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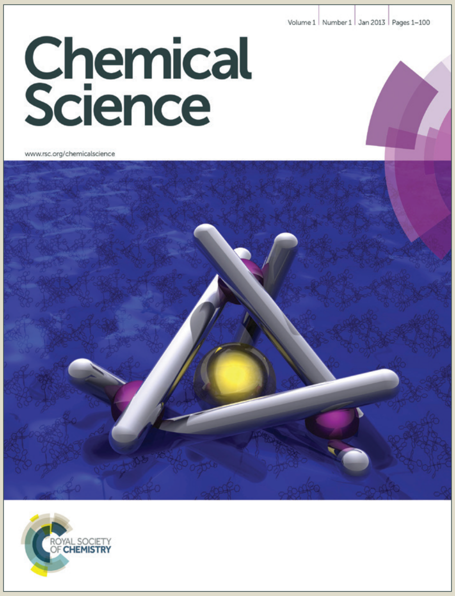

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Ultra-Sensitive pH Control of Supramolecular Polymers and Hydrogels: pK_a Matching of Biomimetic Monomers

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Achieving self-assembly in water with small molecules that disassemble in response to minimal changes in environmental conditions is important for the development of many promising materials. We report the most sensitive possible pH control of a supramolecular polymer assembly – demonstrated with hydrogel-forming monomers that mimic nucleic acid base pairing. Bidirectional pH-responsive supramolecular polymers are formed upon the self-assembly of three monomers with two pK_a matched recognition units (one acidic and one basic). These supramolecular assemblies are most stable when the pH equals the pK_a of the monomers, but disassemble in response to small pH changes near neutrality (exhibiting the theoretical limit for pH-dependent stability). At pH 7 a hydrogel forms in solutions that are 0.7% by weight in monomer, however, the hydrogel dissolves at pH 6 and pH 8. Additionally, we show that hydrogel stability is finely tuned by employing monomer mixtures that frustrate formation of insoluble aggregates. These results illustrate the advantages of using pK_a matched recognition elements and polymer heterogeneity in the development of responsive materials. Finally, these same recognition elements have recently been proposed as possible ancestors of the current bases of RNA. In this context, the assemblies described here might also provide clues to how the first genetic polymers were driven between states of base pairing and non-base pairing by periodic changes in environmental pH.

Introduction

Precise control over the self-assembly of small molecules in water underlies developmental strategies for many promising materials,[1, 2] including those for use in drug delivery and tissue engineering.[3, 4] Control of supramolecular polymer assembly and hydrogel formation by low molecular weight gelators can be achieved by using monomers with intermolecular interactions that are altered by environmental changes, such as pH, temperature and the addition of other molecules or ions.[5-8] Unfortunately, it is the case with many low molecular weight gelators that the same interactions that enable hydrogelation also lead to the formation of precipitates and, overtime, gel collapse.[9, 10] Therefore, new approaches that allow precise control over the supramolecular assembly process and increased gel lifetime are critical for supramolecular systems to be integrated into functional materials.

An appealing approach to the design of self-assembling structures for biological applications is the creation of supramolecular polymers that respond in a highly non-linear manner to modest pH changes within the physiological range.[11] Bidirection pH control of self-assembly has been demonstrated for monomers that contain acidic and basic groups that promote assembly through charge pairing within the pH range between the pK_as of the charged groups.[12, 13] The pH range over which such an assembly is stable and responsive, typically defined as $\Delta pH_{10\text{-}90}$ (where 10-90% of the assemblies are present in solution), is coupled to the pK_as of the acidic and basic recognition elements. The response of a system to changes in pH can therefore be enhanced by minimizing the ΔpK_a of the recognition elements (Fig. 1a and ESI). While bidirectional systems commonly employ amines and carboxylic acids to enable charge pairing, these functionalities do not have pK_as near pH 7 and have a ΔpK_a of at least 5, making these systems mostly insensitive to pH changes near neutrality (Fig. 1a). We hypothesized that maximum pH sensitivity for a supramolecular polymerization process that responds to pH changes that are physiologically relevant could be achieved with assemblies that employ recognition elements with pK_as matched near pH 7 (Fig. 1b).

Work on the coassembly of monomers containing the triazines melamine and cyanuric acid (**Cy**), or their related pyrimidines triaminopyrimidine (**TAP**) and barbituric acid, as complementary hydrogen-bonding recognition units has fundamentally advanced our understanding of artificial self-assembly,[9-11] particularly in organic solvents where initial investigations were performed. In water, precipitation occurs immediately upon combining any two of these complementary heterocycles, but the addition of a hydrophilic side chain to one or both of the recognition elements promotes the formation of water soluble supramolecular assemblies.[14, 15] Of these heterocycles, **TAP** and **Cy** are unique as they are complementary and pK_a matched near neutrality.[14] Critically, **Cy** is predominantly negatively charged above this shared pK_a, while **TAP** is positively charged below it, precisely the properties necessary for a self-assembling system with ultimate possible sensitivity to pH. Additionally, **TAP** has recently been shown to readily react with ribose to form nucleosides under plausibly prebiotic conditions that assemble with **Cy** to form supramolecular polymers and hydrogels, which has implications regarding the origin of the nucleobases of the hypothetical 'pre-RNA world'.[16]

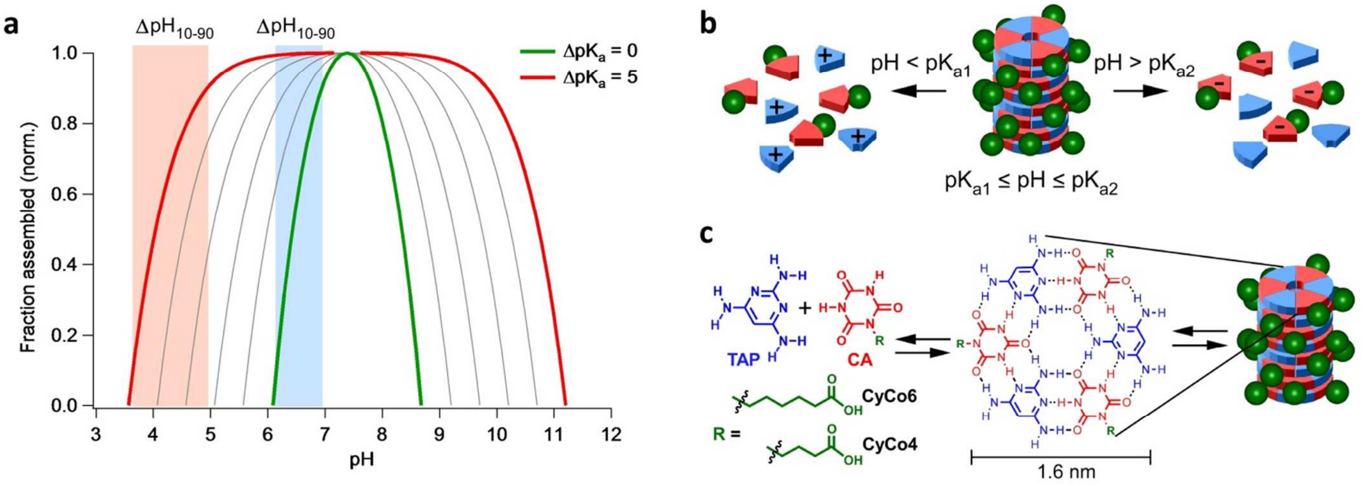

Fig. 1 (a) Theoretical limits for pH-dependent bidirectional assembly systems with ΔpK_as ranging from 0 to 5. The sensitivity of the assembly to change in pH, $\Delta pH_{10\text{-}90}$, is highlighted in blue for $\Delta pK_a = 0$ (pK_a matched), and pink for $\Delta pK_a = 5$ (the minimal ΔpK_a for assembly systems that use amines and carboxylic acids for charge pairing). Note that the pH range over which assemblies are present increases with increasing ΔpK_a; the system with $\Delta pK_a = 0$ shows the greatest sensitivity to changes in pH. Calculations were performed using idealized model systems that all have a minimum assembly concentration (MAC) of 15 mM in each monomer at pH 7.4, and a total sample concentration of 35 mM in each monomer. See Notes and References† for additional model details and ESI for derivation and example calculation. (b) Graphical representation of pH-mediated assembly and disassembly by monomers with pK_a-matched recognition units. (c) Chemical structures of triaminopyrimidine (**TAP**), cyanuric acid (**CA**, R = H), **CyCo6**, **CyCo4**, and proposed rosette and stacked rosette assemblies.

We surmised that reversible supramolecular polymers could be formed from a mixed system of monomeric units having an integrated bidirectional pH-sensitive switch. To test this approach, we prepared water soluble monomers composed of **Cy** substituted with a hexanoic acid (**CyCo6**) or a butyric acid (**CyCo4**) (Fig. 1c).[17] Mimicking nucleic acid base pairing, hydrogen bonding directs the assembly of **TAP** and the **CyCo** monomers into hexameric rosette structures, and hydrophobic interactions cause these rosettes to stack into supramolecular polymers that can then non-covalently crosslink to form a hydrogel matrix. Supramolecular assemblies were found to be most stable when composed of all three monomers and to exhibit the maximal sensitivity to change in pH for a supramolecular polymerization system.

Results and discussion

A shear-thinning, thermally reversible supramolecular hydrogel forms spontaneously when one equivalent of **TAP** is added to a 20 mM solution of **CyCo6** (0.7% by weight in total monomer), at pH 7 and 20 °C. Gelation is not observed below pH 6 or above pH 8 (Fig. 2a); demonstrating the pH sensitivity of the gel-forming process. At pH 7 and pH 8 **TAP** and **CyCo6** remain in solution for times greater than six months, but precipitation occurs at pH 6 within minutes. Under the same conditions **CyCo4** forms a precipitate immediately when mixed with **TAP** (Fig. S1). Incorporation of a cogelator has been shown to enhance the lifetime of the hydrogel phase.[18] We hypothesized that non-uniform structures formed by the inclusion of closely related **CyCo6** as a co-monomer into **TAP-CyCo4** assemblies would frustrate regular interactions between linear stacks of monomers. As predicted, mixtures of **CyCo4** and **CyCo6** form hydrogels with **TAP** whose gel lifetime at pH 7 increases as the ratio of **CyCo6** to **CyCo4** increases (Fig. S1). For example, a solution containing **TAP**, **CyCo4** and **CyCo6** at a ratio of 1:0.8:0.2, respectively, forms a gel that begins to precipitate within five minutes while a solution at a 1:0.33:0.67 ratio in each molecule, respectively, forms a gel that is stable for greater than 5 hours. At pH 6 the trimolecular assemblies are stable against precipitation for over an hour, while the bimolecular assemblies precipitate rapidly, demonstrating a synergistic relationship among the monomers.

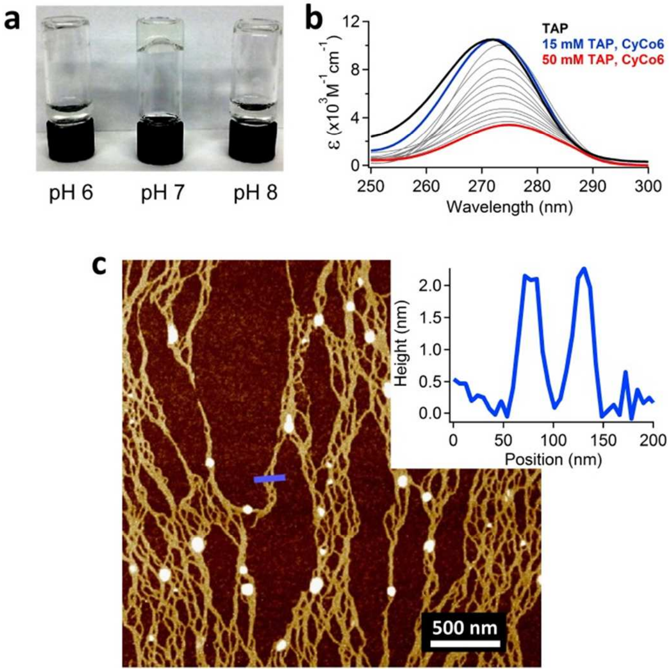

Fig. 2 (a) Inverted bottle test showing gelation of **TAP** with **CyCo6** at pH 7 and no gelation at pH 6 or pH 8. All three solutions are 20 mM in both monomers. (b) UV spectra of solutions of **TAP** alone and **TAP** with **CyCo6** at equimolar concentrations from 15 to 50 mM. (c) AFM topographic image of **TAP-CyCo6** fibers. Insert shows the profile delineated by the blue line in the panel.

The gels' mechanical properties were further studied by oscillatory rheology with solutions that were 1.4% by weight in monomer. Hydrogels consisting of either **TAP-CyCo6** or **TAP-CyCo4-CyCo6** (1:0.33:0.66) exhibited a predominant elastic character over the time scales examined, as evident by a greater storage modulus, G', than loss modulus, G'' (Fig. S2). Frequency sweeps were performed at a constant strain value of 1.0%, corresponding to the linear regime as revealed by strain sweeps (Fig. S3). In addition, strain sweeps indicate that G' values are similar at pHs near neutrality (ca. +/- 0.5 pH units) for the biomolecular and trimolecular systems, while G'' values are found to vary more markedly with these subtle pH variations and between the systems. More substantial effects were observed when the pH of the solutions was increased to 8.5, as both dynamic moduli decreased by over an order of magnitude. These results indicate that the mechanical properties of the gel phase can be altered with modest pH change.

The nature of **TAP-CyCo** assemblies at the molecular level was investigated using spectroscopic and microscopic techniques. The UV/vis absorption spectrum of **TAP** in the gel phase is red shifted relative to the spectrum of free **TAP**, indicative of intermolecular π-π electron interactions that are typical of J-type associations by planar molecules (Fig. 2b). The spectrum of samples containing 1:1 **TAP-CyCo6** and **TAP-CyCo4-CyCo6,** at various ratios where the total **Cy** containing monomer is equal to the concentration of added **TAP**, revert to that of free **TAP** upon dilution from 40 mM to 15 mM (in **TAP**) (Fig. S4), indicating the absence of intermolecular stacking interactions at concentrations below 15 mM. AFM imaging of the **TAP-CyCo6** and the trimolecular gel phase shows micron-length linear structures with heights of approximately 2 nm (Fig. 2c and Fig. S5). These structures are consistent with the expected width of the stacked rosette assembly. The observation of extended networks of fibers by AFM is also consistent with the gelation properties of the assemblies.

^{1}H NMR spectroscopy indicates the incorporation of these monomers into supramolecular assemblies by exhibiting a concentration-dependent loss of integrated resonance intensity.[19] Specifically, ^{1}H spectra of **TAP-CyCo6** solutions below 15 mM in each monomer show **CyCo6** resonances with chemical shifts that are identical to **CyCo6** alone in solution and with integrated intensities that are directly proportional to concentration (Fig. 3). In contrast, raising **TAP** and **CyCo6** concentrations above 15 mM results in no further increase of the integrated intensity of **CyCo6** resonances, nor are changes observed in ^{1}H chemical shifts. This observation indicates that at the higher concentrations, **CyCo6** and **TAP** are slowly exchanging between their free (unassembled) states and incorporation into large assemblies.

NMR and UV/vis experiments both reveal a cooperative assembly process,[20, 21] with a minimal assembly concentration (MAC) for **TAP** and **CyCo6** of 15 mM in each monomer at 20 °C, pH 7. In agreement with the proposed cooperative assembly, NMR and AFM analysis also indicate an absence of intermediate assemblies, i.e., only free monomers and long noncovalent polymers are present. The driving force of this highly cooperative assembly, and thus the formation of long noncovalent polymers, is apparently the hydrophobic effect. The exposure of the two faces of a rosette to water is predicted to have a large positive free energy (ca. 27 kcal/mol).[15, 22] Moreover, the favourable assembly between **TAP** and **Cy** may be enhanced by pK_a matching of these recognition elements, as both experimental and theoretical studies have indicated that smaller pK_a differences between H-bond donor and acceptor groups leads to stronger H-bonding.[23-26]

Trimolecular systems were next investigated by ^{1}H NMR spectroscopy. The analysis shown in Fig. 3 reveals that the MAC for solutions that contain **TAP**:**CyCo4**:**CyCo6** in ratios of 1:0.5:0.5 is identical to the MAC measured for the bimolecular **TAP-CyCo6** system when the sum of the integrated intensity of **CyCo4** and **CyCo6** resonances in the trimolecular system are compared to the integrated intensities of **CyCo6** resonances in the bimolecular solutions (Fig. 3b). The same is true for solutions in which the ratio of **TAP**:**CyCo4**:**CyCo6** is 1:0.33:0.66 (Fig. 3c). The amount of **CyCo4** and **CyCo6** incorporated into large assemblies with **TAP** is simply proportional to the ratio of these monomers present in solution; equal concentrations of **CyCo4** and **CyCo6** are incorporated in the 1:0.5:0.5 samples (Fig. 3a and 3b), and a 1:2 ratio of **CyCo4** and **CyCo6** are incorporated in the 1:0.33:0.66 samples (Fig. 3c). Additionally, the MAC determined for the mixed systems is the same as that measured for the **TAP-CyCo6** system at pH 7 for temperatures ranging from at least 5 to 35 ºC (Fig. S6). These observations confirm that the mixed trimolecular system assembles with the same propensity as the **TAP-CyCo6** system and demonstrates that formation of soluble co-assemblies with **CyCo4** and **CyCo6** are insensitive to the differences in the lengths of their carboxylic acid tails.

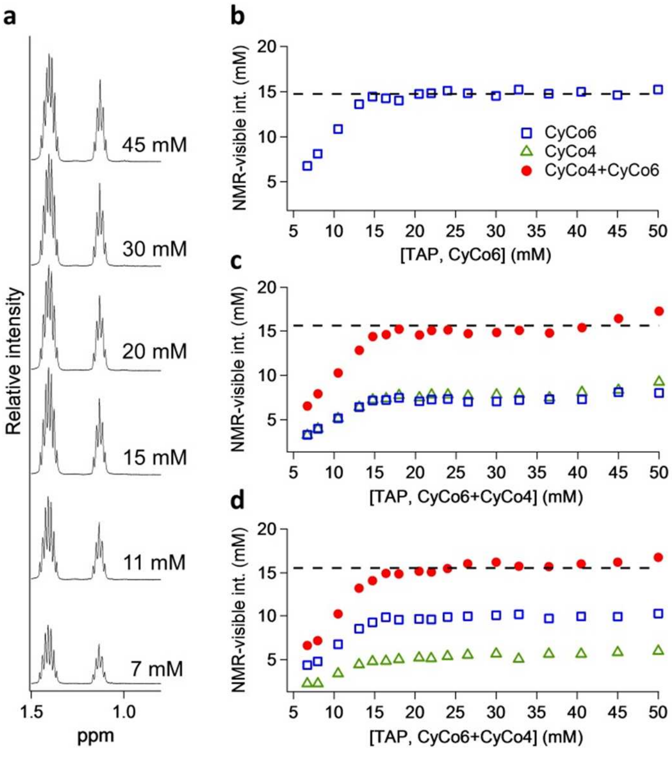

Fig. 3 (a) Representative ^{1}H NMR spectra of the **CyCo6** methylene protons for samples containing various concentrations of **TAP** and **CyCo6** (in equimolar ratios). (b-d) Plots of NMR-visible resonance intensity of unassembled **CyCo4** and/or **CyCo6** vs actual monomer concentrations in (b) 1:1 **TAP** and **CyCo6** solutions, (c) 1:0.5:0.5 solutions of **TAP**:**CyCo4**:**CyCo6**, and (d) 1:0.33:0.66 solutions of **TAP**:**CyCo4**:**CyCo6**. Dashed lines in plots indicate minimal assembly concentrations of the total **CA**-containing species.

We note that the precipitate of a 1:0.5:0.5 ratio **TAP**:**CyCo4**:**CyCo6** sample is enriched in **CyCo4** (Fig. S7), which is likely the result of monomer rearrangement producing regions enriched in **TAP-CyCo4** assemblies that precipitate due to the greater propensity of this particular assembly to aggregate. This proposed mechanism of precipitation and observed precipitate enrichment in **CyCo4** is consistent with a recent report by Meijer and coworkers, which showed that rearrangement of monomers within and between noncovalent polymers in water can require on the order of hours.[27]

Precipitation of **TAP-CyCo4** and **TAP-CyCo6** assemblies below pH 7 likely results from intermolecular hydrogen bonding of the neutral carboxylic acid groups that bundle the linear assemblies into large aggregates, as has been seen with other supramolecular polymerization systems that contain monomers with carboxylic acid groups.[9] Cooperative interactions of the mixed monomers are highly synergistic, enabling both the **CyCo4** monomer to form long-lived gels and extending the lifetime of **CyCo6** assemblies under unfavorable conditions (i.e., low pH). It has been previously shown that substantial modifications in the peripheral groups of recognition elements that also form rosette assemblies can result in the adoption of alternative assembly modes.[28, 29] However, less substantial changes are known to lead to soluble rosette nanotubes or insoluble tightly packed rosette assemblies.[30-32] As demonstrated here, the addition of a structurally similar co-monomer allows the rosette assembly unit to be maintained while simultaneously leading to large differences in assembly solubility due to the frustration of regular packing (and thus crystallization) between the assemblies.

The pH dependent assembly of both bimolecular (**TAP-CyCo6**) and trimolecular (**TAP-CyCo6-CyCo4**) supramolecular polymers were investigated by ^{1}H NMR spectroscopy. Any deviation in pH away from 7.4 corresponded to disassembly of the supramolecular polymer (Fig. 4 and S8). Complete disassembly occurs when the pH is below 5.7 or above pH 9.0. As anticipated, precipitation was evident when the bimolecular system was evaluated at any pH below 6.5 (Fig. S8). However, for the three-component system, no precipitation was observed over this entire pH range.

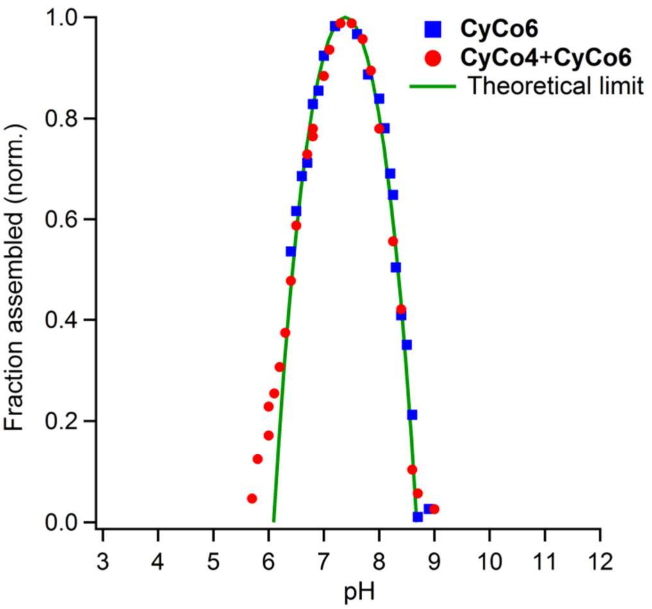

Fig. 4 Plot of the normalized fraction of monomer assembled in solutions that were 35 mM in **TAP** and **CyCo** monomer based on the ^{1}H NMR visible concentration of unassembled **CyCo6** (in 1:1 **TAP**:**CyCo6** solutions) (blue) and **CyCo4+CyCo6** (in 1:0.33:0.66 **TAP**:**CyCo4**:**CyCo6** solutions) (red) as a function of pH. The theoretical limit governing supramolecular assembly for a pK$_a$-matched system as a function of pH is shown as a green line. See Notes and References† for additional model details and ESI for derivation. Note that below pH 6.3 the solution containing only **TAP** and **CyCo6** immediately formed a precipitate, so data points for mixtures below this pH could not be obtained.

The behaviour of the trimolecular system quantitatively illustrates the high level of structural control that can be achieved with pK$_a$-matched monomers. Specifically, a comparison of the experimental data to the theoretical limit for a multimolecular assembly as a function of pH demonstrates that the **TAP-CyCo** system has the theoretically maximum sensitivity to change in pH that can be obtained for a supramolecular polymerization system (Fig. 4). An observation that is consistent with the **TAP-CyCo** assemblies being the most pH responsive supramolecular polymers ever reported. We note that our observation of the **TAP-CyCo** system being maximally assembled at pH 7.4 indicates pK$_a$-matched monomers at this pH, which is about 0.5 pH units above the reported pK$_a$s of **TAP** and **Cy**. Consistent with this observation, the apparent pK$_a$s of **TAP**, **CyCo6** and **CyCo4** were individually determined to each be around pH 7.4 in the buffer conditions of our experiments (Fig. S9).

Conclusions

We present here a new design strategy for the formation of supramolecular structures that are optimally responsive to changes in pH by utilizing pK$_a$ matched complementary monomeric recognition elements, acidic and basic monomers. Systems that are pK$_a$ matched enable dual, highly non-linear pH control of the assembly, which is most favoured in a solution in which the pH equals the pK$_a$ of the monomers. The **TAP**-**Cy** assembly motif is exceptional because both recognition elements have pK$_a$s near neutrality, making systems that employ these complimentary heterocycles attractive for the development of materials useful for biological applications, as small pH changes near physiological pH will elicit an appreciable and predicable response.[11, 33, 34] For example, materials that can undergo a structural transition upon encountering slightly acidic conditions, as found in the extracellular microenvironment of some tumours (pH 6.3-7.2), could find applications in drug delivery and diagnostics.[11, 35] A significant observation of this study is that heterogeneity within the supramolecular polymers stabilizes the hydrogel phase against precipitation. The ability of mixed assemblies formed by multiple monomers with the same recognition elements but different side chains that suppress the interactions that cause precipitation further supports this approach as a general principle for the design of well-behaved, self-assembling noncovalent polymer systems. In addition to their importance for materials development, the self-assembly properties of these pK$_a$-matched recognition elements, and their resemblance to RNA nucleobases, have implications regarding the search for the original nucleobases of the pre-RNA world; which we have previously discussed elsewhere.[16, 36]

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Notes and references

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† Theoretical limits for the assembly of monomers with various ΔpK_as (0-5) were determined using idealized model systems with 15 mM MACs (at pH 7.4) and solution concentrations (free and assembled) of 35 mM in each monomer. Curves of fraction assembled as a function of pH were generated by taking the product of the pairing monomers at pH 7.4 (the ΔpK_a midpoint) as the solubility constant of each system. This constant and the calculated monomer ionization equilibrium were then used to calculate the maximum expected assembly as a function of pH (normalized to fraction assembled at pH 7.4). See ESI for derivation and example.

Electronic Supplementary Information (ESI) available: Materials, methods and supplemental images. See DOI: 10.1039/b000000x/

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