A new class of ratiometric small molecule intracellular pH sensors for Raman microscopy†

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Intracellular pH (pHi) homeostasis is intertwined with a myriad of normal cellular behaviors as well as pathological processes. As such, small molecule probes for the measurement of pHi are invaluable tools for chemical biology, facilitating the study of the role of pHi in cellular function and disease. The field of small molecule pHi sensors has traditionally been dominated with probes based on fluorescent scaffolds. In this study, a series of low molecular weight (<260) oligoyne compounds have been developed which exhibit pH sensitive alkyne stretching frequencies (ν_{alkyne}) in Raman spectroscopy. The modular design of the compounds enabled tuneability of their pK_a(H) through simple structural modification, such that continuous pH sensitivity is achieved over the range 2–10. Alkyne stretching bands reside in the ‘cell-silent’ region of the Raman spectrum (1800–2600 cm⁻¹) and are readily detectable in a cellular environment with subcellular spatial resolution. This enabled the application of a pH sensitive oligoyne compound to the ratiometric sensing of pHi, in prostate cancer (PC3) cells in response to drug treatment. We propose that probes based on Alkyne Tag Raman Imaging offer an entirely new platform for the sensing of pHi, complementary to fluorescence microscopy.

Introduction

pH homeostasis is essential for normal physiology. Regulation of both intracellular (pHi) and extracellular (pHe) pH plays a key role in many processes including cell proliferation and differentiation, protein synthesis, the acetylation and deacetylation of histones and metabolism. Given the importance of pH in many critical functions it is unsurprising that many diseases are associated with aberrant pH profiles. For example, pHi and pHe dysregulation have been linked to Alzheimer’s disease and cancer. The development of methods to determine the role of pH homeostasis in cellular processes and its relationship to disease is therefore of great interest.

pHi can be determined using fluorescent probes, the most commonly used being BCECF (2′,7′-bis-(2-carboxyethyl)-5′-(and-)carboxyfluorescein) and its derivatives. BCECF has been used extensively in mammalian and plant cells and living tissues, providing accurate pH measurement and excellent sensitivity at concentrations as low as 0.1 μM. However, there are limitations of BCECF and related fluorescent sensors such as photostability, phototoxicity and probe retention. These deficiencies limit the use of many probes, particularly in extended real time monitoring of live cells. Whilst efforts have been made to address these problems, significant challenges remain. In addition, diffuse absorption and emission spectra hinder the analysis of multiple fluorescent probes simultaneously (multiplexing), since spectral overlap limits the number of resolvable features within the spectra (especially where ratiometric sensors are used).

Intracellular pH sensing has also been achieved by Surface Enhanced Raman Scattering (SERS) using functionalised metal nanoparticles. These nanoparticles bear a surface coating of organic molecules with pH-dependent Raman spectra, which are detectable inside cells with excellent sensitivity, owing to the SERS effect. Nanoparticle sensors, however, can suffer from reproducibility and accuracy issues arising from nanoparticle inhomogeneity, and the possible

†Electronic supplementary information (ESI) available: Analytical data, experimental procedures and 1H, 13C and 19F NMR spectra for all compounds reported. Raman spectra of all compounds reported, pK_a(H) determination plots, and additional Raman data pertaining to pH measurement in PC3 cells. See DOI: 10.1039/d0an00865f
off target effects and toxicity of nanoparticles within cells remain unclear.\textsuperscript{27} We propose that small-molecule Raman pH sensors have the potential to address these issues.

An emerging technique, which is complementary to fluorescence microscopy, is Alkyne Tag Raman Imaging (ATRI).\textsuperscript{28,29} Alkynes have key vibrational modes that occur in the ‘cell-silent’ region of the Raman spectrum (1800–2600 cm\(^{-1}\)), in which few vibrational modes from endogenous biomolecules exist.\textsuperscript{29} Alkyne bands can be detected in a cellular environment using spontaneous Raman microscopy or nonlinear variants such as Stimulated Raman Scattering (SRS).\textsuperscript{30} The first example of ATRI in cells was the use of ethynyldeoxyuridine (EdU, Fig. 1A) which was metabolically incorporated into DNA for its visualization in cells.\textsuperscript{28} Since then, functionalisation of small biomolecules with alkyne ‘tags’ has enabled visualization of the intracellular distribution of drugs,\textsuperscript{31} natural products,\textsuperscript{32,33} sugars\textsuperscript{34,35} and lipids\textsuperscript{36–38} using Raman and SRS microscopy. Alkyne-tags have also been metabolically incorporated into macromolecules such as RNA and proteins for single-step imaging by SRS.\textsuperscript{39}

An application of ATRI that remains unexplored is the development of sensors and probes for the quantification of endogenous biomolecules and ions. A pioneering report by Min and co-workers demonstrated the suitability of chemically reactive oligoyne scaffolds in the development of a ratiometric sensor for H\(_2\)S (\textsuperscript{2}, Fig. 1B).\textsuperscript{20} This sensor contained an azide group in conjugation with a bisarylbutadiyne scaffold which was reduced to the corresponding aniline in the presence of H\(_2\)S. This chemical transformation induced a Raman shift change in the alkyne band, enabling the detection of intracellular H\(_2\)S by SRS microscopy. Whilst this clearly demonstrates that Raman microscopy can be used to develop intracellular sensors, it relies on an irreversible chemical process rendering the detection semi-quantitative. The development of quantitative probes for use in a cellular environment would require any change in the shift of a Raman band to be bought about through a rapid and reversible chemical transformation, such as protonation/deprotonation. Sodeoka and Fujita reported the simultaneous imaging of the protonated and deprotonated forms of carboxyfuchsin p-trifluoromethoxyphenylhydrazone (FCCP) in HeLa cells, using a pH sensitive C=\textsuperscript{N} stretch, suggesting pH did represent a potential strategy for quantitative sensing.\textsuperscript{40} Within this work we report bisarylbutadiynes sensitive to environmental pH, and their application to ratiometric pH determination by Raman microscopy (3, Fig. 1C). These molecules rely on reversible protonation/deprotonation events to induce a Raman shift change, enabling quantitative measurement of pH. These small molecule ratiometric pH sensors that are low molecular weight (MW <260), modular, readily tuneable, and possess good photostability,\textsuperscript{21} represent a novel platform for pH sensing at the cellular level. Using Raman microscopy as the method for detection provides additional experimental data including a direct measure of distribution for endogenous biomolecules (proteins, lipids and nucleic acids).

**Results and discussion**

**Electron delocalization influences alkyne stretching frequency**

The design of our pH-sensitive probes was based around the fact that 4-ethynylaniline 4 (Fig. 2) has a lone pair of electrons on the aniline nitrogen that is in conjugation with the alkyne. Protonation of the aniline to give 4H\(^+\) would result in the nitrogen and the alkyne no longer being conjugated. We reasoned this could manifest itself in a detectable change in the Raman...
shift observed for the alkyne group. In support of this we performed DFT calculations on 4 and 4H⁺ which clearly showed that 4 had a significant contribution of the aniline lone pair to the calculated HOMO of the molecule whereas the aniline nitrogen made no contribution to the calculated HOMO of 4H⁺. Additionally, 4 displayed an unsymmetrical distribution of the HOMO across the alkyne triple bond, while distribution was more symmetrical in 4H⁺. The calculations suggested that the difference in the Raman stretching frequency of the two species 4 and 4H⁺ was ∆νalkyne = 21 cm⁻¹. We hypothesised that the calculated sensitivity of the Raman spectrum of 4 to protonation could be exploited to develop alkyne-based probes for the determination of environmental pH.

**Development of pH sensitive diynes**

A bisarylbutadiyne scaffold was selected for our compounds, as it has been shown that the increased hyperpolarizability associated with the alkyne stretching vibrations of this functionality results in high Raman-scattering intensity, giving rise to lowered detection limits in solution. We synthesised aniline 5 (Fig. 3A) and aimed to assess the sensitivity of its Raman spectrum to pH. Due to the experimental complications associated with thermodynamic pKₐ(H) determination in water for hydrophobic molecules, we generated pKₐ(H) estimates for all compounds prepared in mixtures of 30% w/w EtOH/H₂O under conditions of constant ionic strength (I = 0.2) and temperature (20 °C). The buffer solutions used (diyne analysis buffers), and their preparation, are described in the Experimental section. The experimental pKₐ(H) derived for 5, and all other compounds, is valid only under the conditions of solvent, ionic strength and temperature studied. Fig. 3B shows overlays of the alkyne region of the Raman spectra of 5 at pH 1.1, 2.7 and 4.6. At pH 4.6, the unprotonated microspecies 5 dominates, and the observed νalkyne is at a minimum (2212.8 cm⁻¹) due to π-electron donation from the aniline lone pair.

Conversely, at pH 1.1, the protonated microspecies 5H⁺ dominates and the observed νalkyne is near maximum (2221.0 cm⁻¹) due to diminished π-electron donation. At pH 2.7, near the experimental pKₐ(H), the spectrum observed is a combination of contributions from both microspecies, and the apparent νalkyne is intermediate in value (2216.4 cm⁻¹). Fig. 3C shows a plot of the apparent νalkyne of compound 5 across a range of indicated pH values. Asymptotes of the sigmoidal curve provided the maximum and minimum alkyne stretching frequency values (νmax = 2221.0 and νmin = 2212.8 cm⁻¹) corresponding to the peak centres of the protonated 5H⁺ and parent microspecies 5, respectively. The difference between these two values is designated ∆νalkyne (8.2 cm⁻¹). In order to determine pKₐH we assumed the observed alkyne peak at any given pH value was a linear additive combination of the peaks arising from 5 and 5H⁺. As shown in Fig. 3D, the combined fits effectively described the observed peaks at different pH values. The peak areas of 5 (A₅) and 5H⁺ (A₅H⁺) were determined, correct for differing intrinsic peak intensities, allowing the mole fraction of conjugate base (χbase) to be determined as a function of pH (Fig. 5E).

Fitting the data to a Boltzmann function enabled the estimation of pKₐH from the inflection point of the sigmoid.
(Fig. 3F), giving a value of $2.72 \pm 0.02$. This $pK_a$ measurement of 5 was validated using UV-Visible spectroscopy under the same conditions of ionic strength and temperature (Fig. S3†), which indicated a $pK_a$ of 2.66, consistent within 0.06 units of the value obtained by Raman, providing strong confidence in the methods used.

To test the hypothesis that the pH sensitivity of $\nu_{\text{alkyne}}$ exhibited by 5 could be attributed to aniline protonation, we synthesised the corresponding acetamide 6 (Fig. 3G). Pleasingly, 6 showed no change of the $\nu_{\text{alkyne}}$ over the pH range 1.1–5.0 (Fig. 3H). In line with our hypothesis we attribute this to the absence of a basic nitrogen lone pair.

Encouraged by the positive results obtained with aniline 5, we synthesised a series of diyne compounds 7–18 which exhibited pH sensitivity in different ranges (Fig. 4). Compounds were designed to incorporate aromatic substituents in conjugation with the diyne moiety which could be expected to influence $\pi$-electron density within the molecule. In addition, substituents were selected which had an expected $pK_a(H)$ 2–10, with the rationale that protonation/deprotonation events in solution would influence $\pi$-electron density in the alkyne chain, and in turn the observed $\nu_{\text{alkyne}}$. Plots of $\nu_{\text{alkyne}}$ variance as a function of pH were constructed for compounds 7–18 (Fig. S4†). As predicted, compounds 5 and 7–18 showed sigmoidal relationships between $\nu_{\text{alkyne}}$ and environmental pH, and $pK_a(H)$ values could be determined using the multiple curve fitting method discussed previously. Plots of $\chi_{\text{Base}}$ as a function of pH can be found in Fig. S5†.

Fig. 4 details the apparent $pK_a(H)$, $\nu_{\text{max}}$, $\nu_{\text{min}}$ and $\Delta \nu_{\text{alkyne}}$ values for compounds 5 and 7–18. As discussed above, aniline 5 showed a $pK_a$ of 2.7 and a decrease in $\nu_{\text{alkyne}}$ with increasing pH, consistent with an increasing proportion of the microspecies 5 (decreasing $5H^+$). Aniline 7 showed a similar trend. Notably, 7 exhibited an increased $pK_a(H)$ (3.2) and diminished $\Delta \nu_{\text{alkyne}}$ (4.6, compared to 8.2 for 5), likely arising from reduced conjugation due to the meta-substitution pattern.

Unlike most compounds studied, pyridine derivative 8 ($pK_a(H) = 3.3$) showed an increase of $\nu_{\text{alkyne}}$ with increasing pH.
We reason that, given the π-deficient electron withdrawing nature of the pyridine ring, protonation of the nitrogen atom induced increased conjugation with the alkynes, which resulted in a decreased stretching frequency for the microspecies $8H^+$. Imidazoles 9 and 10 showed the opposite trend to the pyridine species 8, as the protonated microspecies $9H^+$ and $10H^+$ exhibited increased $\nu_{\text{alkyne}}$ upon protonation. We reason that, as imidazoles are π-excessive and electron donating, protonation induced decreased conjugation with the alkyn. We were unable to accurately determine a $pK_a$ value for imidazole 9 as multiple curve fitting reported inconsistent values for $A_{\text{Base}}$ and $A_{\text{Acid}}$, likely due to the small $\Delta\nu_{\text{alkyne}}$ of 2.8.

Triflamide 11 ($pK_a = 4.1$) displayed a decrease in $\nu_{\text{alkyne}}$ on increasing pH, as the deprotonated analogue of 11 is expected to have increased conjugation with the alkyn chain. Aminopyridine 12 exhibited a pH dependence in line with that of pyridine 8, displaying a predictably higher $pK_a$ (5.0). Phenols 13–16 exhibited a decrease in $\nu_{\text{alkyne}}$ upon increasing pH consistent with an increased π-electron delocalization upon deprotonation to the phenolate. It is noteworthy that the observed $pK_a$ values of these compounds were readily tuneable by incorporation of inductively withdrawing fluorine atoms in proximity to the phenolic oxygen allowing variance in the observed $pK_a$ of >3 pH units (cf. compounds 13 and 16).

It also proved possible to include two pH sensitive groups within the molecule providing two $pK_a(H)$ values for a single compound. For example, the bifunctional molecule 17, which contains an aniline and a phenol group on different aromatic rings displayed two discrete inflection points, the first at the $pK_a$ of the aniline (2.8) and the second at the $pK_a$ of the phenol (9.6). The related compound 18, where the two functional groups are on the same aromatic ring, also showed inflection points for the aniline ($pK_a = 2.7$) and the phenol ($pK_a = 9.7$). With the ability to finely tune $pK_a(H)$ values by the introduction of either electron donating or electron withdrawing groups on the aromatic ring[s], bespoke pH probes can be designed to provide sensitivity over multiple pH ranges.

The modular design of compounds 5 and 7–18 enabled rational tuning of their $pK_a(H)$ by substitution of the pH responsive group. Between the 14 diyne compounds prepared, there are compounds capable of pH-sensitivity for any pH value between 2–10 and this work provides the foundation for the design of oligoyne pH sensors for Raman microscopy across the physiological pH range. We have shown that detectable changes in $\nu_{\text{alkyne}}$ (3–18 cm$^{-1}$) can be induced by protonation/deprotonation events. Other researchers have shown$^{22,24,37}$ that larger changes in the base $\nu_{\text{alkyne}}$ values of oligoyynes can be engineered by isotopic labelling of alkynes (>20 cm$^{-1}$), and increasing the number of alkyn groups present in the molecule (>40 cm$^{-1}$). It stands to reason, therefore, that the use of pH sensitive oligoyynes with different alkyn chain lengths could enable visualization of multiple species simultaneously, with complete resolution, to enable multiplexing of multiple pH sensors, designed to report on discrete microenvironments.

To illustrate the potential for multiplexing, we captured spectra of phenol 16 and commercially available dimethyl-aniline derivative 19 (Fig. 5A) in a single solution at different pH values (Fig. 5B). The Raman peaks corresponding to the alkyn group(s) of both compounds are clearly resolved at pH 12.1 (blue line), pH 6.3 (green line) and pH 1.1 (red line), enabling visualization of pH sensitivity for both compounds. The spectra indicate that at high pH (12.1) a different microspecies of compound 16 is present in solution when compared to the spectra obtained at lower pH (6.3 and 1.1). They also indicate that at low pH (1.1) dimethyl-aniline derivative 19 is present as a different microspecies to the spectra recorded at higher pH (6.3 and 12.1). These experiments show that through judicious design multiplexing of pH sensitive Raman probes should be possible.

**Intracellular pH sensing**

For the purposes of developing an intracellular pH sensor, compounds with $pK_a(H)$ values close to physiological pH ranges were desirable.$^4$ The apparent $pK_a(H)$ values reported in Fig. 4 served as a guide for assessing suitability of the diyne compounds for intracellular pH sensing. Based on the data accrued, phenol 13 appeared to be a promising candidate for use as a cytosolic pH sensor due to its apparent $pK_a$ of 6.2 (Fig. 6A). Prior to investigation within a cellular environment, the photostability of 13 in solution was established by acquiring 120 consecutive spectra (acquisition time = 0.5 s) followed by integration of the alkyn peak using OriginPro 2018 (Fig. S6†). In addition, 13 was evaluated for any potential cellular toxic effect. Exposure of PC3 cells to 25 μM 13 for 6 h showed no change in cell count when compared to a DMSO control (Fig. S7†).

To quantitatively determine pH, 13 required calibration within the environment of interest. For the purposes of cellular pH sensing, the method of Thomas$^{42}$ was used to calibrate our selected compound. Nigericin is an H$^+/K^+$ ion transporter which is commonly used to calibrate fluorescent pH probes.$^{18,43,44}$ Cells were treated with 13 (25 μM, 30 min), and nigericin (25 μM) was used to equilibrate pH, with pH$_c$ in high
K⁺ buffer solutions at pH values of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The cells were then imaged using a Raman microscope. Under these conditions $\nu_{\text{max}}$ and $\nu_{\text{min}}$ were determined to be 2221 cm⁻¹ and 2210 cm⁻¹ respectively. Fig. 6B shows sample spectra from PC3 cells treated with 13 (25 μM, 30 min) and fixed to the pH values of 5.5 (orange) and 7.5 (blue), illustrating the change in spectroscopic properties of 13 in response to pH. Fig. 6C shows a plot of average 2221/2210 cm⁻¹ signal intensity ratios derived from Raman maps of PC3 cells across the pH range measured ($n = 5$ cells per condition, 5 μm step size in $x$ and $y$, ±standard deviation). The data were fitted to a Boltzmann function to generate a calibration curve, enabling us to relate the 2221/2210 cm⁻¹ ratio of 13 to pH. Fig. 6D and E show false colour images of the 2221/2210 cm⁻¹ ratio in PC3 cells fixed to pH 7.5 and 5.5 respectively. The Raman maps from which these derive were acquired at a resolution of 1 μm in $x$ and $y$. These false colour images, and all others reported in this manuscript and ESI† were generated using a custom MATLAB® script reported in previous publications. As with fluorescent sensors, a minimum intensity cut-off should be established prior to ratiometric analysis, because ratios vary significantly as signal intensity decreases toward the threshold of detection. We established that the signal intensity of 13 was lowest within cell nuclei (Fig. S9†), due to minimal nuclear penetration of the probe compounds. This distribution pattern is consistent with analogous oligoyne molecules. As such, although the 2221/2210 cm⁻¹ ratio appears to be consistent throughout the cells in Fig. 6D and E, we chose to exclude nuclear regions from interpretation and these are highlighted by black bands.

It is clear, however, from Fig. 6D and E that the 2221/2210 cm⁻¹ ratio is consistent throughout the cytoplasm of the PC3 cells and varies as a function of pH. Having calibrated 13 within a cellular environment, we aimed to determine whether this probe could be used to monitor and quantify changes in pH in response to drug treatment. It has been reported that apoptosis is accompanied by a decrease in pH. Treatment of 6 plates of PC3 cells with etoposide (80 μg mL⁻¹, 30 min) induced apoptosis. Following incubation periods of 0.5, 1.5, 2.5, 3.5, 4.5 or 5.5 h, 13 (25 μM) was added and the cells were imaged after a further 30 minutes ($n = 6$ cells per time point, 532 nm, 0.5 s, 5 μm step size in $x$ and $y$). From these data the 2221/2210 cm⁻¹ ratio was extracted from average spectra at each time point and related to pH using the calibration curve shown in Fig. 6C. Fig. 7A shows a plot of pH over time. The average 2221/2210 cm⁻¹ ratio at 0 h (no etoposide treatment) of 0.843 ± 0.027, corresponds to a pH of 7.34 ± 0.05. The pH steadily decreased over time, reaching a minimum at 4 h of 7.13 ± 0.08 (***p < 0.01), before showing signs of recovery. The change in pH and cellular recovery is consistent with observations with HL-60 cells. This experiment was repeated, with consistent results (Fig. S10†). For visual clarification, high resolution maps (1 μm step size in $x$ and $y$) were acquired at the 0 and 4 h time points. Fig. 7B and C show false colour representations of the 2221/2210 cm⁻¹ ratio in PC3 cells at 0 h and 4 h respectively. As before (cf. Fig. 6), low alkyne signal was observed in the nuclear regions of these cells (Fig. S11†), and so pH data in these regions (highlighted by black bands) was excluded from interpretation. It is clear that the cell at 4 h post etoposide treatment (Fig. 7C) has a higher 2221/2210 cm⁻¹ ratio and thus lower pH than the cell at 0 h (Fig. 7B), in agreement with the data in Fig. 7A, showing that 13 can be used to effectively monitor and quantify changes in pH of live cells in response to drug treatment.

A distinct advantage of Raman spectroscopy over other cellular imaging techniques is the rich information provided on compartmentalisation, the location of organelles and the distribution of endogenous biomolecules such as lipids and proteins. This is determined in a label free manner, therefore, each piece of experimental data gathered within this investigation provides considerable further knowledge, as demonstrated by identification of nuclear regions through lipid to protein ratio analysis (Fig. S9 and S11†). This data could not be obtained using spontaneous Raman microscopy in conjunc-
tion with a fluorescent probe, due to photobleaching, and the obscuring of Raman data by the fluorescence response.

To demonstrate the compatibility of 13 with advanced Raman imaging techniques, live PC3 cells were treated with 13 (25 µM, 30 min) and imaged by SRS microscopy for improved spatial resolution. Fig. 7D shows false colour images of 2933 cm⁻¹ (CH₃, proteins), 2851 cm⁻¹ (CH₂, lipids), 2221 cm⁻¹ (alkyne, 13), and 2321 cm⁻¹ (off-resonance). Images acquired at 512 × 512 pixels, 10 µm pixel dwell time with false colours applied to different detection wavenumbers.

Experimental

DFT calculations

DFT calculations were performed using the Gaussian09 suite of programs. Structures were optimised and then characterised using frequency calculations at the B3LYP/6-311(G)++(d,p) level of theory.

Buffer preparation

Dyne analysis buffers. A series of Britton–Robinson buffers were prepared in 30 wt%. EtOH to a constant ionic strength (I) of 0.2 by addition of KCl, according to the method of Mongay and Cedra. The pH values of the prepared solutions were measured using a Jenway Model 2510 pH meter, and adjusted for the solvent effect according to correction values reported by Gelsema et al. Final, corrected pH values for buffer solutions were: 1.99, 2.32, 2.69, 3.07, 3.41, 3.82, 4.57, 4.95, 5.34, 5.77, 6.27, 6.85, 7.51, 8.17, 8.74, 9.35, 9.94, 10.45, 10.90, 11.50 and 12.05. In order to access the pH range 1–2, HCl/KCl buffers were prepared from 3:1 and 1:1 mixtures of 0.2 M HCl and 0.2 M KCl in 30 wt%. EtOH, to give corrected pH values of 1.05 and 1.45 respectively.

High K⁺ buffers for nigericin calibration. Aqueous buffers containing 135 mM KCl, 2 mM K₂HPO₄, 20 mM HEPES, 1.2 mM CaCl₂, and 0.8 mM MgSO₄ were prepared, and adjusted to the pH values of 5.50, 6.00, 6.50, 7.00, 7.50, 8.00 and 8.50 by the addition of 1 M HCl or NaOH.

Cell culture. PC3 cells (human prostate adenocarcinoma) were gifted from the Strathclyde Institute of Pharmacy and Biomedical Sciences (Glasgow) as a subculture from a stock received from the European Collection of Authenticated Cell Cultures (ECACC).

pH sensor calibration. PC3 cells were plated on 35 mm glass-bottomed culture dishes (Ibidi) in DMEM at a concentration of 5 × 10⁵ cells per mL and incubated at 37 °C and 5% CO₂ for 24 h prior to treatment. Cells were treated with 25 µM etoposide from a 10 mM stock solution in DMSO and incubated for 30 min at 37 °C and 5% CO₂. Dishes were then aspirated and washed twice with high K⁺ buffer solution at the required pH. High K⁺ buffer solution containing 25 µM nigericin at the required pH was then added to the plates, which were incubated at 37 °C and 5% CO₂ for 5 min prior to imaging.

pH measurement. PC3 cells were plated on 35 mm glass-bottomed culture dishes (Ibidi) in DMEM at a concentration of 5 × 10⁵ cells per mL and incubated at 37 °C and 5% CO₂ for 24 h prior to treatment. Cells were treated with 25 µM etoposide from a 10 mM stock solution in DMSO and incubated for 30 min at 37 °C and 5% CO₂. Dishes were then aspirated and washed twice with PBS, before refilling with PBS prior to imaging.

For etoposide treatment experiments, cells in 6 separate dishes were treated with 80 µg mL⁻¹ etoposide from a 16 mg mL⁻¹ stock solution in DMSO for and incubated for 30 min at 37 °C and 5% CO₂. Dishes were aspirated and washed twice with PBS and refilled with DMEM. Each dish was incubated for a further time of 0.5, 1.5, 2.5, 3.5, 4.5 or 5.5 h at 37 °C and 5% CO₂. Incubation periods were interrupted 30 min prior to
their end point and cells were treated with 25 μM 13, then returned to the incubator for 30 min. Then they were then aspirated and washed twice with PBS, before refilling with PBS prior to imaging.

**SRS microscopy.** PC3 cells were plated on high precision glass cover slips (#1.5H Thickness, 22 × 22 mm, Thorlabs) in a 6-well plate in DMEM at a concentration of 5 × 10^5 cells per mL and incubated at 37 °C and 5% CO2 for 24 h prior to treatment. Cells were treated with 25 μM 13 from a 10 mM stock solution in DMSO and incubated for 30 min at 37 °C and 5% CO2. Plates were then aspirated and washed twice with PBS. Cover slips were then affixed to glass microscope slides with a PBS boundary between the glass layers prior to imaging.

**Raman spectroscopy**

All Raman spectra were acquired on a Renishaw inVia Raman microscope equipped with a 532 nm Nd:YAG laser giving a maximum power of 500 mW, 1800 lines per mm grating. Prior to spectral acquisitions, the instrument was calibrated using the internal silicon standard at 520 cm⁻¹.

**Neat compounds.** A small amount of sample compounds 5–19 were transferred onto a CaF2 window and spectra were acquired using an Olympus 20×/NA 0.40 NPlanEPI objective, an acquisition time of 1 s, a laser power of 1% (ca. 0.2 mW) and 10 accumulations with a spectral centre of 1600 cm⁻¹. 3 measurements were taken for each sample.

**Experimental pHₐ(H) determinations.** Wells of a 96 well plate were charged with a stock solution of alkyne compound 5–19 (0.7 μL, 50 mM, EtOH) and diluted to 350 μL using dye analysis buffers at the desired pH values. The resulting final concentrations of 5–19 were 100 μM (unless otherwise stated). Samples were equilibrated to a room temperature of 20 °C prior to spectral acquisition. Spectra were acquired of each solution using a 20×/NA 0.40 NPlanEPI objective, a laser power of 100% (ca. 20 mW), acquisition time of 1 s, with 20 accumulations and a spectral centre of 1800 cm⁻¹. 3 measurements were taken per condition.

**pH sensor calibration cell maps.** PC3 cells in glass bottomed culture dishes (Ibidi), prepared as described previously were mapped using a Nikon 60×/NA 1.0 NIR Apo water immersion objective (HC PL IRAPO 40×, N.A. 1.10 water immersion lens) with a 5 μm step size in x and y, 0.5 s acquisition time, 100% laser power (ca. 17 mW) and a spectral centre of 2500 cm⁻¹. 5 maps of different cells were acquired from a single culture plate for each condition. Note: to ensure reproducibility, the spectrometer was calibrated regularly (every 30 min) using the internal silicon standard during these experiments.

**pH measurement cell maps.** PC3 cells in glass bottomed culture dishes (Ibidi), prepared as described previously were mapped using a Nikon NIR Apo 60× water immersion objective, with a 5 μm step size in x and y, 0.5 s acquisition time, 100% laser power (ca. 17 mW) and a spectral centre of 2500 cm⁻¹. 6 maps of different cells were acquired from a single culture dish per condition. For the high-resolution images presented in the Fig. 6 and 7, additional maps were generated using a 1 μm step size in x and y. Note: to ensure reproducibility, the spectrometer was calibrated regularly (every 30 min) using the internal silicon standard during these experiments.

**Stimulated Raman scattering microscopy**

An integrated laser system (picoEMERALD™ S, Applied Physics & Electronics, Inc.) was used to produce two synchronised laser beams at 80 MHz repetition rate. A fundamental Stokes beam (1031.4 nm, 2 ps pulse width) was intensity modulated by an electro-optic-modulator with >90% modulation depth, and a tunable pump beam (700–960 nm, 2 ps pulse width, <1 nm (10 cm⁻¹) spectral bandwidth) was produced by a built-in optical parametric oscillator. The pump and Stokes beams were spatially and temporally overlapped using two dichroic mirrors and a delay stage inside the laser system and coupled into an inverted laser-scanning microscope (Leica TCS SP8, Leica Microsystems) with optimised near-IR throughput. SRS images were acquired using 40× objective (HC PL IRAPO 40×, N.A. 1.10 water immersion lens) with a 10 μs pixel dwell time over a 512 × 512 frame. The Stokes beam was modulated with a 20 MHz EoM (Zurich Instruments). Forward scattered light was collected by a S1 N.A. 1.4 condenser lens (Leica Microsystems). Images were acquired at 8-bit image depth. The laser powers measured after the objective lens were in the range 10–30 mW for the pump beam only, 10–50 mW for the Stokes beam only and 20–70 mW (pump and Stokes beams).

**UV-Visible spectroscopy**

Solutions of 5 and 11 were prepared using by diluting an aliquot from a stock solution (2 μL, 12.5 mM, EtOH) to a volume of 1 mL using dye analysis buffer at the desired pH. The resulting final concentration of 5 or 11 was 25 μM. Samples were equilibrated to a room temperature of 20 °C prior to spectral acquisition. Spectra were recorded using an Agilent Cary 60 UV-Visible spectrometer between 250 and 800 nm at a resolution of 0.5 nm.

**Data processing**

**Single spectra.** All spectra were processed using WiRE 4.4™. Spectral baselines were subtracted, and smoothing was achieved using Savitzky–Golay filtering with a polynomial order of 3 and a frame length of 9. Peak centres and intensity values were ascertained using the Lorentzian function built into the curve-fit tool.

**Experimental pHₐ(H) determinations by Raman spectroscopy.** Alkyne stretching frequency values of 5 and 7–18 as a function of pH were plotted in OriginPro2018 and fitted to a Boltzmann function. From the output, Vₘₐₓ and Vₖₐᵣₙ values were determined from asymptotes. Spectra of 5, and 7–18 at different pH values were subjected to curve fitting analysis using the peak analysis tool in OriginPro 2018. For each spectrum, analysis was performed on the 2100–2300 cm⁻¹ region, and spectral baselines were established by interpolation over 20 points. 2 peaks were fitted using Gaussian functions, at fixed peak centres defined by the Vₘₐₓ and Vₖₐᵣₙ values of the
compound. Bounds were set such that the area of each peak must be ≥0, and all other curve parameters were left variable. The resulting peak areas arising from the conjugate acid (\(A_{\text{Acid}}\)) and base (\(A_{\text{Base}}\)) microspecies were recorded for each spectrum. The parameters \(A_{\text{HighpH}}\) and \(A_{\text{LowpH}}\) were derived from average total peak areas \((n = 3)\) at extreme pH values for each compound. Average \(x_{\text{Base}}\) values \((n = 3)\) were then plotted as a function of pH for each compound according to eqn (1), where \(c\) is \(A_{\text{HighpH}}/A_{\text{LowpH}}\).

\[
x_{\text{Base}} = \frac{A_{\text{Base}}}{A_{\text{Base}} + c(A_{\text{Acid}})}
\]

These data were fitted to Boltzmann functions in OriginPro 2018, and \(pK_a(H)\) values were derived from inflection points.

**Experimental \(pK_a(H)\) determinations by UV-Visible spectroscopy.** Data were exported as an Excel file and transferred to OriginPro 2018, within which intensity values were extracted, and the 340/306 nm ratio (5) or 340/293 nm ratio (11) were recorded. Data were then plotted according to eqn (2).

\[
pH = pK_a + c \left[ \log \frac{R - R_{\min}}{R_{\max} - R} \right] + \log \frac{I_a}{I_b}
\]

\(R\) is the ratio of emission intensity at two wavelengths. \(R_{\max}\) and \(R_{\min}\) are maximum and minimum limiting values of \(R\) (determined from the asymptotes of plots of \(R\) vs. \(pH\)), and \(c\) is the slope. \(I_a/I_b\) is the ratio of the absorption intensity in acid to the absorption intensity in base at the wavelength chosen for the denominator of \(R\). \(pK_a(H)\) was determined from the y intercept minus \(\log(1/I_b)\).

**Cell maps.** Cosmic rays were removed using a nearest neighbour algorithm and noise filtering was carried out prior to other processing. To determine average pH values in cells, average spectra were computed from map data, from which intensities at 2210 and 2221 cm\(^{-1}\) were extracted. The intensity ratio maps shown in Fig. 6, 7, S6 and S7† were generated using a custom MATLAB® script reported in previous publications.38,45

**SRS images.** Images were assigned false colours using ImageJ, and scale bars were applied. Brightness settings were unadjusted for the 2933 cm\(^{-1}\) and 2851 cm\(^{-1}\) images, while the 2221 cm\(^{-1}\) and 2321 cm\(^{-1}\) images were scaled between 0 and 125 units.

**Conclusions**

In summary, we have reported the development of a bespoke suite of low molecular weight diyne compounds which exhibit pH sensitive Raman spectra. Using a modular design strategy, the \(pK_a(H)\) values of the series were tuned to achieve sensitivity across a physiologically-relevant pH range (2–10) in order to detect and monitor changes in pH. Following calibration, we demonstrated that compound 13 could be used to ratiometrically determine pH\(_i\) in PC3 cells and, significantly, to monitor change in pH\(_i\) in response to a homeostatic disruptor, in this case exemplified by administration of the chemotherapeutic agent etoposide. The probe molecule 13 proved to be photo-stable, non-toxic and cell penetrant, providing the first example of an environmentally sensitive small molecule to enable accurate pH determination by Raman microscopy. Central to the success of these quantitative probes was the use of a rapid and reversible chemical equilibrium to influence the Raman spectrum of the bioorthogonal alkyne. The ability to use Raman microscopy for quantitative sensing using ratiometric probes represents an exciting new application of ATRI which could be used to detect other important ions and biomolecules of relevance to disease. We believe that the work presented here represents an entirely novel platform for pH sensing in a cellular environment which will find broad applicability in the chemical and life sciences.

**Conflicts of interest**

There are no conflicts to declare.

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