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COMMUNICATION

Internal pH indicators for biomolecular NMR

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Abstract We describe a non-invasive technique for determining pH in biomolecular NMR sample using buffer components (formate, tris, piperazine, and imidazole) as internal pH indicators, whose ^{1}H NMR chemical shifts are sensitive to pH in a range from 2.5 to 9.8. This method is suitable for a wide range of applications where samples are handled intensively during NMR titrations or in high throughput analysis in structural genomics or metabolomics.

Keywords Internal indicator · Biomolecular NMR · pH

Abbreviations

NMR	Nuclear magnetic resonance
HSQC	Heteronuclear single quantum coherence

The vast majority of biological processes occur under a stringent control of pH, which should be reproduced during in vitro experiments. The most common method to report pH in vitro employs a pH sensitive glass electrode. This method, although well established and widely used, has significant disadvantages in solution state biological NMR experiments. First, positively charged proteins and peptides are frequently nonspecifically absorbed by a negatively charged glass surface of an electrode, which leads to the loss of a protein, contamination of the glass surface, and as a result to the distortion of pH calibration. Second, due to the moistening of the electrode surface, a fraction of protein solution is retained on the walls of an electrode, leading to an error in the determination of sample volume, which is especially significant when amplified during repeated pH measurements. Third, the measurement of pH with an electrode is an invasive technique, during which the impurities affecting equilibria and promoting sample degradation might be introduced. This is especially important for rare samples or for the samples particularly sensitive to denaturants and degradation. Fourth, in the case of high throughput analysis, pH measurements with an electrode amount to a substantial time cost.

The idea of monitoring pH using NMR chemical shifts of ^{19}F, ^{13}C, and ^{31}P has found numerous biological (DeFronzo and Gillies 1987; Taylor and Deutsch 1988; Pietri et al. 2000, 2001) and chemical applications (Vistad et al. 2003). Several compounds were proposed as pH indicators with their ^{1}H NMR chemical shifts sensitive to pH: hydroxylammonium chloride (Rabenstein and Fan 1986), which is a reducing agent that might react with protein solution; phosphoric acid (Szakacs et al. 2004), which has limited solubility in the presence of biologically relevant metals, precipitating as a salt; mixture of methylated imidazoles (Sorkin and Miller 1997), that covers mostly basic pH range; guanidinium chloride (Rabenstein and Fan 1986), which might promote highly undesirable protein unfolding; cacodylate (Valcour and Woodworth 1986), which is an arsenic compound and potentially toxic. While many compounds can be employed, common buffers are the most convenient to use as pH indicators. In this work, tris, formate, piperazine, and imidazole were employed as internal reporters of pH, since they demonstrate a dependence of ^{1}H NMR chemical shifts on pH and their 1D ^{1}H NMR spectra are relatively simple, showing

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one or two peaks, so that the resonances of a protein or a peptide of question would be minimally obstructed. These compounds are also biologically inert including the lack of interactions with metal ions such as Ca^{2+} and Mg^{2+}, which is important in case of Ca^{2+} binding proteins for example troponin C. We have previously developed an imidazole based buffer (T.C. Williams, unpublished data), sensitive to pH in a region of 5.6–8.6, with a pK_a of imidazole equal to 7.11 (100 mM KCl, 10 mM imidazole, 30°C). This buffer has proved to be useful in a large number of NMR studies of troponin C performed in our laboratory over a period of more than a decade (Sia et al. 1997; Li et al. 1999; Lindhout and Sykes 2003). In order to control pH over a wider range, in this work we used a combination of four pH reporters: tris, formate, piperazine, and imidazole.

This method requires a calibration that needs to be performed only once for a particular set of conditions, such as temperature, salt concentration, etc. Parameters pK_a, δ_{HA}, and δ_A for the buffers chosen do not change greatly over the typical range of conditions for biological NMR samples. For example, over the temperature of 25–40°C, the pK_a of piperazine changes by 0.3, the pK_a of tris changes by 0.2, the pK_a of formate changes by 0.2, and the pK_a of imidazole, the most stable among studied compounds, changes by 0.1. The changes in pK_a's with ionic strength ranging from ~20 to 100 mM were negligible within the errors of experiment; however, it is desirable to calibrate against a glass electrode for a particular set of conditions to achieve the best detection accuracy. Once the calibration has been performed, pH can be determined from the ^{1}H chemical shifts of buffer compounds.

The buffer containing 100 mM KCl, 2 mM tris, 2 mM formate, 2 mM piperazine, 2 mM imidazole, 0.2 mM DSS, and 5% D_2O was calibrated using a standard pH-sensitive glass electrode (purchased from Radiometer America Inc.) in a 30°C thermostat. Solutions of 1 M HCl or NaOH were added to adjust the pH of a buffer. 1D ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz spectrometer at 30°C using a standard 1D pulse sequence provided by BioPack. Calibrations were repeated three times with an example calibration shown in Fig. 1. ^{1}H chemical shifts for formate were found to range from 8.236 to 8.442 for protonated and deprotonated forms correspondingly (Fig. 1a, Table 1), and the value of pK_a was found to be 3.56 ± 0.05, as determined using xcrvfit program (http://www.bionmr.ualberta.ca/bds/software/index.html). ^{1}H chemical shift for the H2 proton of imidazole ranged from 8.672 to 7.765 for protonated and deprotonated forms (Fig. 1a, Table 1) with the pK_a of 7.08 ± 0.08. ^{1}H chemical shifts for tris ranged from 3.732 to 3.509 for protonated and deprotonated forms (Fig. 1b, Table 1) with the pK_a value of 8.23 ± 0.08. ^{1}H chemical shifts for piperazine ranged from 3.585 to 3.113 for protonated and deprotonated forms (Fig. 1b, Table 1) with the pK_{a_1} value of 5.61 ± 0.07.

The values of pK_a's and ^{1}H chemical shifts for protonated and deprotonated forms determined during calibration were used in a macro written for VnmrJ and available on-line (http://www.bionmr.ualberta.ca/mc/). The macro utilizes

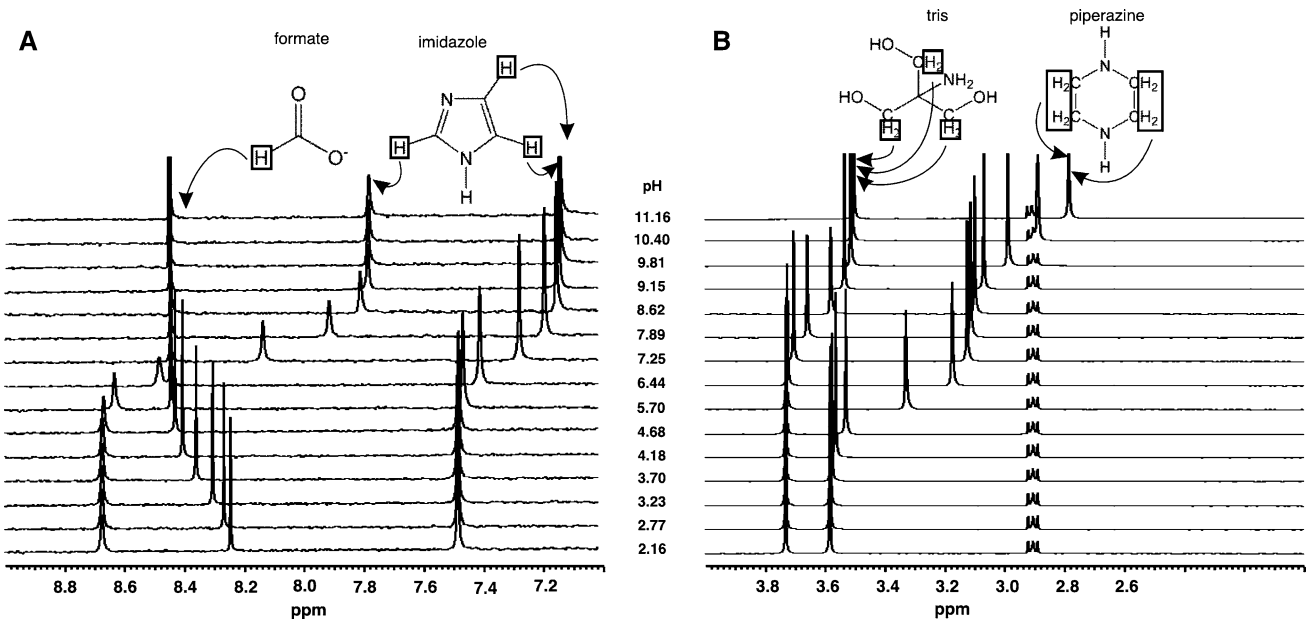

Fig. 1 1D 500 MHz ^{1}H NMR spectra of the buffer containing four internal pH indicators (formate (**a**), imidazole (**a**), tris (**b**), and piperazine (**b**)) as a function of pH. Protons that give rise to peaks in ^{1}H 1D NMR spectra are shown in boxes and connected to the corresponding resonances with arrows. The triplet on panel B corresponds to a CH_2 resonance arising from DSS that served as a reference compound

Table 1 pK_a values, ^1H chemical shifts for protonated and deprotonated forms of internal pH reporters and the limits of their applicability

	pK_a	σ_{HA}	σ_A	Applicability regions	
Formate	3.56 ± 0.05	8.236	8.442	2.5 (δ of 8.246)	4.7 (δ of 8.427)
Piperazine	5.61 ± 0.07	3.585	3.113	4.4 (δ of 3.542)	6.4 (δ of 3.175)
Imidazole	7.08 ± 0.08	8.672	7.765	5.8 (δ of 8.635)	8.6 (δ of 7.803)
Tris	8.23 ± 0.08	3.732	3.509	6.7 (δ of 3.725)	9.8 (δ of 3.516)

the following relationship: $pH = pK_a - lg(\delta_{obs} - \delta_{HA}/\delta_A - \delta_{obs})$, where δ_{HA} and δ_A, are the chemical shifts for protonated and deprotonated forms correspondingly, and δ_{obs} is the ^1H chemical shift observed for the solution with unknown pH. The most precise determination of pH is achieved when δ_{obs} lies within the linear portion of chemical shift dependence on pH (Fig. 1). The limits within which pH indicators provide the most reliable information were truncated for formate to pH of 2.5 (δ of 8.246) and 4.7 (δ of 8.427), for piperazine to pH 4.4 (δ of 3.542) and 6.4 (δ of 3.175), for imidazole to pH 5.8 (δ of 8.635) and 8.6 (δ of 7.803), and for tris to pH 6.7 (δ of 3.725) and 9.8 (δ of 3.516) (Table 1). Thus, the overall pH range covered by these four compounds spans from 2.5 to 9.8, which can be easily extended if needed by adding another buffer component with an appropriate pK_a value. Within these limits, the pH of solution can be determined with the error of ±0.05 provided that the spectrum was correctly referenced to DSS and the error of a standard glass electrode used during calibration was of the same order or less.

It might seem that the measuring of pH using NMR chemical shifts vs. glass electrode is a more time consuming during the initial preparation of the sample due to the necessity for shimming and re-equilibrating each time after adjusting pH. However, the use of a glass electrode also requires that the sample and buffer are equilibrated at all temperatures, and that pH meter calibration curves are done at all temperatures, which is tedious and often not done properly. Once the sample is prepared, the NMR method is far easier to quickly measure the pH within the sample due to any changes that might occur with time, reaction, or degradation, for example.

The method described here utilized the pH dependence of ^1H chemical shifts of buffer components such as formate, tris, imidazole, and piperazine to determine pH of the NMR sample. This method is devoid of many disadvantages of a glass electrode. It affords a better reproducibility of results, consistency among users, availability for after the fact validation, and although known before, it deserves a wider application. It can be conveniently used for measuring pH in kinetic experiments or when monitoring long-term stability of a sample. It is indispensable when proteins are in shigemi style tubes or when pH is measured against temperature. Most importantly this method is highly beneficial during NMR titrations when samples are intensively handled or during high throughput analysis in proteomics, metabolomics, and structural genomics.

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