparticles has obvious advantages like higher photostability and better signal-to-noise ratio, etc.^[15] Despite these advantages, often fluorescent labelling of nano/microparticles with pH-sensitive small molecules has been known to cause modification in the fluorescence properties of the probes. Sometimes these modifications cause a drastic reduction in pH sensitivity or overall emission intensity or both. These potential problems are thus important to be considered before designing a nano-/micro-particle-based sensor and choosing the combination of dye molecules and nano/microparticle composition. A very appropriate example of these undesired changes in pH sensitivity could be observed with pyranine, which is also known as HPTS.

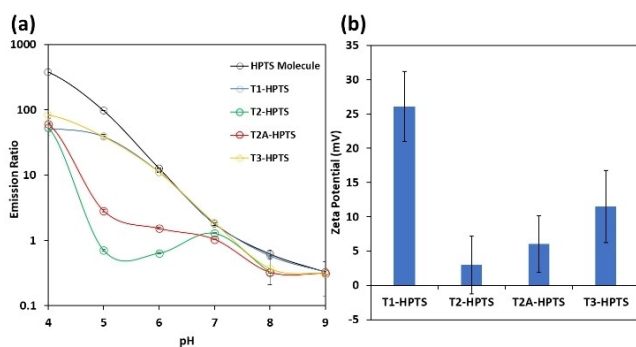


Figure 1. a) Comparison of the pH sensitivity of HPTS-based fluorescent microsensors by using different strategies. b) Surface charge of different HPTS-based microparticles due to the difference in synthesis methodology.

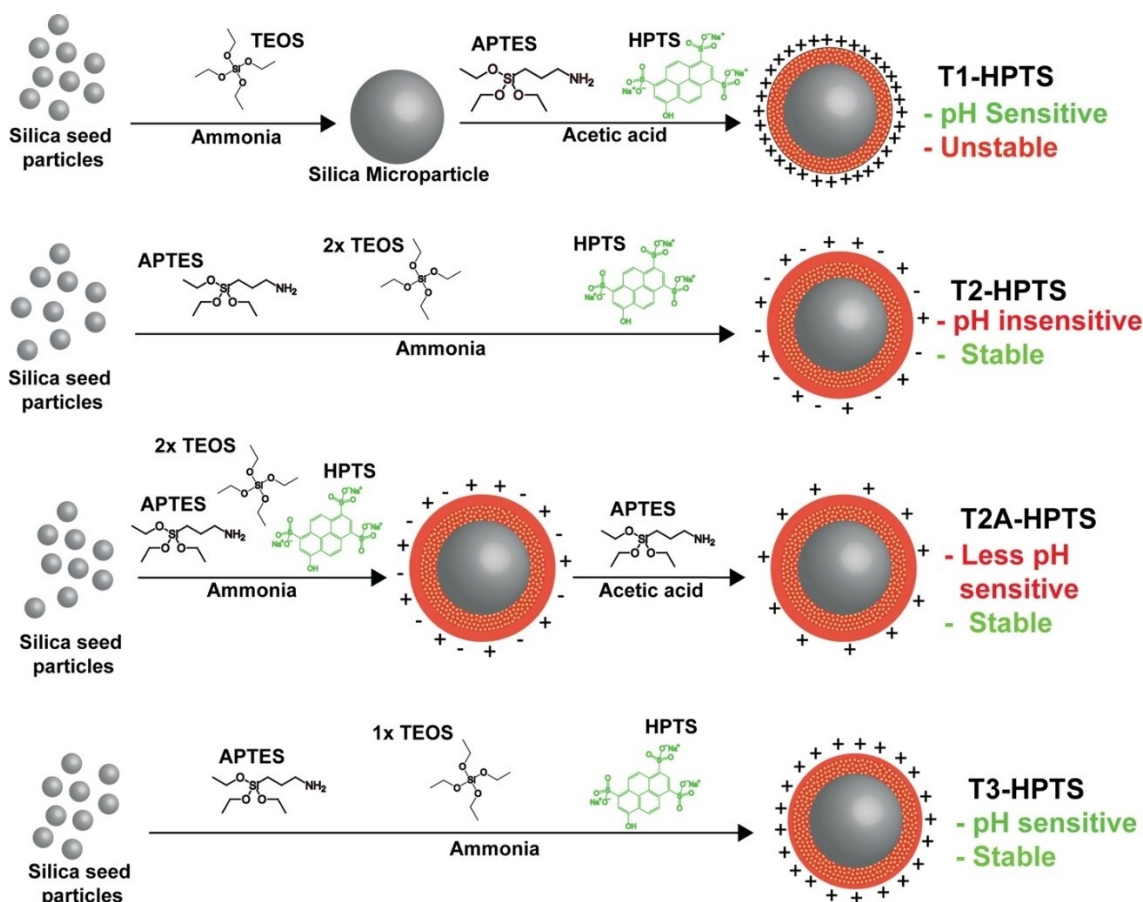
Sensor types	Sensitivity to pH	Emission ratio pH 8	pH 4	% change w.r.t. HPTS	pK_a	Stability
HPTS (ref)	extremely high	0.61	380.15	100	7.25 ± 0.17	–
T1-HPTS	very high	0.58	52.05	13.5	6.53 ± 0.12	low
T2-HPTS	low	0.32	53.55	14	5.76 ± 1.21	high
T2A-HPTS	very high	0.32	60.92	15.9	6.15 ± 0.59	high
T3-HPTS	very high	0.36	84.04	22	6.97 ± 0.19	high

HPTS is a highly sensitive, inexpensive, water-soluble and membrane-impermeant pH indicator with a pK_a of ~ 7.3 in aqueous buffers. In addition, its large Stokes shift, and possibility of ratiometric pH sensing capability makes it special compared to other pH sensitive fluorophores, which either require a reference fluorophore or have a very narrow Stokes shift.^[16] The ratiometric pH sensing that exists due to pH-dependent shift in its absorption can be realized by exciting HPTS at 405 and 450 nm, where both excitations cause emission with maxima at 511 nm and the ratio of emission at respective excitation wavelength can be correlated to pH to create a standard curve.^[17]

In the past, the reports on HPTS-based silica nanoparticles for pH sensing exhibited shifted or less sensitive pH response compared to the HPTS molecule dissolved in solution.^[7,18] The reason for the modification was associated with the change in HPTS fluorescence properties caused by the confinement of the dye in the silica matrix or its closeness to the surface that can affect the degree of ionization of the dye against different pH values. Here, we have used physical entrapment and electrostatic interaction to hold the HPTS molecules on the silica substrate instead of a covalent linkage. The reason lies in the fact that covalent binding of HPTS molecules through its sulfonic acid groups is known to decrease the pK_a for each bond.^[12,19] Therefore, it can strongly hold the dye molecules but

will significantly change the pH sensitivity, which is not desired. In this work, we have investigated unique noncovalent methodologies to incorporate HPTS dye molecules on SMPs and studied the reason behind associated advantages and disadvantages for each of them. The outcome of these studies resulted in an extremely sensitive and stable HPTS-based pH micro-sensor.

We have investigated four different methodologies immobilizing the HPTS molecules on SMPs (Scheme 1) and we studied the corresponding variation in both the stability and sensitivity of the resulting pH microsensors. We started with the synthesis of highly positively charged SMPs, where HPTS molecules were held onto the surface by electrostatic interactions. These microparticles were named T1-HPTS. In terms of pH sensitivity, T1-HPTS were observed to be most sensitive but highly unstable, as the dye molecules easily wash out in a few washing steps. For each respective pH value, the emission ratio was calculated by dividing the fluorescence emission intensities for excitation wavelengths of 405 and 450 nm ($Em: \lambda_{ex}: 405 \text{ nm}/Em: \lambda_{ex}: 450 \text{ nm}$). The sensitivity of T1-HPTS microparticles was 13.5% to that of free dye molecules, indicating minimum possible hindrance caused by SMPs (Figure 1a). The excellent pH sensitivity observed for these sensors can be attributed to the fact that the dye molecules are existing mostly in an electro-positive environment with the zeta potential of the T1-HPTS



Scheme 1. Different methodologies of HPTS immobilization on SMPs, the difference in their pH sensitivity and stability.

particles as +26.1 mV (Figure 1b). Such condition is known to help in balancing the negative charge of the silica surface that can interfere with the HPTS pH sensitivity. Hence to a large extent, the pH-dependent ionization behavior and the corresponding fluorescence emission change for T1-HPTS was unperturbed.

Although the sensitivity in the case of T1-HPTS was exceptionally good, its stability was an issue. Therefore, we decided to protect the dye molecules by embedding it in a thick layer of the silica shell. This second type of microparticle sensor was named T2-HPTS, where the coating of HPTS was formed around silica seed particles by slowly growing layers of silica using APTES and TEOS as monomers in presence of HPTS. Here, TEOS provided strength to the growing silica layer but brought negative charge to the surface due to hydroxyl groups that can significantly repel negatively charged HPTS molecules. Therefore, to reduce the overall negative charge of TEOS, APTES was used as comonomer, to allow sequestration of negatively charged HPTS molecules during silica microparticle growth. APTES in addition to becoming part of the growing silica shell has a primary amine group, therefore in its ionized form, its positive charge attracts and holds HPTS molecules. The combined use of TEOS and APTES layer polymerization prevented leaching of HPTS molecules and significantly enhanced the stability of T2-HPTS microparticles, however, the pH sensitivity was significantly perturbed. The T2-HPTS particles showed no clear trend in fluorescence emission change as visible for T1-HPTS (Figure 1a). Whereas T1-HPTS showed an exponential increase in fluorescence emission ratio from 0.36 at pH 8 to 52 at pH 4, T2-HPTS showed a fluctuating emission ratio, which increased from pH 8 to 7 then decreased for pH 6 and again increased for pH 5 and 4.

To understand the reason behind the perturbed pH sensitivity in T2-HPTS, we assessed the zeta potential and found that zeta potential of T2-HPTS was +2.97 mV which was 88.6% less than that for T1-HPTS (+26.1 mV). Thus, it was suspected that the presence of just positive surface charge is not enough and there needs to be a minimum threshold surface charge for the desired pH sensitivity. To test our hypothesis, we decorated the surface of T2-HPTS with additional APTES molecules to make the surface charge more electropositive and tested its effect on the pH sensitivity. As supposed, the pH sensitivity increased significantly after the positive charge enhancement in addition to the irregularity of pH against fluorescence emission rectified. The modified T2-HPTS has been therefore named as T2A-HPTS, where A represented the presence of additional primary amine groups from APTES. The comparative analysis showed that T1-HPTS was still most electropositive with a zeta potential of about $+26.1 \pm 5$ mV followed by T2A-HPTS and T2-HPTS with zeta potentials as $+6.02 \pm 4$ and $+2.97 \pm 4$ mV, respectively. Here, it is to be emphasized that the stability of T2A-HPTS microparticles was similar to that of T2-HPTS particles, thus the reaction to enhance the positive charge on the particle surface caused no change in stability of the immobilized dye molecules.

Motivated with the attainment of both pH sensitivity as well as stability with T2A-HPTS pH sensors, we decided to further

optimize the synthesis protocol to attain best pH sensitivity without losing stability. These microparticles were synthesized similar to that of T2-HPTS, however, the concentration of TEOS is halved. The rationale behind the reduction in TEOS concentration was to increase the overall positive charge on the particle's surface while still leaving enough TEOS to physically lock the HPTS molecules on the surface. This strategy significantly enhanced the pH sensitivity of the resulting microparticles (T3-HPTS). Comparatively, T3-HPTS were observed to be much better than T1-HPTS in terms of stability and more sensitive compared to T2A-HPTS, where the emission ratio changes from 0.36 to 84 for pH change from 8 to 4. Therefore, the percent change in emission ratio with pH change is highest for T3-HPTS (233 times) among stable microparticles (Table 1). As expected, the reason for the enhanced pH sensitivity was also evident from the zeta potential which increased to $+11.5 \pm 5.2$ mV.

Thus, we concluded that charge of the immediate environment of the dye molecule can significantly affect its sensitivity by perturbing the ionization efficiency of the charged dyes as also reported elsewhere.^[20] Although T1-HPTS was highly positive and showed superb pH sensitivity, it was not stable and showed loss of dye molecules, whereas T2-HPTS was very stable but showed poor pH sensitivity. Therefore, protection of the HPTS dye molecule without disturbing its pH sensitivity required a balance of charge on the microparticle surface. While a high positive charge is beneficial for enhancing the pH sensitivity of the HPTS dye on the surface of microparticle, preparing a microparticle with that much charge by reducing the concentration of TEOS during synthesis can significantly affect the strength of shell holding the dye molecules on the microparticle surface. Therefore, it can cause poor dye retention resulting in significant loss of fluorescence from the microparticles, leaving it blank and non-fluorescent. As observed from different forms of sensors prepared in our study, we found that zeta potential of more than +10 mV is enough for keeping the sensitivity equivalent to that provided by zeta potential of +26 mV (for T1-HPTS).

T2A-HPTS and T3-HPTS sensors reversibility and ageing were also investigated (Figure 2). Reversibility was evaluated by a series of three pH switches between pH 7 and 5. At least 3 samples for each type of sensor were tested. T3-HPTS sensors

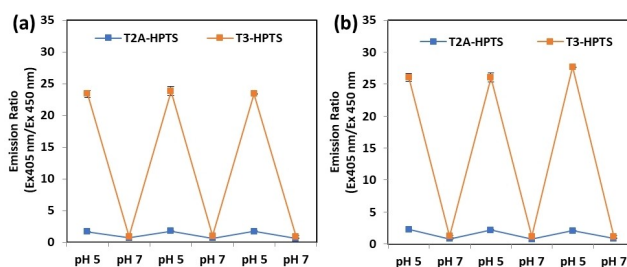


Figure 2. a) Reversibility of T2A-HPTS and T3-HPTS evaluated by conducting sequences of 3 pH switches between pH 5 and 7 ($n=3$). b) Ageing of HPTS-aminated and HPTS sensors evaluated by repeating sequences of 3 pH switches between pH 5 and 7 ($n=3$) after 7 days on the samples adopted to evaluate the reversibility.

appeared more sensitive compared to T2A-HPTS showing higher ratios values, with average emission ratios equals to 21.86 and 0.13 for pH 5 and 7, respectively (Figure 2a). As regard the sensors ageing for T2A-HPTS and T3-HPTS, series of three pH switches between pH 7 and 5 were repeated after 7 days on the same samples employed to evaluate reversibility to assess their sensing ability despite the ageing. Again, all observed data resulted statistically significant accordingly to ANOVA with single factor ($\alpha=0.05$) with very limited standard deviation and variance values on both T2A-HPTS and T3-HPTS, thus proving robustness and response reliability of both systems.

The two graphs can be considered directly comparable as the back calculated pH values from the emission ratios would be very similar in both the cases. This robustness is observed due to the fact that the change in the emission ratio with pH change is exponential as can be observed in Figure 1a. For T3-HPTS the observed change in emission ratio is 233 folds when the pH is changed from pH 8 to 4. For this large change in emission ratio, even a minuscule fluctuation in pH due to instrumental error of pH meter, can result in an observable change in emission ratio.

Reversibility studies with T1-HPTS and T2-HPTS were not conducted as T1-HPTS particles were not very stable causing significant amount of dye molecule being lost during washing as well as pH balance steps. Therefore, it was unreliable to test the pH reversibility for T1-HPTS particles. T2-HPTS was excluded from reversibility study because its range of pH sensitivity was not following a trend as shown in Figure 1a.

To assess the monodispersity of the pH microsensors, we selected the best two pH sensor types, that is, T2A-HPTS and T3-HPTS, and analyzed their morphology by means of scanning electron microscopy (Figure 3). The analysis showed appreciably monodispersed spherical microparticles with a diameter of $\sim 962 \pm 78$ and $\sim 790 \pm 31$ nm for T2A-HPTS and T3-HPTS, respectively. Notably, the microparticles due to their large size compared to nanoparticles have a unique advantage in fluorescence microscopy, that they can be resolved very easily to observe each microparticle individually. The larger size also brings with it a lower surface area to volume ratio that reduces the probability of aggregation in an experimental setup and during long time storage. Additionally, SMPs have been observed to be more cytocompatible as compared to their nanoparticle counterparts that have higher surface energies

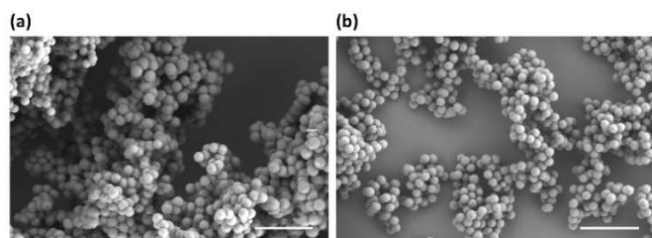


Figure 3. Scanning electron micrographs of a) T2A-HPTS and b) T3-HPTS microparticles with diameter of ~ 962 and ~ 790 nm, respectively; scale bars: 5 μ m.

that make them more interactive against cellular organelles and biomolecules.

Motivated by the encouraging results for T3-HPTS, we also tried to assess the pH sensitivity of T3-HPTS by using confocal laser scanning microscopy (CLSM), where a significant change in emission intensity was observed when pH was changed from pH 8 to 4 (Figure 4). The panels represent sensor particles imaged at different pH values. Using images acquired under different excitation wavelength, merged and ratiometric images were generated and color coded for showing ratiometric pixel values. Ratiometric images were obtained by dividing the intensity of individual pixels of images acquired using 405 and 458 nm excitation and creating a third image with the obtained pixel ratios.

Owing to the cytotoxicity caused by particle-based sensors, viability of HCT116 cells were assessed after their incubation with T3-HPTS sensors. Compared to the positive control (untreated cells), the cells incubated with the T3-HPTS sensors showed equal viability at different incubation time points as indicated in Figure S1. The statistical analysis proved that there was no significant difference between viability of treated and untreated cells. This nontoxic behavior is most likely due to bioinert behavior of silica^[21] and the micrometer size of these particles that significantly reduce the surface energy that is primary cause of interaction of the nanoparticles with biomolecules^[22] and cell organelles.^[23]

Next, the pH sensitivity and cytocompatibility analyses of T3-HPTS, we tested the uptake of T3-HPTS sensors by mammalian cells. To this aim, we incubated colon cancer cell line HCT116 with T3-HPTS pH sensors for 24 h followed by fluorescence imaging. At time 0 h, the HPTS particles were mainly distributed in the cell medium (neutral pH) and, in agreement with the HPTS pH-sensitivity, showed fluorescence emission from both 405 and 458 nm excitation (Figure S2). At 24 h, the majority of T3-HPTS pH sensors were detected in close proximity or inside the cells and showed a diminished fluorescence emission at 458 nm excitation. In agreement with

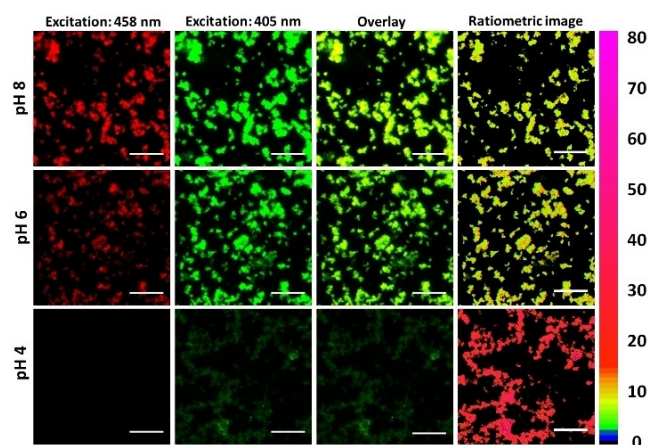


Figure 4. Fluorescence confocal microscopy images showing pH-dependent fluorescence emission of T3-HPTS in independent emission channels, overlay and ratiometric images ($\lambda_{\text{ex:405 nm}}/\lambda_{\text{ex:458 nm}}$). The emission was detected over the range 474–570 nm; scale bars: 20 μ m.

other studies based on different pH probes,^[1a,b,24] the change of fluorescence of HPTS might reflect the intracellular localization of the T3-HPTS particles in an acidic environment^[25] (e.g., endosomes or lysosomes), causing the observed decrease of emission from excitation at 458 nm. Additional experiments by electron microscopy aimed at deeply investigating the cell uptake of the T3-HPTS sensors and their intracellular localization are currently underway in our laboratories.

Table 1 shows the main properties of the different types of HPTS-based sensors. The relative pH sensitivity for each type of sensor was calculated using the sensitivity of molecular HPTS as a reference. This comparison showed that T1-HPTS had the mean sensitivity of 13.5% and pK_a of about 6.53 which was less than that of free HPTS. T2-HPTS on the other hand showed similar mean sensitivity but large standard deviation owing to its irregular pH versus emission ratio behavior. T2A-HPTS showed pH sensitivity of almost 16%, which was higher than T2-HPTS, but less than the sensitivity of T3-HPTS (22%). As can be observed, for all the sensors the pK_a is less than that of free HPTS pK_a which is ~ 7.25 .^[11,26] However, among all the analyzed systems, the change in pK_a is minimum for T3-HPTS (pK_a : 6.97). Thus, the final optimized methodology for pH sensor development is suitable for sensitivity, stability as well as maintaining the intrinsic behavior of the fluorophore.

In conclusion, we have devised ways to successfully use the negatively charged pH-sensitive HPTS molecule with SMPs without compromising its pH sensitivity. These sensors could be used in sensing pH in *in vitro* as well as *in vivo* systems due to the cytocompatibility of HPTS molecules and SMPs. Due to their stability and sensitivity, these HPTS-based pH microsensors could potentially be used for endosomal pH tracking as well as extracellular pH assessment in *in vitro* studies involving spheroids and organoids. In future we will be exploring their utility in these directions.^[27]

The extreme change in emission ratio with even a small change in pH will help in investigating very subtle changes in pH. The methodology used here can be employed to immobilize various other anionic dye molecules and even anionic drugs on the silica surface. New sensing platforms could be developed by using this methodology to build sensitive optical fiber probes that have potential in *in vivo* applications like measuring tumor tissue acidity. In a similar fashion, planar surfaces could be layered with electrostatically incompatible charged dye molecules by using the current methodology. This would help in the development of miniature sensor surfaces integrated into microfluidic devices where the glass substrate could be modified with dye molecules that are difficult to tether.^[27c]

As emphasized previously, a balance between tailoring the surface charge and protecting the dye molecule against leaching must be maintained to have a stable as well as sensitive pH sensor. This approach would ultimately help in realizing more sensitive and robust sensing devices and platforms.

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Conflict of Interest

The authors declare no conflict of interest.

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- [1] a) L. L. del Mercato, M. Moffa, R. Rinaldi, D. Pisignano, *Small* **2015**, *11*, 6417–6424; b) I. L. Moldero, A. Chandra, M. Cavo, C. Mota, D. Kapsokalyvas, G. Gigli, L. Moroni, L. L. del Mercato, *Small* **2020**, *16*, 2002258; c) J. Li, Y. Liu, Y. Xu, L. Li, Y. Sun, W. Huang, *Coord. Chem. Rev.* **2020**, *415*, 213318; d) P. Wang, H. Yang, C. Liu, M. Qiu, X. Ma, Z. Mao, Y. Sun, Z. Liu, *Chin. Chem. Lett.* **2021**, *32*, 168–178; e) Y. Sun, F. Ding, Z. Chen, R. Zhang, C. Li, Y. Xu, Y. Zhang, R. Ni, X. Li, G. Yang, Y. Sun, P. J. Stang, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 16729–16735; f) Q. Ouyang, L. Tu, Y. Zhang, H. Chen, Y. Fan, Y. Tu, Y. Li, Y. Sun, *Anal. Chem.* **2020**, *92*, 14947–14952; g) F. Ding, Z. Chen, W. Young Kim, A. Sharma, C. Li, Q. Ouyang, H. Zhu, G. Yang, Y. Sun, J. Seung Kim, *Chem. Sci.* **2019**, *10*, 7023–7028.
- [2] L. Jiao, F. Song, B. Zhang, H. Ning, J. Cui, X. Peng, *J. Mater. Chem. B* **2017**, *5*, 5278–5283.
- [3] L. Pla, B. Lozano-Torres, R. Martínez-Mañez, F. Sancenón, J. V. Ros-Lis, *Sensors* **2019**, *19*, 5138.
- [4] a) X. Ji, H. Wang, B. Song, B. Chu, Y. He, *Front. Chem.* **2018**, *6*; b) A. Chandra, S. Prasad, G. Gigli, L. L. del Mercato in *Fluorescent Nanoparticles for Sensing*, Vol. 16 (Eds.: W. J. Parak, N. Feliu), Elsevier, **2020**, pp. 117–149.
- [5] S. Prasad, A. Chandra, M. Cavo, E. Parasido, S. Fricke, Y. Lee, E. D'Amone, G. Gigli, C. Albanese, O. Rodriguez, L. L. del Mercato, *Nanotechnology* **2020**, *32*, 062001.
- [6] L. D. Lavis, R. T. Raines, *ACS Chem. Biol.* **2008**, *3*, 142–155.
- [7] a) J. Liang, Z. Lu, J. Xu, J. Li, H. Zhang, W. Yang, *J. Mater. Chem.* **2011**, *21*, 1147–1152; b) I. Acquah, J. Roh, D. J. Ahn, *Chem. Asian J.* **2017**, *12*, 1724–1729.
- [8] H. Mbarak, R. T. Ghahrizjani, S. M. Hamidi, E. Mohajerani, Y. Zaatari, *Sci. Rep.* **2020**, *10*, 5110.
- [9] H.-H. Ho, A.-T. Nguyen, Y.-C. Chen, L.-Y. Chen, H.-P. Dang, M.-J. Tsai, H. Cheng, S.-F. Horng, C.-S. Huang, H.-W. Zan, H.-F. Meng, *ACS Omega* **2020**, *5*, 23021–23027.
- [10] R. Kumar, R. Yadav, M. A. Kolhe, R. S. Bhosale, R. Narayan, *Polymer* **2018**, *136*, 157–165.
- [11] S. Ulrich, A. Osypova, G. Panzarasa, R. M. Rossi, N. Bruns, L. F. Boesel, *Macromol. Rapid Commun.* **2019**, *40*, 1900360.
- [12] R. M. Kenney, M. W. Boyce, N. A. Whitman, B. P. Kromhout, M. R. Lockett, *Anal. Chem.* **2018**, *90*, 2376–2383.
- [13] a) E. P. O'Brien, B. R. Brooks, D. Thirumalai, *J. Am. Chem. Soc.* **2012**, *134*, 979–987; b) A.-S. Yang, B. Honig, *J. Mol. Biol.* **1993**, *231*, 459–474.
- [14] a) M. Naghavi, R. John, S. Naguib, M. S. Siadaty, R. Grasu, K. C. Kurian, W. B. v. Winkle, B. Soller, S. Litovsky, M. Madjid, J. T. Willerson, W. Casscells, *Atherosclerosis* **2002**, *164*, 27–35; b) S. Ono, R. Imai, Y. Ida, D. Shibata, T. Komiya, H. Matsumura, *Burns* **2015**, *41*, 820–824; c) X. Qiao, J. Xu, Q.-J. Yang, Y. Du, S. Lei, Z.-H. Liu, X. Liu, H. Liu, *Oxid Med Cell Longev* **2013**, *2013*, 1–6.
- [15] a) B. F. Soerawidjaja, M. Kang, H. Kim, H. K. Yang, J. H. Kim, M. Kwak, *Mol. Cryst. Liq. Cryst.* **2019**, *687*, 53–59; b) A. Reisch, A. S. Klymchenko,

- Small* **2016**, *12*, 1968–1992; c) X. Wu, W. Zhu, *Chem. Soc. Rev.* **2015**, *44*, 4179–4184.
- [16] O. S. Wolfbeis, E. Furlinger, H. Kroneis, H. Marsoner, *Fresenius Z. Anal. Chem.* **1983**, *314*, 119–124.
- [17] M. Renganathan, E. Pfundel, R. A. Dilley, *Biochim. Biophys. Acta Bioenerg.* **1993**, *1142*, 277–292.
- [18] a) I. Acquah, J. Roh, D. J. Ahn, *Macromol. Res.* **2017**, *25*, 950–955; b) H. D. Duong, Y. Shin, J. I. Rhee, *Microchem. J.* **2019**, *147*, 286–295; c) Z. Bai, R. Chen, P. Si, Y. Huang, H. Sun, D.-H. Kim, *ACS Appl. Mater. Interfaces* **2013**, *5*, 5856–5860; d) F. Gao, X. Chen, Q. Ye, Z. Yao, X. Guo, L. Wang, *Microchim. Acta* **2011**, *172*, 327–333.
- [19] H. Offenbacher, O. S. Wolfbeis, E. Furlinger, *Sens. Actuators* **1986**, *9*, 73–84.
- [20] a) S. Hulth, R. C. Aller, P. Engström, E. Selander, *Limnol. Oceanogr.* **2002**, *47*, 212–220; b) N. Opitz, D. W. Lübbers in *A Correction Method for Ionic Strength-Independent Fluorescence Photometric pH Measurement* (Eds.: D. W. Lübbers, H. Acker, E. Leniger-Follert, T. K. Goldstrick), Springer, Boston, **1984**, pp. 907–912.
- [21] J. F. Popplewell, S. J. King, J. P. Day, P. Ackrill, L. K. Fifield, R. G. Cresswell, M. L. di Tada, K. Liu, *J. Inorg. Biochem.* **1998**, *69*, 177–180.
- [22] a) A. A. Vertegel, R. W. Siegel, J. S. Dordick, *Langmuir* **2004**, *20*, 6800–6807; b) W. Shang, J. H. Nuffer, V. A. Muñoz-Papandrea, W. Colón, R. W. Siegel, J. S. Dordick, *Small* **2009**, *5*, 470–476.
- [23] a) H. Jaganathan, B. Godin, *Adv. Drug Delivery Rev.* **2012**, *64*, 1800–1819; b) J. G. Croissant, Y. Fatieiev, N. M. Khashab, *Adv. Mater.* **2017**, *29*, 1604634; c) S. P. Hadipour Moghaddam, R. Mohammadpour, H. Ghandehari, *J. Controlled Release* **2019**, *311–312*, 1–15.
- [24] L. L. del Mercato, A. Z. Abbasi, M. Ochs, W. J. Parak, *ACS Nano* **2011**, *5*, 9668–9674.
- [25] O. Kreft, A. M. Javier, G. B. Sukhorukov, W. J. Parak, *J. Mater. Chem.* **2007**, *17*, 4471.
- [26] A. Hakonen, S. Hulth, *Anal. Chim. Acta* **2008**, *606*, 63–71.
- [27] a) J. Gong, M. G. Tanner, S. Venkateswaran, J. M. Stone, Y. Zhang, M. Bradley, *Anal. Chim. Acta* **2020**, *1134*, 136–143; b) S. Berneschi, A. Barucci, F. Baldini, F. Cosi, F. Quercioli, S. Pelli, G. C. Righini, B. Tiribilli, S. Tombelli, C. Trono, A. Giannetti, *Optics* **2020**, *1*, 213–242; c) D. Măriuța, S. Colin, C. Barrot-Lattes, S. Le Calvé, J. G. Korvink, L. Baldas, J. J. Brandner, *Microfluid. Nanofluid.* **2020**, *24*, 65.

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