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Co-assembly of Tetrapeptides into Complex pH-Responsive Molecular Hydrogel Networks

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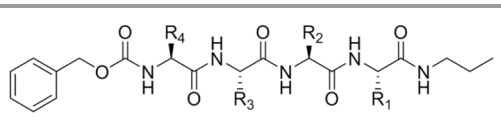
Here we prepare pH-responsive complex molecular hydrogels from oppositely charged tetrapeptidic components that can be pH-tuned resulting in interconversion between different networks. Two different systems are described based on tetrapeptides with an alternating sequence of non-polar (F) and polar (D or K) residues. Co-aggregated hydrogels are easily formed in situ at neutral pH whereas one-component networks are maintained after changing into acidic or basic pH. These systems have been applied for the pH selective release of two hydrophobic dyes – methylene blue and bromothymol blue – as drug models. Different release profiles have been observed depending on the characteristics of the network as well as the pH of the media. These materials offer great potential as multidrug carriers.

Introduction

Molecular hydrogels – supramolecular hydrogels formed by self-assembly of low-molecular weight compounds – have been widely studied in the last years because of their potential application in fields as diverse as materials science, biomedicine or catalysis.[1] These materials are formed in a programed hierarchical process from small building blocks assembled by weak non-covalent interactions. In this way, they have been shown to be responsive to different physicochemical stimuli and this property can be exploited for instance in their application in drug release, regenerative medicine or as optoelectronic materials among others.[2] Usually, molecular hydrogels are formed from a solution of the gelator that is obtained either by heating or starting from a soluble pre-gelator system that is activated *in situ* by a chemical process, an enzymatic reaction or even a physical stimulus such as light or ultrasounds.[3] For instance, pH responsiveness has been used in order to form hydrogels from compounds that present acid-base behavior.[4] Two-component hydrogels can be also formed by ion pair formation between oppositely charged compounds.[5] This is a convenient strategy as it starts from two independent solutions of pre-gelators that would immediately form a hydrogel after mixing without the need of heating or the addition of other reagents.

Here we present a system based on tetrapeptidic derivatives bearing ionisable groups of opposite charge at the side chain (Figure 1) for which we aim to prepare hydrogels following a simple injection methodology. Additionally, owing to the presence of pH sensitive groups, we aim to modulate the hydrogel features with pH which could be of interest for the pH selective release of loads.[6]



1 (ZFDFD) $R_1 = R_3 = -CH_2COOH$ $R_2 = R_4 = -CH_2Ph$
2 (ZFKFK) $R_1 = R_3 = -(CH_2)_4NH_2$ $R_2 = R_4 = -CH_2Ph$
3 (ZKFKF) $R_1 = R_3 = -CH_2Ph$ $R_2 = R_4 = -(CH_2)_4NH_2$

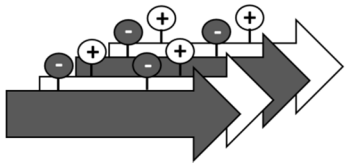


Figure 1. Tetrapeptidic gelators and proposed self-assembly scheme.

Results and discussion

It is known that peptides of opposite charge combined with hydrophobic residues can co-assemble to form different aggregates.[7] We have shown recently that small peptides bearing alternating phenylalanine (F) and aspartic acid (D) residues such as **1** are able to form molecular hydrogels at very low concentration.[8] Additionally, compounds **2** and **3** have been designed as charge complementary analogues bearing alternated phenylalanine (F) and lysine (K) residues that are able to aggregate at low concentration (1 mM) in basic media (see ESI for details). All three compounds are soluble and ionized at neutral pH as their pKa values have been determined to be ca. 6 for compound **1** and ca. 9 for compounds **2** and **3** (see ESI for details). Nevertheless, when a 5 mM solution of compound **1** in Na_2CO_3 10 mM was added over an acidic solution of compound **2** or **3** in HCl 10 mM a slightly translucent hydrogel was obtained immediately (Figure 3A, inset; see ESI for details). Aggregates were also formed at a

lower concentration (1 mM) although they were not effective on percolating the solvent. As can be seen in Figure 2, ^{1}H NMR of these aggregates was silent, namely, no signals from any of the single components could be detected. Apparently, the increase in hydrophobicity after charge screening drives the formation of the aggregates as can be demonstrated by the thioflavin T (ThT) binding assay[9] in which an enhancement of ThT emission band is observed upon binding on the hydrophobic aromatic surfaces for the co-aggregates **1**:**2** whereas no emission is observed for the starting solutions of pure precursor compounds **1** and **2**. Interestingly, for co-aggregates of compound **3** such an enhancement of ThT fluorescence is not observed suggesting smaller aromatic surfaces (see Figure S1). As reported before,[8] the presence of a Z-protected phenylalanine at the N-terminus creates an extended aromatic binding site for ThT and other flat aromatic guests such as methylene blue, as will be discussed later on.

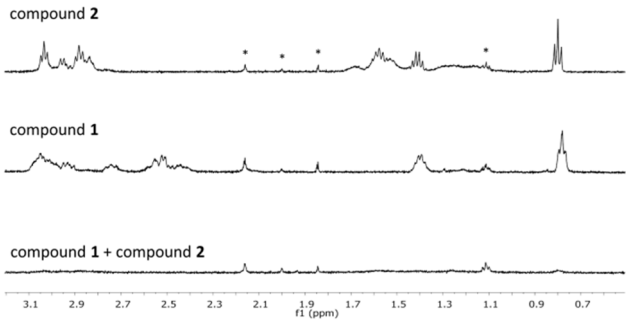


Figure 2. ^{1}H NMR (D_2O) spectra of ionised compounds **1** and **2** at basic and acidic pH respectively, and spectrum of their equimolar mixture at neutral pH. (*: solvent impurities)

Interestingly, the presence of ionisable groups makes these aggregates pH-responsive (Figure 3A). It is expected that after addition of an acidic solution over the neutral co-aggregate, compound **2** will be protonated and will become soluble whereas compound **1** will remain as a neutral species. On the other hand, the addition of a basic solution over the neutral co-aggregate should result into the deprotonation and solubilisation of compound **1** leaving lysine derivative **2** as a neutral species. These neutral species are poorly soluble in aqueous solution and will then aggregate or precipitate depending on the resulting concentration. Indeed, as can be seen in Figure 3B, the addition of an HCl solution to a mixture at 5 mM prepared in the presence of Bromocresol Green as a pH indicator shows a progressive diffusion of an acidic band and the maintenance of the macroscopic hydrogel aspect during the whole process (final pH=1.5). A similar process was observed for the addition of a Na_2CO_3 solution over a hydrogel formed in the presence of Bromothymol Blue (final pH=10.5). These hydrogels, that remained stable with time, were analysed in order to see if the primitive co-aggregated network persisted after the change on the ionisation state of one of the components or it had evolved *in situ* into a new one-component network. This fact should be easy to monitor by ^{1}H NMR as the soluble ionised component should appear in the spectrum whereas the networked one should stay silent. As can be seen in Figure 4, the diffusion-limited colour change of the dye after addition of HCl was accompanied with an increase of the amount of compound **2** free in solution. Although the naked-eye observation should be taken as qualitative the local change in pH and the expulsion of **2** from the network are initially happening in the same time scale of hours. Similar results were obtained after the addition of base to co-aggregates **1**:**2** (see Figure S2).

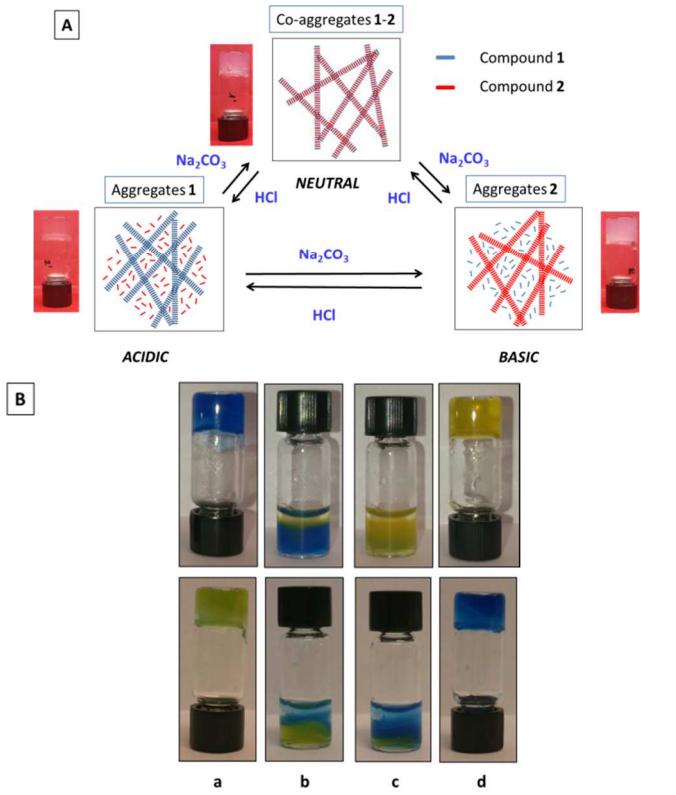


Figure 3. A) Scheme of the pH-responsiveness of mixtures **1**:**2**. Insets: macroscopic aspect of the hydrogels B) Hydrogels of co-aggregated **1** and **2** (5 mM) in the presence of Bromocresol Green (top) and Bromothymol Blue (bottom) before (a) and after addition of 100 µL HCl 0.1 M and 100 µL Na_2CO_3 0.1 M respectively (b: 5 min, c: 25 min. and d: 50 min).

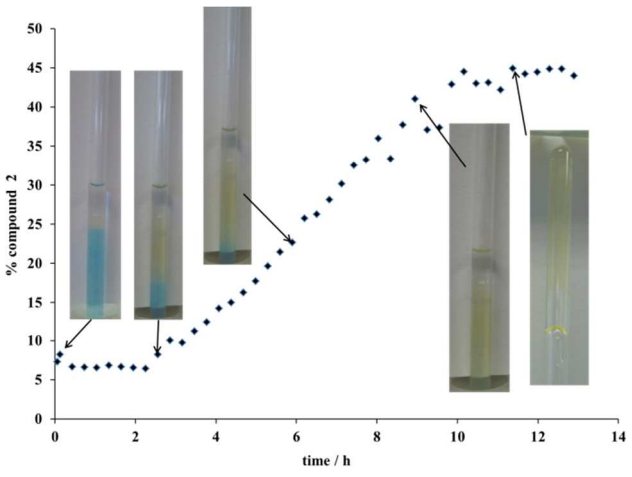


Figure 4. Kinetics of release of protonated compound **2** and change in Bromocresol Green colour after the addition of HCl to a neutral two-component hydrogel (5 mM).

In order to avoid diffusion artefacts, the amount of free compounds in solution for different samples at a total concentration of 1 mM was determined by ^{1}H NMR (see

Figures S3). Blank samples prepared for each compound at basic and acidic pH showed all the compounds in solution for their ionised species and less than 5% in solution for their neutral species. These values that are at the detection limit of the NMR correspond to the solubility of these neutral compounds. In the case of co-aggregates, starting from an equimolar mixture of **1** and **2** at neutral pH, samples were observed after acidification with HCl or basification with Na_2CO_3. In both cases, the ionised component was released in solution up to a 65-75% whereas the neutral species remained NMR silent being the main component of the aggregated network. This process of reversible pH switching between three different aggregated states as schematised in Figure 3A could be performed several times. It is remarkable first, the instantaneous formation of a co-aggregated network by simply mixing of solutions of the two components, and secondly the time scale of network exchange of hours and the dynamics of interconversion at the molecular level that is not far from the diffusion limit for the molecular motion of the studied dyes. A residual amount of compound **2** remains adsorbed to the aggregates after pH change probably due to hydrophobic interactions. Similar results were obtained for co-aggregates of compounds **1** and **3** (see Figure S4). Circular dichroism was performed on samples of both co-aggregates at different pH and, as can be seen in Figures S5 and S6, significant differences could be observed between them. Co-assembly **1**:**2** shows a negative band centred at 218 nm that could be assigned to a β-sheet conformation and that is only slightly shifted upon changing the pH. However, in the case of co-assembly **1**:**3** relevant differences on the band position were observed from 227 nm for the neutral aggregates to 219 nm at acidic pH and 208 under basic conditions.

Figure 5 collects the microscopic morphology of the aggregates at different pH for both mixtures **1**:**2** and **1**:**3** studied by TEM. All the samples showed a fibrillar aspect typical for a hydrogel network with long fibres of several micrometers length and thin fibrils of ca. 20-50 nm of width. It can be noticed that, neutral co-aggregates **1**:**2** present a more crowded network than neutral co-aggregates **1**:**3**. Besides, after the addition of either acid or base the aspect of the samples corresponds to that of the blank of the pure compound that remains within the network (**1** at acidic pH and **2** or **3** at basic pH, see Figure S7) Interestingly, samples of aggregates at basic pH revealed the presence of twisted fibres for both mixtures **1**:**2** and **1**:**3** in accordance to blank samples of pure **2** and **3** (see Figure S7).

To better understand the mechanical properties of the two networks at different pH values, rheological measurements were carried out on hydrogel samples equilibrated at the required pH for 24 h at room temperature. As can be seen in Figures S8 and S9, all the samples showed the viscoelastic behaviour typical of a molecular gel with storage modulus (G') values larger than loss modulus (G'') ones. The differences in moduli were of about one order of magnitude and together with low values of yield stress suggest that these gels are only moderately strong. Besides, as can be seen in Figure 6, differences could be observed among the six different samples. For instance, gels formed by neutral co-assembled networks **1**:**2** and **1**:**3** showed quite similar G' values. On the other hand, the addition of either acid or base to co-assembly **1**:**3** resulted in an evident weakening of the network as a result of the expulsion of one of the components. However, this effect was not as clear in the case of co-assembly **1**:**2** in which the addition of acid lead to a significant decrease on G' whereas the change was not so important at basic pH being G' even slightly higher than the original co-assembly. From these results it seems apparent that several factors could be affecting on mechanical properties of the gels, not only the composition of the network at a given pH but also the supramolecular structure of the starting co-assembled network. In this sense, kinetics of network exchange could play a relevant role and deserve a deeper study that will be undertaken in the near future.

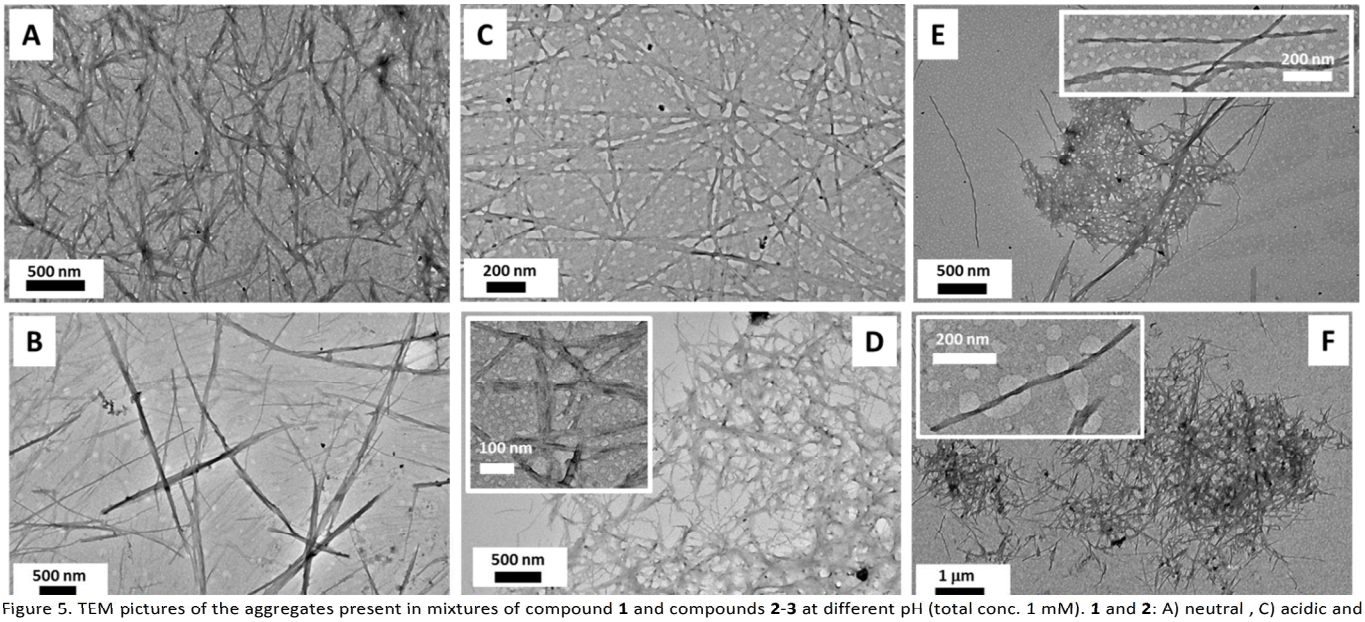


Figure 5. TEM pictures of the aggregates present in mixtures of compound **1** and compounds **2**-**3** at different pH (total conc. 1 mM). **1** and **2**: A) neutral , C) acidic and E) basic; **1** and **3**: B) neutral, D) acidic and F) basic.

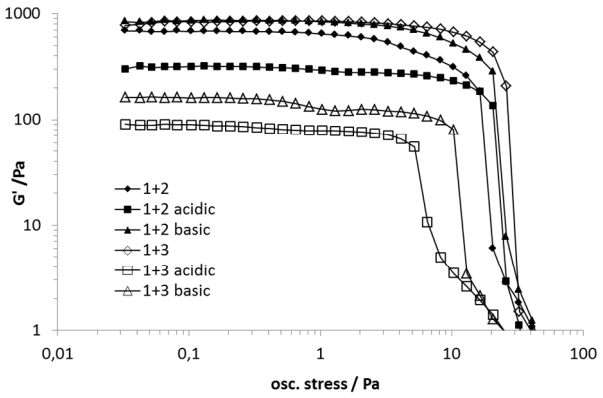


Figure 6. Storage modulus (G') data under an oscillatory stress experiment at a frequency of 1 Hz. (See ESI for full details on G', G'' and frequency sweep experiments).

The particular features displayed by these two-component hydrogels can be of great interest in the field of drug delivery as a hydrogel loaded with drugs can be prepared easily by injecting two solutions at near physiological pH and moreover different drugs could be released at will by tuning the pH of the environment. In order to evaluate their potential use in the field of drug release two pH sensitive dyes were studied as models of hydrophobic drugs – Methylene Blue (MB) and Bromothymol Blue (BTB). MB is a flat aromatic compound with a permanent positive charge and a tertiary amine that is protonated in acidic medium (ammonium pKa = 3.8). BTB is a pH dye indicator that turns from yellow below pH 6 (apolar cyclic form) into blue above pH 7.6 (anionic form). Co-assembled hydrogels containing MB (25 μM) were prepared by adding an acidic solution of compound **2** and MB to a basic solution of compound **1** and left to stabilise for 24 h (final gel concentration 5 mM). After this period of time 1 mL of a solution of the required pH was added and the release of MB from the gel was monitored *in situ* following the absorbance at λ = 624 nm of the supernatant solution. Conversely, hydrogels loaded with BTB (32 μM) were prepared by mixing a basic solution of compound **1** and BTB, and an acidic solution of compound **2**. After 24 h of stabilisation of the hydrogels and addition of supernatant solutions, released BTB was monitored at λ = 435 nm for acidic pH and water and at λ = 616 nm for basic pH (see ESI for details). Three different solutions were added on top of the hydrogels: a) 0.1 M HCl, b) water and c) 0.1 M Na_2CO_3 and as it can be seen in Figure 7 in the case of MB, after 12 h the release was more efficient at acidic pH. Under this conditions protonated MB is excluded from the hydrogel network together with protonated compound **2**. The release of MB is much slower upon addition of neutral water and almost null at basic pH. It has to be noticed that the absorption bands of released MB appear blue shifted compared to the expected spectrum (λ_{max} = 665 nm for free monomer, see SI for details) in all the samples starting from **1**:**2** co-aggregates despite of the different pH. This observation points to a remarkable interaction between MB and the neutral co-aggregate and its release as a complex with compound **2**. On the other hand, BTB is preferentially released under basic conditions, in parallel with the expulsion of deprotonated compound **1** (Figure 8). In this case no shifts were observed suggesting that the interactions with the aromatic fragments are not as intense and specific as in the case of MB.

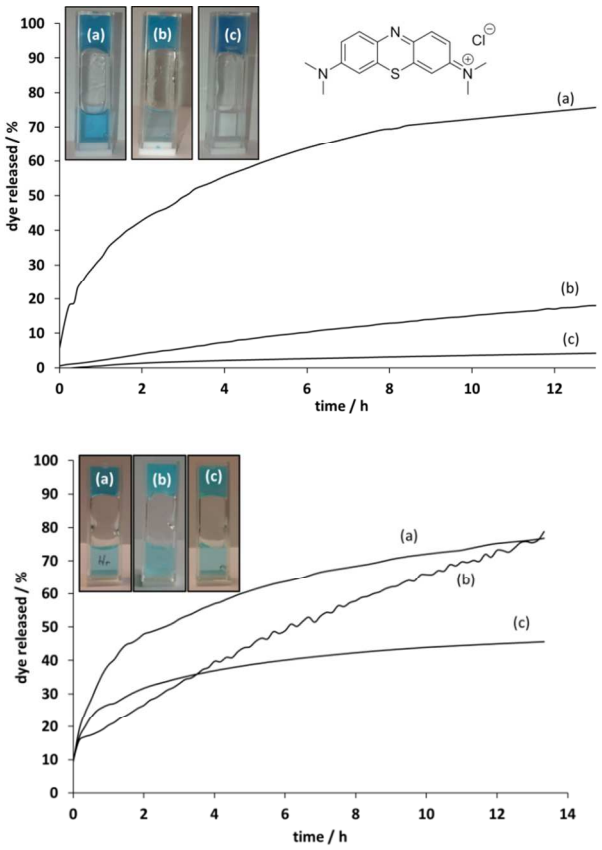


Figure 7. Release profile of Methylene Blue from co-assembled hydrogels **1:2** (top) and **1**:**3** (bottom) after addition of 1 mL of solutions: a) 0.1 M HCl, b) water and c) 0.1 M Na_2CO_3. (Inset: macroscopic aspect of the system 24 h after the addition).

Similar experiments were performed for co-aggregate **1**:**3**. In this case, the maximum of absorption of released MB was coincident with the free monomer (λ = 665 nm) discarding any interaction of the dye with that network as well as with compound **3**. Besides, although the release under acidic conditions was slightly better, the addition of water or basic solution also released a considerable amount of dye. Taking into account that rheology did not show any remarkable difference in network strength between neutral 1:2 and 1:3 co-assemblies the difference on MB release should be mainly ascribed to the difference in MB binding features of the two systems. In the case of BTB, similarly to **1:2** networks, the release from co-aggregated **1**:**3** was only efficient at basic pH and negligible release was observed either at neutral or acidic pH (see Figure 8). In difference with MB, which has a permanent charge, the apolar cyclic form of BTB is not released from any of the networks unless a basic solution is added leading to its anionic form. Besides, there is no evidence of any specific binding of BTB to the networks.

In summary, these experiments reveal that several factors are playing a role; in one hand the intrinsic differences between components of the networks **1**:**2** and **1**:**3** and the pH tuned interconversion from two- to one-component hydrogels, and on the other hand the intermolecular interactions between network components and released compounds together with the change of polarity of the dyes. A fine tuning of all these factors may allow the loading of multiple drugs for a convenient pH controlled distribution.

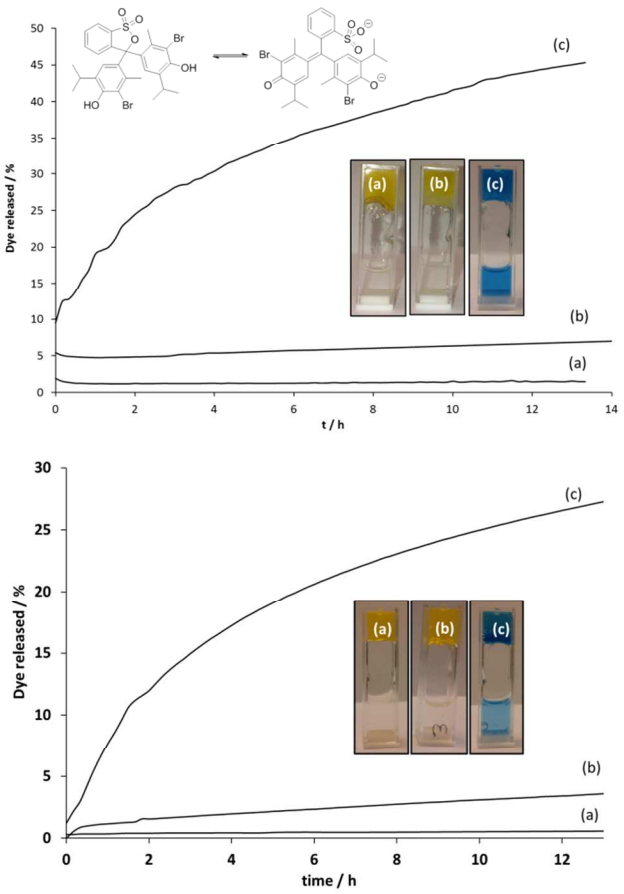


Figure 8. Release profile of Bromothymol Blue from co-assembled hydrogels **1**:**2** (top) and **1**:**3** (bottom) after addition of 1 mL of solutions: a) 0.1 M HCl, b) water and c) 0.1 M Na_2CO_3. (Inset: macroscopic aspect of the system 24 h after the addition).

Conclusions

In conclusion, we have demonstrated that pH-sensitive co-assembled networks can be easily obtained from oppositely charged small self-assembling peptides. We have shown that it is possible to switch between two-component networks at neutral pH to one-component networks at either basic or acidic pH with a concomitant change on the features of the material. Therefore, two component hydrogels provide adaptation to environmental changes, affording gel stability over the whole range of pH values (Figure 9). It could be said that these complex hydrogels present a chameleonic behaviour, changing its internal structure upon variation of external pH but preserving its outward appearance. Overall this case highlights how introducing complexity by means of incorporating an additional hydrogelator provides with outstanding new properties. Moreover, the results indicate that these systems can be applied to the tuneable release of loads based on their molecular structures as well as on the particular environmental parameters. It is envisaged a broad possibility for the application in multidrug delivery by tuning the release of different drugs by adapting the hydrogel composition to the required media (i.e. pH gradients found between different tissues). Besides, we have shown that differences in dye release appear by slightly changing the peptide sequence of the positively charged partner from **2** to **3**, and we foresee a wide chance of modulation of the release behaviour by using other isomeric analogues of **1** recently reported by us. Moreover, compound **1** and analogues have been shown to interact with amyloid fragment Aβ40 which combined with the current release properties could be of great interest from a therapeutic point of view.[8]

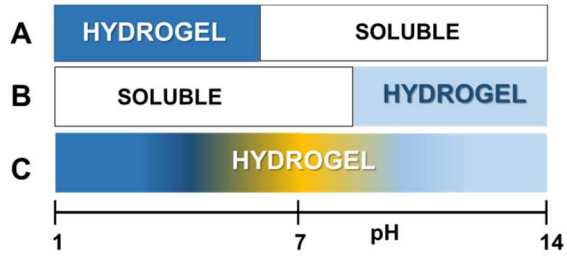


Figure 9. Schematic representation of the chameleonic behaviour of the networks formed by compounds **1** (A), **2**-**3** (B) and mixtures **1**:**2** and **1**:**3** (C).

Acknowledgements

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Electronic Supplementary Information (ESI) available: Experimental procedures, characterization of compounds and additional graphs. See DOI: 10.1039/b000000x/

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