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## Thermoresponsive properties of polyacrylamides in physiological solutions†

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Polymer solutions with a lower critical solution temperature (LCST) undergo reversible phase separation when heated above their cloud point temperature ( $T_{CP}$  or CPT). As such, they have been proposed for a wide range of biomedical applications, from injectable drug depots to switchable coatings for cell adhesion. However, in systematic studies, the  $T_{CP}$  of these thermoresponsive polymers has been mostly measured in non-physiological solutions, thereby hindering the development of their medicinal applications. Here, we analysed the thermoresponsive properties of four acrylamide-based polymers with LCST, namely poly[(*N*-2,2-difluoroethyl)acrylamide] (**pDFEA**), poly[(*N*-isopropyl)acrylamide] (**pNIPAM**), poly[(*N,N*-diethyl)acrylamide] (**pDEA**), and poly[(*N*-acryloyl)pyrrolidine] (**pAP**). As shown by turbidimetry, their  $T_{CP}$  in phosphate saline buffer (PBS) and foetal bovine serum (FBS) were consistently lower than those reported in the literature, typically assessed in pure water, even when using the same setup. In addition, these physiological solutions affected the variation of  $T_{CP}$  as a function of polymer concentration (1.25 to 10.0 mg mL<sup>-1</sup>) and molar mass (20 to 50 kg mol<sup>-1</sup>). As shown by isothermal calorimetry, interactions between proteins in FBS and polymer aggregates were predominantly exothermic, which indicates that protein–polymer complexes are formed through enthalpically driven processes. In conclusion, the  $T_{CP}$  of thermoresponsive polymers strongly depends on solvent composition and therefore should be measured under physiological conditions for future medicinal applications.

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## Introduction

Many hydrophilic polymers with a lower critical solution temperature (LCST) form homogeneous solutions below a threshold temperature (cloud point temperature,  $T_{CP}$  or CPT), but they separate into two phases with high and low concentrations when heating above this  $T_{CP}$ .<sup>1</sup> Numerous applications for such “smart polymers” have been proposed, across various fields, including switchable substrates for cell/tissue culture,<sup>2–5</sup> drug delivery systems,<sup>6,7</sup> *in situ* depot formation, controlled drug release (therapy/theranostics),<sup>7–11</sup> gene delivery/therapy,<sup>12–14</sup> tissue engineering,<sup>4,15–17</sup> wound dressing,<sup>18,19</sup> biosensors,<sup>20</sup> vaccines/immunotherapy,<sup>21</sup> and injectable brachytherapy,<sup>22,23</sup> among others.<sup>24,25</sup> Therefore, developing such applications requires understanding the properties of these polymers, particularly their  $T_{CP}$ .

$T_{CP}$  depends on polymer molar mass (dispersity)<sup>1,26–29</sup> and concentration,<sup>1,26,30</sup> on solution pH<sup>9,31–33</sup> and on the concentration and type of ions<sup>33–41</sup> (due to the Hofmeister effect<sup>34</sup>), proteins and other macromolecules<sup>33,42–45</sup> in solution (due to the excluded volume/molecular crowding effect,<sup>46</sup> competition for solvent, or non-covalent protein–polymer interactions<sup>47</sup>), among

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other factors.<sup>1,27,48</sup> Polymer tacticity<sup>1,49</sup> and terminal moiety (*e.g.*, a chain transfer agent, CTA)<sup>1,50,51</sup> and even fairly subtle environmental changes, including changes in solution pH,<sup>32,40,52</sup> can also affect the  $T_{CP}$ . Several theories, especially the lattice fluid theory with hydrogen-bonding corrections (LFT-HB)<sup>53</sup> and other (more advanced) models,<sup>45,54–57</sup> reliably describe  $T_{CP}$  as a function of polymer concentration, but they are rather complicated and heavily rely on empirical data.<sup>45,53–57</sup> Unsurprisingly, many authors agree with the need to assess the thermoresponsive properties of a polymer experimentally.<sup>26,27,34,36,45,54,58</sup>

Many techniques can be used to determine the  $T_{CP}$  of a given polymeric system, such as turbidimetry/spectrophotometry,<sup>26,59</sup> rheology,<sup>60</sup> refractometry,<sup>61</sup> infrared spectroscopy,<sup>62</sup> small-angle neutron scattering,<sup>63,64</sup> dynamic light scattering and small-angle/wide angle X-ray scattering (DLS, SAXS WAXS),<sup>60,65,66</sup>  $^1\text{H}$  or  $^{19}\text{F}$  NMR (nuclear magnetic resonance) spectroscopy,<sup>63,64</sup> isothermal and differential scanning (micro)calorimetry,<sup>67,68</sup> among others. Spectrophotometry (turbidimetry) is arguably the most common method for determining the  $T_{CP}$  of a solution of a thermoresponsive polymer because this method is widely available, quick, sensitive, and robust, accurately providing the  $T_{CP}$  at both high and relatively low concentrations. However, beam wavelength,<sup>59</sup> heating and stirring rates,<sup>59,66</sup> as well as the selected threshold absorbance,<sup>26,59</sup> can alter the measurement sensitivity and thus shift the experimental  $T_{CP}$ .<sup>59</sup> Moreover, this method may be inaccurate for absorbing (coloured), non-homogeneous samples<sup>32,59</sup> and for polymer solutions with a very low refractive index increment ( $dn/dc$ ) – for such samples, DLS is a more reliable option.<sup>59</sup> In turn, DLS may provide additional information about the variation in the size of molecular assemblies as a function of temperature (with some bias concerning larger structures),<sup>65</sup> but this method is more time demanding and laborious than turbidimetry.<sup>65</sup>

Notwithstanding the wide range of methods for determining the  $T_{CP}$  of thermoresponsive polymers and for their thorough characterisation, most previous systematic studies have only done so in non-physiological solutions. Furthermore, the solvent (PBS, FBS, plasma, serum, and interstitial fluid) effect on the  $T_{CP}$ s of such polymer solutions has never been systematically assessed, with most researchers analysing the effects of molar weight or salts/proteins on  $T_{CP}$  separately and under different conditions. Similar homopolymers prepared/measured under different conditions may display vastly different thermoresponsive properties, as shown by meta-analysis.<sup>1,26</sup> Therefore, understanding how molar mass and salts/proteins affect  $T_{CP}$  requires assessing all these effects with one batch of polymers in a head-to-head study. While numerous thermoresponsive homopolymers have already been reported, only poly[(*N*-isopropyl)acrylamide] (**pNIPAM**)<sup>33–37,43,44,67,69</sup> and poly[(*N*-acryloyl)pyrrolidine] (**pAP**)<sup>37</sup> have been extensively studied in both water and (at least partly) physiologically relevant solutions. Those studies have shown that  $T_{CP}$  is significantly lower in those solutions than in water.

Considering the above, we used **pNIPAM** as a benchmark to directly compare its properties, particularly  $T_{CP}$ , to those of

other polyacrylamides which have not been characterised yet in both physiological and non-physiological solvents. For this purpose, we synthesised four acrylamide-based homopolymers by RAFT polymerization in three molar-mass-categories 20 to 25 kg mol<sup>-1</sup>, 30 to 35 kg mol<sup>-1</sup> and 40 to 50 kg mol<sup>-1</sup>, namely poly[(*N*-2,2-difluoroethyl)acrylamide] (**pDFEA**), **pNIPAM**, poly[(*N,N*-diethyl)acrylamide] (**pDEA**), and **pAP**. Subsequently, we compared the  $T_{CP}$ s of their solutions in water to those of their solutions in phosphate saline buffer (PBS) and foetal bovine serum (FBS), as models of physiologically relevant conditions.<sup>70–72</sup> After determining the size of polymer aggregates as function of temperature, we compared the results to those from turbidimetry measurements, also measuring their  $T_{CP}$ s as a function of their molar mass and concentration (1.25 to 10.0 mg mL<sup>-1</sup>). Lastly, we assessed the effect of different physiological solvents (PBS and FBS) on polymer aggregation by isothermal calorimetry.

## Results and discussion

### Polymer selection

The polymers were selected for this study because they are non-ionic homopolymers of acrylamide *N*-derivatives with one or two alkyl moieties, and their aqueous solutions display LCST thermoresponsiveness with various  $T_{CP}$ s at similar polymer concentrations (**pAP**  $\gg$  **pNIPAM**  $\approx$  **pDEA**  $>$  **pDFEA**; Fig. 1).<sup>1,73</sup> While **pAP**, **pNIPAM**, **pDEA** have been studied extensively,<sup>1,26</sup> **pDFEA** is a relatively new and atypical thermoresponsive acrylamide with fluorine atoms.<sup>73</sup> As polyacrylamides, all four polymers act as hydrogen bond acceptors, but only **pDFEA** and **pNIPAM** can also act as hydrogen bond donors because they contain secondary amide moieties. In addition, **pDFEA** contains  $-\text{CF}_2\text{H}$  moieties, *i.e.*, lipophilic hydrogen donors<sup>74</sup> (see Scheme S2† and chapter S10.2). All these polymers are non-toxic and biocompatible as well. Accordingly, their different properties (*e.g.*, hydrophilicity or  $T_{CP}$ ) may be used to tailor the final materials for biomedical applications.<sup>1,73</sup> Nevertheless, we avoided comparing copolymers because their  $T_{CP}$  depends on the content of their individual monomers and on their architecture, thus adding other unknown variables to the equation.<sup>75</sup>

We targeted polymers with properties suitable for biomedical applications. For this reason, **pDFEA**, **pNIPAM**, **pDEA**, and **pAP** had a narrow molecular weight distribution ( $D_M \leq 1.11$ ) and molar masses in three different ranges ( $\approx 20$  to 25 kg mol<sup>-1</sup>,  $\approx 30$  to 35 kg mol<sup>-1</sup> and  $\approx 40$  to 50 kg mol<sup>-1</sup>) but all lower than the renal excretion limit<sup>77,78</sup> so that they would not accumulate in the body.<sup>78</sup> The end groups of these polymers contained a methyl ester (from methyl acrylate) on one end and a carboxyl group (initiator residue) on the other, which can be conveniently used to introduce tracers, dyes, and other moieties.

### Polymer synthesis, purification, and characterisation

We prepared these four polymers *via* controlled reversible addition–fragmentation chain-transfer (RAFT) radical poly-



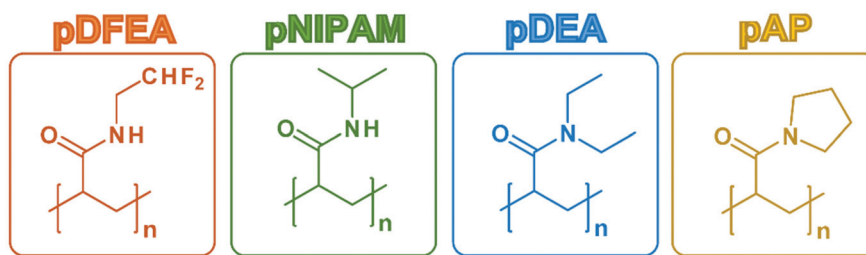


Fig. 1 Structures of the study polymers: poly[(*N*-2,2-difluoroethyl)acrylamide] (pDFEA), poly[(*N*-isopropyl)acrylamide] (pNIPAM), poly[(*N,N*-diethyl)acrylamide] (pDEA), and poly[(*N*-acryloyl)pyrrolidine] (pAP).

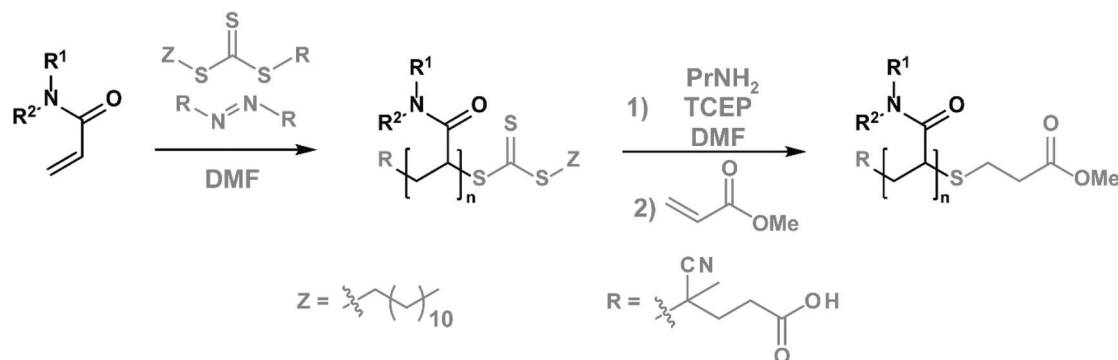


Fig. 2 Polymer synthesis and subsequent modifications.

Table 1 Polymer characteristics

Polymer		$M_w^a$ (kg mol <sup>-1</sup> )	$M_n^a$ (kg mol <sup>-1</sup> )	$D_M^a$	Yield <sup>b</sup> (%)	$dn/dc^c$ (mL g <sup>-1</sup> )
pDFEA	F1	26.2	24.2	1.08	68.3	0.088 ± 0.003
	F2	36.2	35.1	1.03	79.7	0.092 ± 0.005
	F3	49.6	46.9	1.06	79.5	0.095 ± 0.004
pNIPAM	I1	20.2	19.6	1.03	50.6	0.167 ± 0.018
	I2	31.6	30.8	1.03	58.1	0.151 ± 0.012
	I3	48.4	45.2	1.07	55.6	0.143 ± 0.013
pDEA	E1	22.3	21.2	1.06	60.5	0.171 ± 0.004
	E2	34.7	31.7	1.09	61.3	0.176 ± 0.011
	E3	41.3	37.5	1.10	57.5	0.145 ± 0.010
pAP	P1	19.6	17.6	1.11	86.0	0.192 ± 0.007
	P2	36.0	32.9	1.09	77.7	0.180 ± 0.015
	P3	51.2	46.3	1.11	79.1	0.164 ± 0.007

<sup>a</sup> Determined by SEC. <sup>b</sup> Polymerisation yield after the purification procedure based on monomer weight. <sup>c</sup> Determined by differential refractometry in PBS at 29 °C and 620 nm in PBS.

merisation<sup>76</sup> (Fig. 2) using 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid as the CTA and 4,4'-azobis(4-cyanovaleric acid) (ACVA) as the initiator (see Table S1† for the initial quantities of the reagents). Subsequently, we purified and mixed the polymers first with propylamine (aminolysis of the terminal CTA)<sup>76</sup> and then with an excess of methyl acrylate (to mask the reactive thiol moiety by Michael addition),<sup>76</sup> as shown in Fig. 2 and Table S2.† Lastly, we purified and characterised these polymers by size exclusion chromatography and

NMR spectroscopy to determine their purity and to confirm CTA removal (see chapter S10.3†). The properties of these polymers are outlined in Table 1.

#### PBS and FBS affect the $T_{CP}$ of thermo-responsive polyacrylamide solutions

The  $T_{CP}$ s of our thermo-responsive polymers are either known (for pNIPAM<sup>33–37,43,44,67,69</sup> and pAP<sup>37</sup>) or expected (for pDFEA and pDEA) to be lower in physiologically relevant solutions



(FBS and PBS) than in pure water because these buffered solutions have a higher pH and ion concentration (as discussed in the Introduction). However, these polymers also have terminal carboxylic moieties ( $pK_a \approx 4.8$ ), and while they are almost exclusively dissociated at neutral pH, unbuffered water dissolves atmospheric carbon dioxide,<sup>52</sup> lowering the pH to values near the  $pK_a$  of carboxylic acid (Table S7†). As a result, the carboxylic groups will no longer be fully deprotonated, decreasing the hydrophilicity<sup>32,52</sup> and  $T_{CP}$  of these polymers.<sup>52</sup> In contrast, both PBS and FBS (140 mM; pH = 7.4) reliably maintain the pH and osmotic pressure at physiological values.<sup>70,72</sup>

Considering these differences, we have compared the effect of different solvents (water, Dulbecco's PBS<sup>70</sup> and FBS) on  $T_{CP}$  ( $c = 10.0 \text{ mg mL}^{-1}$ ; Fig. 3). In line with previous studies, most  $T_{CP}$  values of our polymers were higher in pure water than in PBS<sup>33–37,40,43,44,67</sup> and FBS<sup>33,43</sup> because the ions and proteins of buffered solutions lower the  $T_{CP}$  of these polymers. Since differences in  $T_{CP}$ s between solvents may be significant for many applications, polymers should be tested in an environment as similar as possible to that of the intended application.

### Thermoresponsive polymers may require DLS for accurate $T_{CP}$ determination

As shown in Fig. 3B, the  $T_{CP}$  of pDFFEA with the highest molar mass (F3) was much higher in PBS than in water. To understand the unexpected  $T_{CP}$  of this polymer, as measured by turbidimetry, we assessed polymer aggregation as a function of temperature (10 to 50 °C) by DLS, in PBS, at a concentration of  $10.0 \text{ mg mL}^{-1}$  (Fig. S45–S56†). Unlike most samples, pDFFEA polymers showed two major changes in population size with the increase in temperature (Fig. S45–S47†). The first change (at 26 to 30 °C) can be ascribed to unimer aggregation (radius 20 to 100 nm), and the second (from 40 to 50 °C) to aggregate coalescence into even larger polymer assemblies (radius

1000 nm or larger). Thus, turbidimetry detects the first thermal change in most samples, but only the second in F3.

Long pDFFEA (co)polymers, such as F3, may form non-typical nanogel-like aggregates with low polymer concentrations, as shown in our previous studies.<sup>63,64,79</sup> In these particles, the aggregation causes only a minor local increase in polymer concentration. When combined with the low  $dn/dc$  of the solute (Table 1), this increase in polymer concentration accounts for the small difference in refractive indices between the phase-separated polymer and the bulk solution, which may prevent an accurate determination of  $T_{CP}$  by turbidimetry.<sup>59</sup> Upon further heating, these nanogel-like particles aggregate/coalesce, increasing the turbidity. Therefore, discrepancies in turbidimetric measurements may be explained by differences in the architecture of polymer aggregates and by the low  $dn/dc$  of pDFFEA.

### The effect of FBS proteins on $T_{CP}$ varies with polymer concentration

Proteins (in FBS) can affect the  $T_{CP}$  of polymers indirectly ('non-specifically', *i.e.*, by competing with polymers for its solvation as well as by excluded volume/crowding effect<sup>34,46</sup>) or directly ('specifically', *i.e.*, by forming complexes with the polymers<sup>34,47</sup>).<sup>34</sup> In turn, inorganic salts (in PBS and FBS) can also indirectly interfere with polymers by interacting with and destabilizing their solvation shell, thus decreasing their solubility and  $T_{CP}$  (Hoffmeister effect), regardless of polymer concentration.<sup>33,40</sup> Unlike inorganic salts, however, proteins affect  $T_{CP}$  as a function of polymer concentration, as shown by our results (Fig. 4).

At low polymer concentrations ( $1.25$  to  $2.50 \text{ mg mL}^{-1}$ ), polymers form soluble protein–polymer complexes (protein binding) in FBS, which prevent them from aggregating, thereby increasing their  $T_{CP}$  above that of PBS solutions, as observed in pDEA and pNIPAM (at both low and high pAP concentrations). Because the polymer-binding capacity of proteins

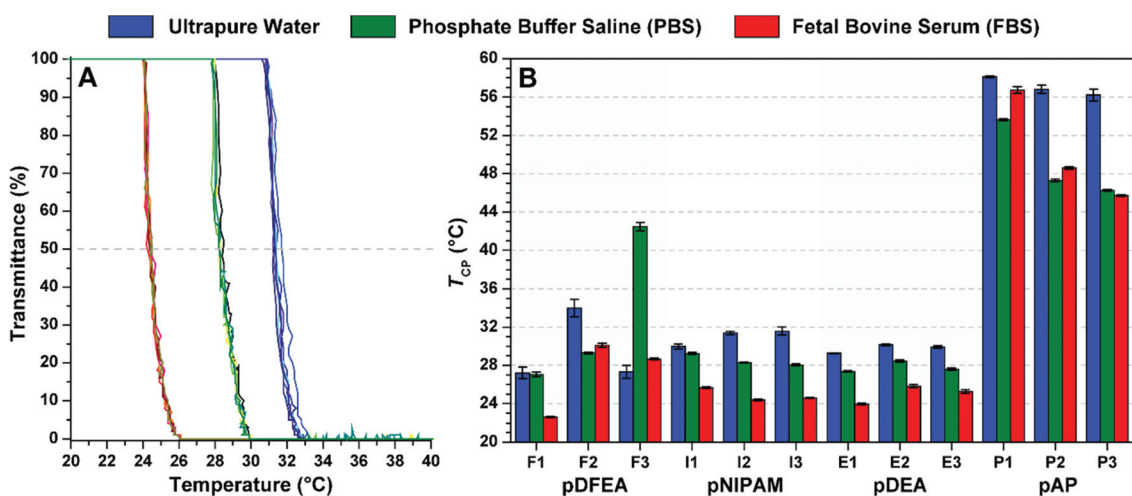


Fig. 3 (A) Comparison of 6 turbidimetric measurements of I2 (pNIPAM) in water, PBS and FBS ( $c = 10.0 \text{ mg mL}^{-1}$ ); (B)  $T_{CP}$  of all polymers in water, PBS and FBS ( $c = 10.0 \text{ mg mL}^{-1}$ ) expressed as a mean of 6 measurements  $\pm$  standard deviation. The results indicate a shift in  $T_{CP}$ .





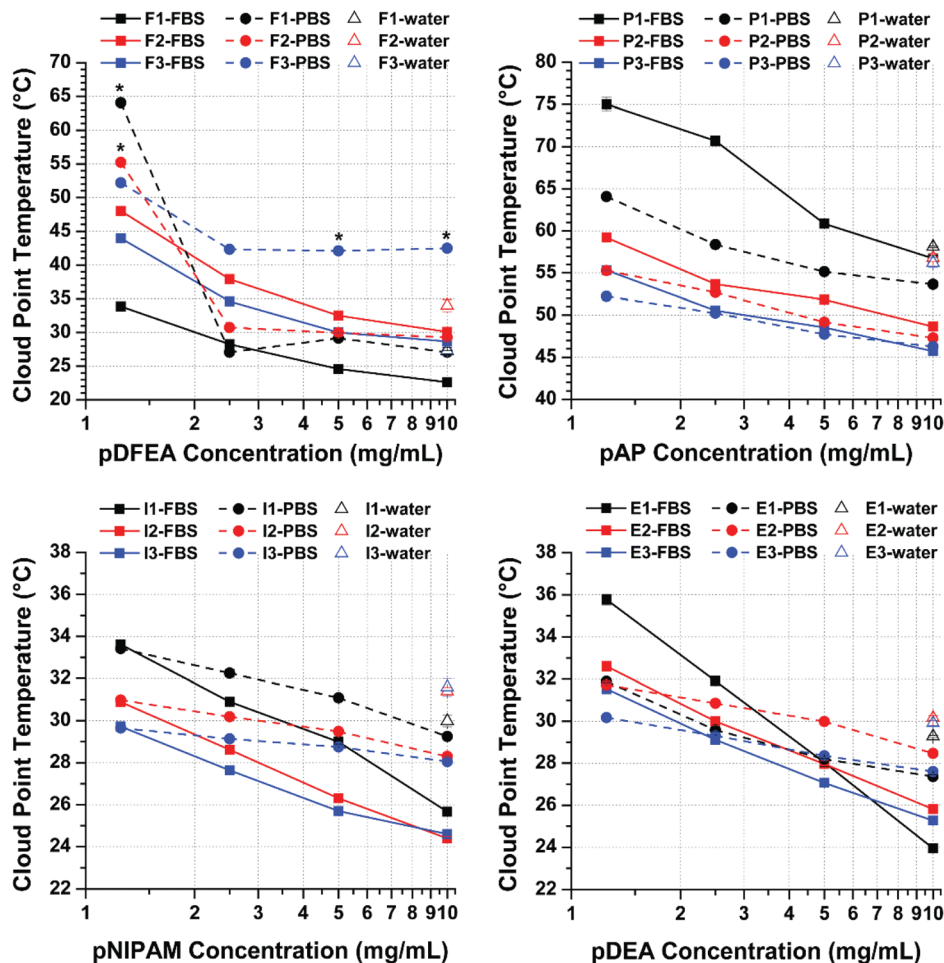


Fig. 4 Plot of  $T_{CP}$ s of the polymers pDFEA (top-left), pAP (top-right), pNIPAM (bottom-left) and pDEA (bottom-right) in FBS and PBS as a function of polymer concentrations. All  $T_{CP}$ s are expressed as the mean of 6 measurement cycles  $\pm$  standard deviation. Asterisks (\*) indicate potential outliers.

is limited (albeit very high for pAP), this effect is only detected at low polymer concentrations and decreases with the increase in polymer concentration (until being offset by ‘non-specific’ effects, which decrease the  $T_{CP}$ ).

At high polymer concentrations (5.00 to 10.0 mg mL<sup>-1</sup>), in contrast, proteins predominantly have a ‘non-specific’ effect by competing with polymer chains for solvation with the polymer<sup>33,34,40,41,80</sup> (similarly to inorganic ions), thereby facilitating aggregation and lowering the  $T_{CP}$ s. Proteins may also stabilize polymer aggregates *via* hydrophobic interactions, further lowering the  $T_{CP}$ s. Under such conditions, most polymers in FBS have the lowest  $T_{CP}$ s of all three media tested in this study (except for F3 in PBS, as discussed above).

For the purpose of this analysis (Fig. 4), we disregarded the transition temperatures of the highest polymer concentrations (20.0 and 40.0 mg mL<sup>-1</sup>) because they differed considerably between independent measurements (low reproducibility), not only in FBS but also in PBS. Nevertheless, the complete dataset is provided in Tables S3–S6.† Furthermore, in the range used in this analysis (1.25 to 10.0 mg mL<sup>-1</sup>), except for a few outliers, all three molar masses of each polymer showed similar

$T_{CP}$  trends, that is,  $T_{CP}$  decreased with the increase in polymer concentration, in line with previous studies.<sup>1,34–37</sup>

#### PBS and FBS differentially affect LCST polymer aggregation

LCST polymer aggregation is an entropy-driven endothermic process<sup>1,26,59</sup> affected by surrounding ions/proteins. Adding these ions/proteins can induce conformational changes in the polymer (aggregation) or affect its solvation shell, both of which can be detected as heat effects.<sup>1,82–84</sup> A decrease in enthalpy after adding ions/proteins indicates an enthalpic effect, *i.e.*, polymers interact with ions/proteins (or new strong polymer–polymer interactions are formed). Conversely, an increase in enthalpy after adding these ions/proteins indicates an entropic effect, *i.e.*, the loss of specific interactions, thereby increasing the entropy, *e.g.*, due to the loss of the solvation shell.<sup>84</sup>

Considering the above, we assessed the enthalpy of interaction of pDFEA, pNIPAM, pDEA, and pAP with PBS and FBS by isothermal titration calorimetry (ITC), which revealed a complex titration isotherm<sup>81</sup> (Fig. 5B), with three phases: I, II and III (Fig. 5A). In phase I, the heat flux can be ascribed to



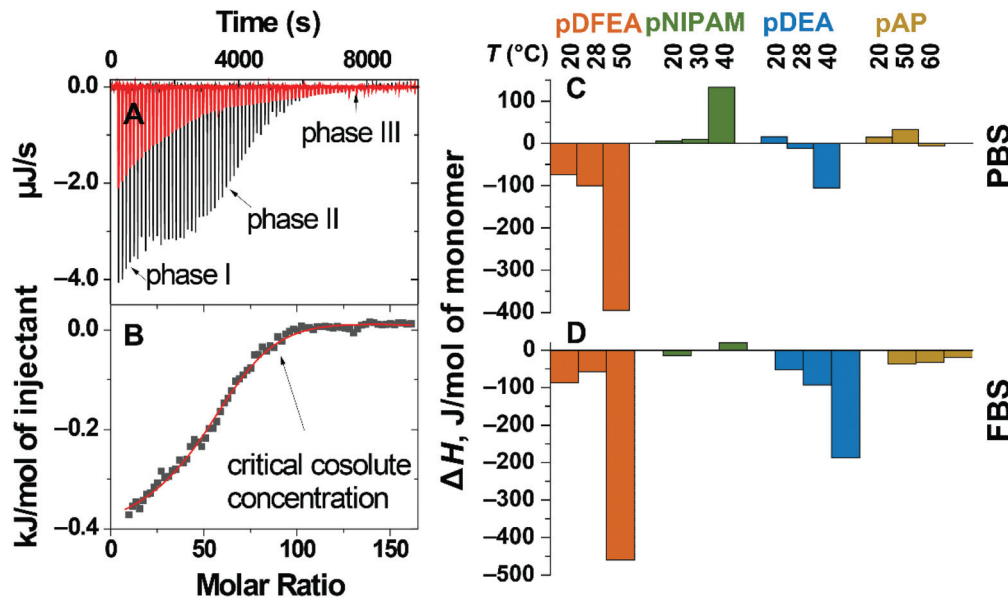


Fig. 5 ITC of 10.0 mg mL<sup>-1</sup> F3 (pDFEA solution in ultrapure water with PBS: heat flux as a function of time, titration of PBS to water (blank, red line), titration of PBS to polymer (black line) (A); integrated heat, normalised to 1 mol of F3 injectant (scatter), fit to model (line) (B). Enthalpies of the titration of 10.0 mg mL<sup>-1</sup> polymer solutions with PBS (C) and FBS (D), normalised to 1 mol of monomer units at three different temperatures. The values of enthalpies are outlined in Table S7.†

the neutralization of the terminal carboxylic acid because adding 3 to 5  $\mu\text{L}$  of PBS (pH = 7.41) or FBS (pH = 7.46) to the solution of benzoic acid had a similar effect on the heat flux (Fig. S41†). For this reason, the corresponding data points were excluded from the titration isotherms. Subsequent adding of titrant induced exo- or endothermic processes, which strongly depended on the titrant concentration (phase II on the titration isotherms). After the critical concentration of titrant (Fig. 5B), a non-zero heat flux, weakly dependent on the titrant, was still detected (phase III). Based on these results, we focused on the heat flux from phase II to analyse the solvent–polymer interactions.

### PBS promotes aggregation in thermoresponsive polymers by decreasing their solvation shell

In non-aggregated polymers (at low temperatures), adding PBS to the solution had a positive enthalpic effect on pNIPAM, pAP and pDEA, as expected based on the Hoffmeister effect, but surprisingly had a significantly negative enthalpic effect on pDFEA. On the one hand, PBS decreases the solvation shell of all polymers (positive enthalpic effect). On the other hand, PBS increases polymer–polymer bonds in the aggregates (negative enthalpic effect). Since PBS had a net negative enthalpic effect on pDFEA, pDFEA must have strong intramolecular interactions (possibly due to hydrogen bonding between CF<sub>2</sub>H moieties<sup>74</sup> – further investigated in chapter S7.4†).

In aggregated polymers (at high temperatures), PBS had a negative enthalpic effect on pDFEA, pDEA and pAP, indicating that this buffer promotes the formation of enthalpically favourable polymer–polymer. However, adding PBS to pNIPAM had a positive enthalpic effect, suggesting further dehydration result-

ing from salting out. Overall, PBS disrupts the solvation shell of thermoresponsive polymers, thereby promoting aggregation.

### FBS proteins stabilize LCST polymer aggregates

Adding FBS to both non-aggregated and aggregated polymers (at both low and high temperatures) had a more negative enthalpic effect than adding PBS due to the additional strong polymers–proteins interactions (Fig. 5). The amphiphilic proteins in FBS may bind to polymers and polymeric aggregates<sup>5,33</sup> via hydrophobic interactions<sup>3,5</sup> and thus stabilize them. In all polymers, adding FBS to polymer aggregates had a stronger negative enthalpic effect than adding FBS to non-aggregating polymers, possibly because polymer aggregates are more prone to interact with proteins through hydrophobic interactions. Consequently, FBS stabilizes thermoresponsive polymer aggregates more strongly than PBS.

## Conclusions

The cloud point temperature ( $T_{\text{CP}}$ ) of thermoresponsive polyacrylamides is considerably lower in physiologically relevant solvents (PBS and FBS) than in water. In particular, FBS proteins stabilize LCST polymer aggregates, but the effect of FBS proteins on  $T_{\text{CP}}$  depends on polymer concentration. At high polymer concentrations, proteins decrease the  $T_{\text{CP}}$  by competing for solvation. At low polymer concentrations, by contrast, proteins form complexes with the polymers, thus increasing aggregation. However, proteins have limited polymer-binding capacities, so their effect decreases with the increase in con-



centration. In turn, PBS promotes aggregation in thermo-responsive polymers by decreasing their solvation shell. Overall, our results suggest that thermo-responsive polymers with a high potential for biomedical applications should be characterised (i) by DLS for accurate  $T_{CP}$  determination and (ii) in physiologically relevant solutions rather than in pure water because they may be otherwise discarded merely for their unsuitable LCST behaviour in water. Moreover, our findings may enable us to better predict the biological properties of thermo-responsive polymers *in vivo* based on their  $T_{CP}$ . Therefore, these results may be used to optimise polymeric drug delivery systems for *in vivo* applications through *in vitro* studies.

## Conflicts of interest

The authors have no conflicts of interests to declare.

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