Cellular Sensing

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A Nanogel for Ratiometric Fluorescent Sensing of Intracellular pH Values**

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Hydrogel nanoparticles (nanogels) are appealing probes for use in chemical and biochemical sensing because of their stability, biocompatibility, and softness.[1] Nanogels have been reported[2,3] for detection of several analytes, but mostly for the macrorealm. Recently, fluorescent nanogels have been reported[4] that are capable of transducing volume changes into a change in fluorescence intensity, and nanoscale sensing of temperature in the cytoplasm of living cells was demonstrated.[5] Fluorescence is by far the most powerful method for detecting the cellular dynamics of low-molecular-weight species, including protons (pH), oxygen, and ions such as calcium(II) and chloride.[6] Most sensing methods, including those based on microscopy, are based on the measurement of fluorescence intensity. Unfortunately, single-intensity-based sensing is compromised by the local distribution of probes, which often bind to proteins, and by drifts of light sources and detectors. More robust signals can be obtained by two-wavelength ratiometric methods, amongst others.[7] Herein we present the first ratiometric fluorescent nanogel capable of sensing pH values in the physiological range, that is, from six to eight. It can be prepared rather simply from an inert but biocompatible polyurethane polymer that was made pH-sensitive by loading it with the pH indicator bromothymol blue (BTB). Furthermore, it was rendered fluorescent by addition of two standard fluorophores that undergo efficient fluorescence resonance energy transfer (FRET) inside the nanogel. The fluorophores coumarin 6 (C6) and Nile Red (NR) were chosen to give a dual (green and red) fluorescent signal that can be easily ratioed.

The nanogel (NG) was obtained by a modified reprecipitation method[8] (see the Experimental Section). In essence, an ethanol solution of a hydrogel containing both hydrophilic and hydrophobic domains was dialyzed against water. As a result, the polymer chains rearrange to form a three-dimensionally stable nanostructure[1] based on mainly hydrophobic interaction. The optical probes used in this work are then entrapped into this network. The polyurethane chosen is well-suited for making such NGs because it contains both hydrophilic and hydrophobic domains, is optically transparent down to 300 nm, commercially available, and widely used in medicine and in contact lenses. Importantly, the volume of the NG particles is hardly affected by pH, which is mandatory with respect to the efficiency of FRET and in terms of in vivo sensing as it will not disturb cellular activities.[9] Figure 1 shows a model of the chemical composition of such a NG-based pH sensor bead.

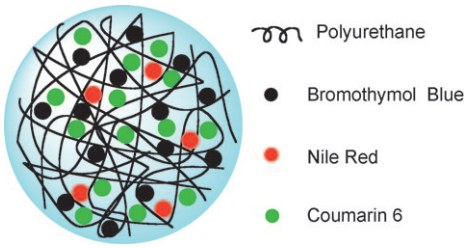

Figure 1. Model of the ratiometric pH sensing nanogel used in this work. Nile Red and coumarin 6 are located, on average, within the distance over which FRET can occur (typically <10 nm) in the nanogel. In contrast, no FRET is observed if the two fluorophores are placed in plain aqueous solution.

The sensing capability of the NG architecture described herein relies on two specific features. The first is the spectral overlap of the absorption of the pH indicator BTB with the dual emission of the fluorophores C6 and Nile Red (Figure 2a). The second feature is the efficient FRET (predominantly red fluorescence at pH 7) that occurs between C6 and NR in an aqueous suspension of NG, but not in aqueous solution alone where they are too far apart (Figure 2b). The mechanism of the pH-dependent FRET can be explained on the basis of the spectra (Figure 2a). Upon photoexcitation of C6 at 440 nm, green fluorescence is induced, with a peak at 520 nm, but part of the emission is transferred to Nile Red by FRET. The red fluorescence of Nile Red (NR) resulting from FRET has a peak at 620 nm. Sensitivity to pH is imparted because BTB is yellow at pH values of less than 6, with an absorption peak at around 435 nm. Therefore, a good fraction of the green emission of C6 (the energy not transferred to NR) is absorbed by the yellow form of BTB (present at low pH), whereas the FRET-induced emission of NR is preserved. In fact, the red fluorescence of NR is easily visible and detectable (Figure 2b). At pH values above 8, however, BTB is blue, with an absorption peak at 628 nm that strongly overlaps the red emission of NR. As a result, it absorbs most of the red fluorescence of NR. Correspondingly, the visible

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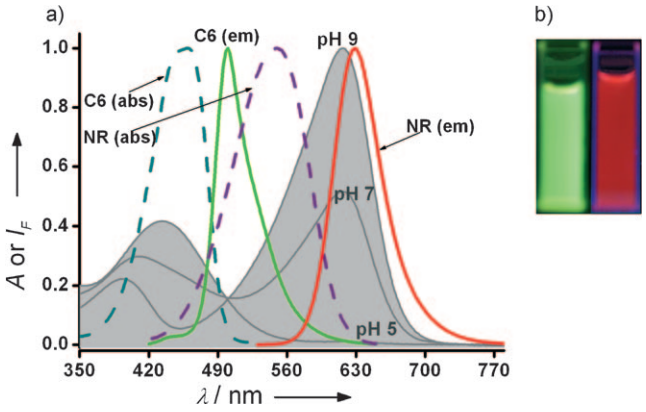

Figure 2. a) pH-dependent absorption of bromothymol blue in aqueous solution at pH 5.0, 7.0, and 9.0 (gray curves), and absorption and emission spectra of coumarin 6 (C6) and Nile Red (NR) in ethanol. b) The green fluorescence of a mixture of C6 and NR in ethanol/water solvent (left), and the same components in the nanogel (NG) in aqueous suspension under 365 nm illumination (right).

fluorescence of the NG is dominated by the green fluorescence of C6.

To elucidate the sensing mechanism, three model polyurethane NGs were prepared and studied. The first (model NG-1) contains BTB and coumarin 6 (C6) only, the second (model NG-2) BTB and Nile Red (NR) only, and the third (model NG-3) C6 and NR only. The results (Supporting Information, Figure S1) underpin the FRET-based sensing mechanism (including optical filter effects). The pH-dependence of the fluorescence of model NG-1 (BTB/C6) is similar to that of the pH-sensing nanogel. Its emission (peaking at 500 nm) increases with pH (Supporting Information, Figure S1 a), and is ascribed to pH-dependent changes in the absorption of BTB as discussed above. The competition in the absorption at around 420 nm between C6 and BTB may play a role in the enhancement of C6 emission but is insignificant in comparison to the decrease in the reabsorption of the C6 emission. Otherwise, the fluorescence intensity of model NG-2 (BTB/NR) at 450 nm excitation would increase initially with pH, then decrease. In fact, the 620 nm emission does not show such an effect (Supporting Information, Figure S1 b). Therefore the pH sensitivity of the pH nanogel solely arises from the pH-dependent reabsorption of the fluorescence of C6 by BTB. The ratio $I_{F(620)}$:$I_{F(500)}$ of the model NG-3 (C6/NR) remains virtually independent of pH (Supporting Information, Figure S1 c).

The ratio of the three dopants in the gel was empirically optimized under the following considerations: 1) doping should not impair the collapse and cross-link of the polymer, and hence the formation of the NG, and 2) the ratio of the indicator BTB to C6 and to NR should result in emissions of comparable intensities. In the optimized system, the ratio of the fluorescence intensities at 620 and 520 nm is about 1.0 at pH 7.4. Transmission electron microscopy images (Supporting Information, Figure S2) reveal that the dried NGs have a well-formed spherical shape, with diameters ranging from 20 to 30 nm. In aqueous suspension, however, the hydrophilic NGs possess a much larger hydrodynamic diameter (137 nm), as revealed by dynamic light scattering (Supporting Information, Figure S3).

Fluorescence emission spectra of the NGs at various pH values under 450 nm excitation are shown in Figure 3 a. The peaks at 500 nm and 620 nm are assigned to C6 and NR,

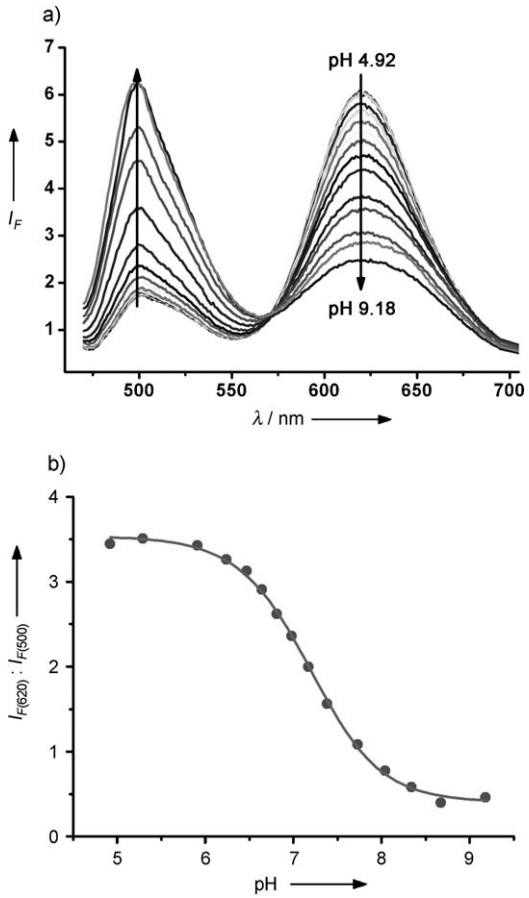

Figure 3. a) Fluorescence spectra of the ratiometric pH-responsive nanogels (NGs) at 450 nm excitation at pH values of 4.92, 5.29, 5.91, 6.24, 6.47, 6.64, 6.81, 6.98, 7.38, 7.73, 8.04, 8.34, 8.67, and 9.18. b) pH calibration plot of the ratiometric NGs. The experimental data (●) were calculated from the ratio of the fluorescence intensities at 620 nm and 500 nm. The line is a fit of the function given in the Supporting Information, Eq. (1), and yields an apparent pK_a of 7.64.

respectively. There is a very strong and inverse change in the dual emission with pH: the emission of C6 increases with pH, whereas that of NR decreases. This effect is quite favorable in that the ratio of the two intensities can be measured and related to pH. The ratio of emission intensities of NR (at 620 nm) and of C6 (at 500 nm) versus pH is plotted in Figure 3 b. An almost ninefold change in the ratio (from 3.5 to 0.4) is observed on going from pH 5 to pH 9. The pK_a value of BTB in the NG was determined to be 7.64 at 22 °C. Interestingly, the pK_a is higher than in water (6.8 at 25 °C),[14] but lower than in plain hydrogel (8.8 at 22 °C).[15]

Ratiometric fluorescent nanoparticles (not NGs) have been reported before that are capable of sensing intracellular pH. They can be classified according to their action: In the first category, the ratio arises from the signals of a pH-

sensitive probe and a pH-insensitive reference fluorophore;[13,16] in the second, the signal ratio arises from two inversely varying bands.[17] If part of a FRET system, they are critically sensitive to the distance of the fluorophores involved. This distance can vary, irrespective of a constant pH value, in the order of 10^{-10} m for various reasons, including changes in conformation, ionic strength, and bivalent metal ions.[18] In the pH-sensitive NGs reported herein, the probes are located inside the polymer matrix. pH-dependent swelling is another cause for erroneous results when sensing pH. Remarkably, the hydrodynamic diameter of the NG used in this work in buffer solutions of pH 4.9 and pH 9.2, respectively, is 127 and 132 nm, which is approximately the same as in aqueous dispersion of pH 7.0 (137 nm).

The response of the pH-sensing NG towards changes in pH is in the order of several seconds, obviously because of its small size and high hydrophilicity. The reproducibility of the fabrication of the NGs is excellent, as demonstrated by determining the pK_a values of several batches. Plots of the ratio $I_{F(620)}$:$I_{F(500)}$ (Supporting Information, Figure S4) are almost identical and yield very similar pK_a values. The NGs are also quite stable in aqueous suspension. The average hydrodynamic diameter of a one-month-old sample decreased to 125 nm (that is, it decreased by 11 nm; Supporting Information, Figure S3, right). Moreover, only minimal dye leakage is found after a period of one month (Supporting Information, Figure S5). On the other hand, on cycling from a rather low (4.98) to a rather high (9.18) pH value results in signal drift and a decrease in relative signal change (Supporting Information, Figure S6), which is probably a result of leaching of BTB from the NG.

The storage stability at near-neutral pH values is assumed to result from several effects, and may partly result from hydrophobic interaction between the hydrophobic chain of the polymer and doped molecules, similar to that in other hydrogels.[10] In addition, hydrogen bonding will contribute to the attractive forces in the NGs, mainly because of the presence of hydroxy, imino, and phenol groups in the hydrogel and in BTB, and because of the hydrogen-accepting nature of the heterocyclic dyes.[11,12] Dye loading of hydrogels based on hydrophobic interaction alone usually results in low loading capacities[10] and leaching.[13]

The NGs were introduced into living epithelial normal rat kidney (NRK) cells grown on glass slides to demonstrate the feasibility of intracellular sensing of pH. Particle uptake was achieved by exposing the cells to the NG particles for 24 h. A good fraction of the particles was actively incorporated by the cells, presumably by vesicular uptake mechanisms. Extracellular NG particles were then washed off prior to microscopic inspection. The micrographs in Figure 4 show an optical x,y section through the center of the cell bodies. The particles are localized inside the cytoplasm but not the nucleoplasm. The inhomogeneous distribution of particles indicates an accumulation in intracellular organelles, such as the endoplasmic reticulum or the Golgi apparatus (see the arrows in Figure 4a).

The cells were imaged in physiological buffer of pH 7.4. The fluorescence of the two fluorophores (coumarin 6 in Figure 4a; Nile Red in Figure 4b) were recorded separately using individual excitation and emission filters. It should be noted that the efficiency of FRET (and thus intracellular pH) cannot be recorded with this experimental setup. The overlay of the two images (Figure 4c) reveals perfect co-localization of the fluorophores inside the nanogel particles.

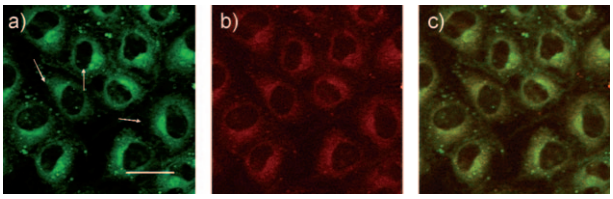

Figure 4. Fluorescence micrographs of NRK cells incubated with a pH 7.4 buffer and loaded with the pH-responsive nanogel. a) Fluorescence of the coumarin dye (C6) acquired in the green channel (scale bar = 20 μm); b) fluorescence of Nile Red acquired in the red channel; c) overlay of (a) and (b).

In conclusion, the first ratiometric fluorescent NG for sensing pH is presented. It can be easily prepared and made pH-responsive by addition of a pH probe and a ratiometric FRET system. We expect that this approach is applicable to the construction of various other kinds of sensing NGs by replacing the respective indicator dyes (probes) by indicators for other ions, provided they have appropriate spectral properties. Thus, this approach is likely to have a wide scope in terms of intracellular chemical sensing.

Experimental Section

For details of the instruments and materials used, see the Supporting Information.

Preparation of the sensing nanogel: The pH probe bromothymol blue (BTB; 0.4 mg), and the dyes 7-diethylamino-3-benzothiazolyl-coumarin 6 (C6; 0.4 mg) and Nile Red (NR; 0.04 mg) were co-dissolved in 20 g of a 500 ppm solution of the polyurethane hydrogel (PU) in an ethanol/water (9:1, v/v) mixture. The resultant ratio of PU/BTB/C6/NR is 200:4:4:0.4 (w/w). The mixtures were thoroughly stirred for 1 h, then dialyzed against distilled water for 24 h, with an interval of 2–3 h to exchange the water. Finally, the aqueous dispersion of the nanogel was filtered through a 0.2 μm filter to remove large aggregates. The resultant suspension was used in further experiments (including spectral characterization, TEM, dynamic light scattering, studies on effects of pH and ageing, and with model nanogels 1, 2, and 3).

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[1] a) S. Nayak, L. A. Lyon, *Angew. Chem.* **2005**, *117*, 7862–7886; *Angew. Chem. Int. Ed.* **2005**, *44*, 7686–7708; b) N. A. Peppas, J. Z. Hilt, A. Khademhosseini, R. Langer, *Adv. Mater.* **2006**, *18*, 1345–1360.

[2] a) J. H. Holtz, S. A. Asher, *Nature* **1997**, *389*, 829–832; b) S. A. Asher, V. L. Alexeev, A. V. Goponenko, A. C. Sharma, I. K. Lednev, C. S. Wilcox, D. N. Finegold, *J. Am. Chem. Soc.* **2003**, *125*, 3322–3329; c) J. Kim, S. Nayak, L. A. Lyon, *J. Am. Chem. Soc.* **2005**, *127*, 9588–9592.

[3] K. Sirkar, A. Revzin, M. V. Pishko, *Anal. Chem.* **2000**, *72*, 2930 – 2936.
[4] a) K. Iwai, Y. Matsumura, S. Uchiyama, A. P. de Silva, *J. Mater. Chem.* **2005**, *15*, 2796 – 2800; b) C. Gota, S. Uchiyama, T. Yoshihara, S. Tobita, T. Ohwada, *J. Phys. Chem. B* **2008**, *112*, 2829 – 2836.
[5] C. Gota, K. Okabe, T. Funatsu, Y. Harada, S. Uchiyama, *J. Am. Chem. Soc.* **2009**, *131*, 2766 – 2767.
[6] a) C. McDonagh, C. S. Burke, B. D. MacCraith, *Chem. Rev.* **2008**, *108*, 400 – 422; b) Y. E. K. Lee, R. Kopelman, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2009**, *1*, 98 – 110.
[7] "Intrinsically Referenced Fluorimetric Sensing and Detection Schemes: Methods, Advantages and Applications": M. Schaeferling, A. Duerkop in *Standardization and Quality Assurance in Fluorescence Measurements* (Ed.: U. Resch-Genger), Springer, Berlin, **2008**, chap. 15, p. 373 (Springer Series in Fluorescence, Vol. 5).
[8] M. K. Yoo, M. K. Jang, J. W. Nah, M. R. Park, C. S. Cho, *Macromol. Chem. Phys.* **2006**, *207*, 528 – 535.
[9] a) J. Rosenzweig, N. Ji, C. Griffin, Z. Rosenzweig, *Anal. Chem.* **2000**, *72*, 3497 – 3503; b) L. Shi, N. Rosenzweig, Z. Rosenzweig, *Anal. Chem.* **2007**, *79*, 208 – 214, and references therein.
[10] a) D. Missirlis, N. Tirelli, J. A. Hubbell, *Langmuir* **2005**, *21*, 2605 – 2613; b) J. K. Oh, D. J. Siegwart, H. I. Lee, G. Sherwood, L. Peteanu, J. O. Hollinger, K. Kataoka, K. Matyjaszewski, *J. Am. Chem. Soc.* **2007**, *129*, 5939 – 5945; c) N. Kato, U. Hasegawa, N. Morimoto, Y. Saita, K. Nakashima, Y. Ezura, H. Kurosawa, K. Akiyoshi, M. Noda, *J. Cell. Biochem.* **2007**, *101*, 1063 – 1070.
[11] a) U. Kosch, I. Klimant, T. Werner, O. S. Wolfbeis, *Anal. Chem.* **1998**, *70*, 3892 – 3897; b) A. Priimagi, S. Cattaneo, R. H. A. Ras, S. Valkama, O. Ikkala, M. Kauranen, *Chem. Mater.* **2005**, *17*, 5798 – 5802.
[12] G. R. Desiraju, T. Steiner, *The Weak Hydrogen Bond in Structural Chemistry and Biology*, Oxford University Press, Oxford, **1999**, pp. 190 – 193.
[13] H. Sun, A. M. Scharff-Poulsen, H. Gu, K. Almdal, *Chem. Mater.* **2006**, *18*, 3381 – 3384.
[14] M. D. DeGrandpre, *Anal. Chem.* **1993**, *65*, 331 – 337.
[15] L. Sun, H. Peng, M. I. J. Stich, D. E. Achatz, O. S. Wolfbeis, *Chem. Commun.* **2009**, 5000 – 5002.
[16] a) E. Allard, C. Larpent, *J. Polym. Sci. Part A* **2008**, *46*, 6206 – 6213; b) S. A. Hilderbrand, K. A. Kelly, M. Niedre, R. Weissleder, *Bioconjugate Chem.* **2008**, *19*, 1635 – 1639; c) T. Doussineau, M. Smaihi, G. J. Mohr, *Adv. Funct. Mater.* **2009**, *19*, 117 – 122.
[17] a) P. T. Snee, R. C. Somers, G. Nair, J. P. Zimmer, G. Mougi, M. G. Bawendi, D. G. Nocera, *J. Am. Chem. Soc.* **2006**, *128*, 13320 – 13321; b) S. W. Hong, C. Ahn, J. Huh, W. H. Jo, *Macromolecules* **2006**, *39*, 7694 – 7700.
[18] a) A. R. Clapp, I. L. Medintz, J. M. Mauro, B. R. Fisher, M. G. Bawendi, H. Mattoussi, *J. Am. Chem. Soc.* **2004**, *126*, 301 – 310; b) Q. H. Xu, S. Wang, D. Korystov, A. Mikhailovsky, G. C. Bazan, D. Moses, A. J. Heeger, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 530 – 535.

