Hydrogel polymer appears to mimic the performance of the GroEL/GroES molecular chaperone machine

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Received 15th March 2006, Accepted 25th April 2006
First published as an Advance Article on the web 23rd May 2006
DOI: 10.1039/b603915d

Controlled protein folding/refolding remains a substantial challenge to the biotechnology industry. Robust and adaptable artificial polymer molecular chaperones could make important contributions towards solving this problem. Taking inspiration from the mechanism of the GroEL/GroES molecular chaperone machine, we report the preparation and testing of a selection of cross-linked thermo-responsive hydrogels, one of which is shown to assist quantitative refolding of a stringent unfolded protein substrate (mitochondrial malate dehydrogenase [mMDH]) during temperature cycling between hydrophobic and hydrophilic states. To our knowledge, this is the first hydrogel-only artificial polymer molecular chaperone to be derived, which is also potentially a generic artificial polymer molecular chaperone for use in a folding bioreactor.

Introduction

Molecular chaperones are proteins that assist the folding of other proteins without being involved in their final folded state. Although the three dimensional structure of a given protein is specified by its sequence of amino acid residues, the kinetic process of protein folding frequently needs assistance *in vivo* as well as *in vitro*.[1] In this context, GroEL and GroES are remarkable (Fig. 1). Both proteins come from the bacterium *Escherichia coli*, but homologues are found in all cells of all organisms. Together they are able to assist the folding or refolding of many unfolded protein substrates, and have therefore been of enormous interest to the specific and general scientific community.

Previously, we developed two model refolding assay systems involving mitochondrial malate dehydrogenase (mMDH) and cytochrome c to study the effect of GroEL and GroES on the kinetic pathway of protein refolding.[2–8] Data acquired from these protein refolding assays interlocked together to support the view that GroEL and GroES promote protein folding/refolding by a passive kinetic partitioning mechanism (Fig. 2).[8] According to this mechanism, the essential purpose of GroEL is to bind, isolate and then release protein folding/refolding intermediates (**U** to $\mathbf{I}_2$) vulnerable to bi-/multimolecular *n*th-order aggregation processes (where $n > 2$), in a controlled and cyclical fashion so as to suppress their free, solution concentrations below the critical threshold for aggregation. This encourages protein folding intermediates to partition kinetically along unimolecular pathway(s) to the native state, **N**, in preference to being trapped in aggregated states [$(\mathbf{I}_1)_m$ and $(\mathbf{I}_2)_m$].

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We have attempted to exploit the capacities of GroEL and GroES previously for protein folding/refolding by including both as part of a folding bioreactor.[5,6] Although the bioreactor was effective, the large quantities of adenosine 5′-triphosphate (ATP) required and problems with GroEL and GroES long-term stability in solution meant it was also expensive and rather impracticable. These problems led us to consider the possibility that alternative robust polymeric agents might be discovered with molecular chaperone-like properties, which would be better suited to biotechnology applications. The passive kinetic partitioning mechanism described above is made possible in large part by the conformational changes taking place in GroEL driven by GroES and ATP binding (Fig. 1). GroEL binds substrate protein folding intermediates in a high-affinity state (**T**-state) characterised by the presence of solvent-exposed hydrophobic amino acid residues and releases them once in a low-affinity (**R**-state) that possesses no exposed hydrophobic resides and so is hydrophilic in character (Fig. 1). We surmised that a polymer capable of similar controlled cyclical oscillations between hydrophobic and hydrophilic states might also be able to act as an artificial molecular chaperone assuming it also had the ability to bind folding intermediates during the hydrophobic (pseudo-**T**) state and release them when in a hydrophilic (pseudo-**R**) state. For this reason, we elected to study a range of polymeric thermo-responsive hydrogels.[9,10] These hydrogels are able to change from a hydrophobic state above a certain lower critical solution temperature (LCST) to hydrophilic states below that LCST. Simple thermal switching between two temperatures allows these hydrogels to cycle between their hydrophobic and hydrophilic states. Our hope was that one or more of the hydrogels under investigation might also be able to harness these thermally driven state changes in order to function as a GroEL/GroES-like artificial polymer molecular chaperone.

Results and discussion

Polymeric thermo-responsive hydrogel design was based upon *N*-isopropylacrylamide (NIPAAm) **1** and 2-hydroxyethyl

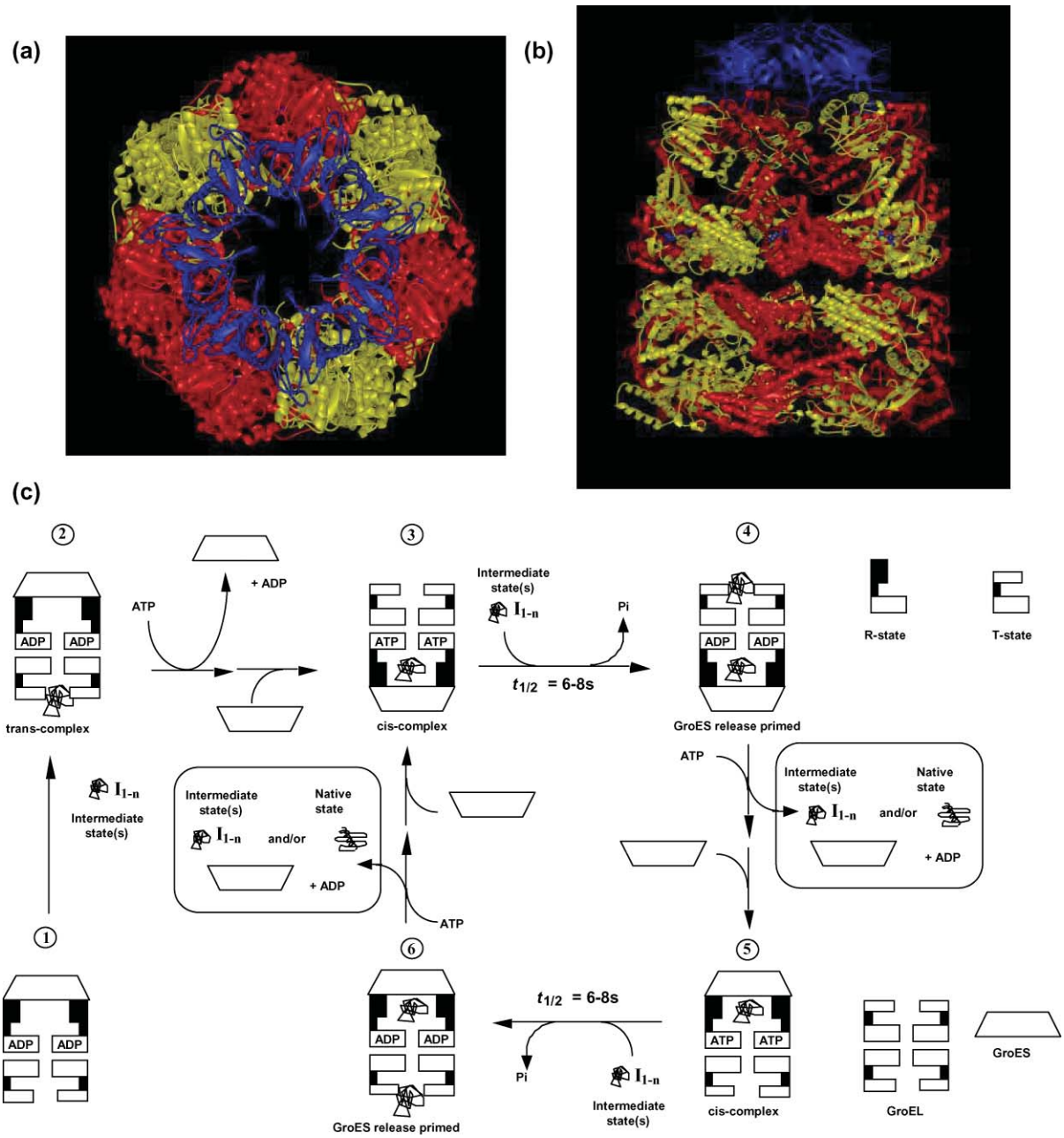


Fig. 1 Top view (**a**) and side view (**b**) of GroEL/GroES/$(ADP)_7$ complex. GroEL is 14mer (yellow and red), each subunit 57 259Da stacked in two rings with central cavities for protein substrate binding. GroES is 7mer (blue), each subunit 10 368Da in a single ring of 7 subunits.[24] (**c**) Complete GroEL/GroES molecular chaperone machine mechanism illustrating the regular cycle of binding and release of protein substrate (in various states of unfolding).[5,25] This regular "two-stroke motor" process of binding and release of protein substrate,[26,27] has been thought at various times to have catalytic effects on protein folding or else provide a means for the folding of substrate proteins at "infinite dilution" in the GroEL central cavities. However, the cycle has much more of a "one-size-fits-all" characteristic that is consistent with the passive kinetic partitioning mechanism for molecular chaperone machine activity described below (see Fig. 2).[8] The **T**-state and **R**-state nomenclature refer to the conformations of individual GroEL subunits in the homo-oligomeric structure. The **T**-state has a high affinity for substrate protein and the **R**-state a low affinity (the affinity for ATP is reversed). GroEL in the **T**-state has solvent-exposed hydrophobic amino acid residues used to interact with substrate protein folding intermediates in the position indicated; GroEL in the **R**-state nomenclature has undergone conformational changes to obscure those hydrophobic residues and adopt a more hydrophilic character. The term $\mathbf{I}_{1-n}$ refers to discrete substrate protein folding intermediates, **I**, of between **1** and ***n*** in number.

methacrylate (HEMA) **2** monomers, cross-linked with *N*,*N*′-methylenebisacrylamide (BAAm) **3** (see Scheme 1).[10] Our initial approach was to consider the creation of hydrogel networks with a pore structure within which protein folding/refolding intermediates could be isolated (by analogy with the existence of the GroEL cavities). Moreover, we expected to take advantage of the fact that the pore structure in such hydrogels can also undergo a volume expansion when brought below the LCST into a hydrophilic (pseudo-**R**) state (by analogy with the behaviour of the GroEL cavities, Fig. 1). However, recent studies

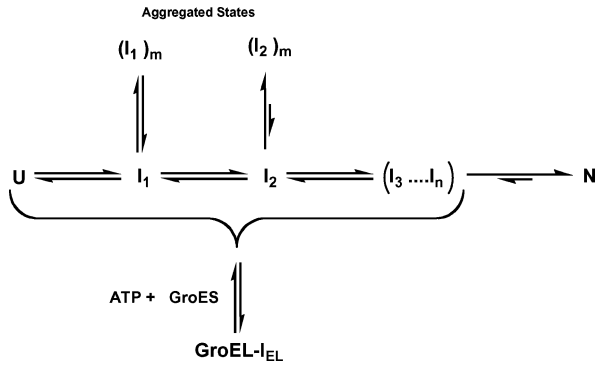


Fig. 2 The GroEL/GroES molecular chaperone machine operates by the passive kinetic partitioning mechanism. This mechanism assumes that protein folding is initiated at an unfolded state, **U**, which folds through a succession of intermediate states $\mathbf{I}_1$, $\mathbf{I}_2$, $(\mathbf{I}_3 \ldots \mathbf{I}_n)$ before reaching the native state, **N**. States $\mathbf{I}_1$ and $\mathbf{I}_2$ are considered arbitrarily to be unstable to aggregation, forming aggregated states $(\mathbf{I}_1)_m$ and $(\mathbf{I}_2)_m$ through interaction of their exposed hydrophobic surfaces. GroEL is potentially able to bind to all vulnerable protein folding intermediate states, except **N**, forming a GroEL-bound state **GroEL-$\mathbf{I}_{EL}$**. The nature of this state is a function of the requirement to optimise the free energy of association between GroEL and the given unfolded protein state under the given set of binding conditions. The binding interaction with GroEL is reversed in a controlled manner with the assistance of first ATP and then GroES binding, after which protein substrate is retained by the GroEL intra-cavity until ATP hydrolysis is complete ($t_{1/2}$ 6–8 s). Thereafter, protein substrate may be released into free solution ready to rebind again if necessary (see Fig. 1). As a result of this cyclical binding and controlled release into a GroEL cavity and then free solution, steady state concentrations of **U**, $\mathbf{I}_1$, $\mathbf{I}_2$ and $(\mathbf{I}_3 \ldots \mathbf{I}_n)$ are maintained below the critical threshold for aggregation so that these states are free to partition kinetically to **N**.[4,8]

have shown that the molecular chaperone machine may also assist the folding/refolding of proteins too large to be isolated within GroEL by cyclical binding and release of protein folding intermediates without encapsulation.[11,12] From this, we surmised by analogy that pore structures in a thermo-responsive hydrogel may also not be required for the hydrogel to promote correct protein folding/refolding. Instead, cyclical surface association and dissociation of protein folding/refolding intermediates might be sufficient (also by analogy with GroEL).

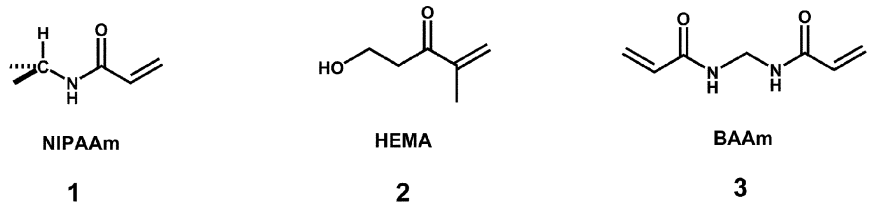
Hydrogels were prepared and screened for their ability to perform assisted refolding of 3 M guanidinium hydrochloride (GuHCl) unfolded-mMDH (see Experimental section), an unfolded substrate protein that is well known to have a stringent requirement for GroEL/GroES assistance in folding/refolding (see above).[2,3] Our approach was to perform a series of experiments in Eppendorf tubes with each different hydrogel in turn. Two thermal cycling programmes were used. **Programme A**, comprised of three 1 h cycles of 10 min at 36 °C and 50 min at 32 °C; **programme B**, comprised of three 1 h cycles of 10 min at 35 °C and 50 min at 25 °C. Both programmes also included a final 15 min phase at the higher temperature. The choice of cycle for a given hydrogel was determined by the central requirement that the LCST should be positioned between the higher and lower temperatures of the chosen thermal cycling programme in order for the hydrogel to have the opportunity to cycle between hydrophobic and hydrophilic states.

The cycle times are a good deal longer than the GroEL hydrophobic–hydrophilic cycle (Fig. 1), primarily because hydrogels cannot currently undergo structural adaptation with temperature as rapidly as GroEL does under the influence of GroES and ATP-binding, although switching times of minutes may be realizable soon.[13,14] Each thermal cycling programme was designed to begin at the higher temperature. In so doing, the hydrogel was expected to be in a fully hydrophobic (pseudo-**T**) state for the first 10 min of either programme in order to ensure optimal capture of vulnerable, aggregation-prone unfolded mMDH substrate protein refolding intermediates at the very beginning. Each programme was also chosen to end with an additional 15 min phase at this higher temperature in order to optimise the release of native mMDH protein from the hydrogel. Omitting this additional phase reduced the yield of active enzyme (results not shown), presumably due to retention of hydrophilic native mMDH in the hydrophilic gel.

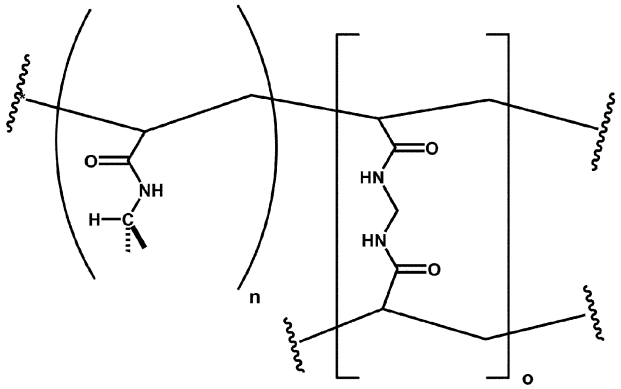
The effect of the thermal cycling programmes **A** and **B** on the yield of enzymically active native and spontaneously refolded mMDH (10 $\mu g m^{-1} l$) was characterised first. The data are shown (Fig. 3a) illustrating that both thermal cycling programmes have a modest effect on yield (particularly reducing that of native mMDH) but this is almost absorbed within the experimental error. Thereafter, as each hydrogel was prepared its effects on the yields of native and spontaneously refolded mMDH were evaluated with and without thermal cycling. The thermal cycling programme for each hydrogel was selected according to LCST as indicated above, with each gel only experiencing one of the two programmes as appropriate (see Table 1). Success was defined as that combination of hydrogel and thermal cycling that reproducibly enabled 100% recovery of mMDH enzyme activity from unfolded mMDH compared with comparable native mMDH controls under the same conditions of pH, temperature and buffer conditions (but in the absence of hydrogel).

Table 1 Summary of some of the hydrogel types prepared (see Scheme 1) and their main q_w and LCST parameters as described in the text

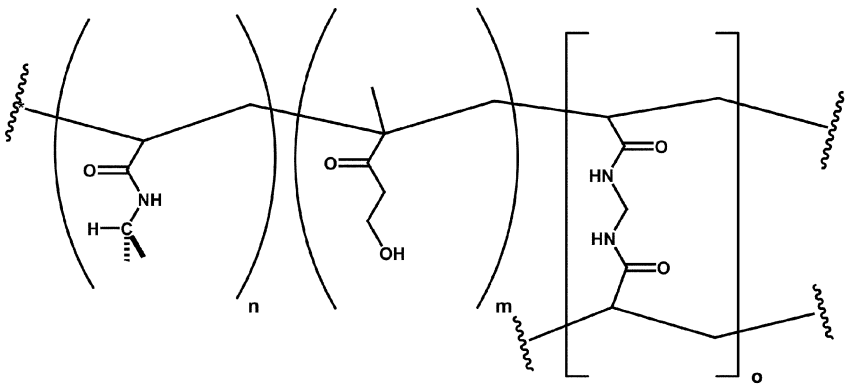
NIPAAm (HEMA)-BAAm hydrogel	q_w	LCST/°C	mMDH refolding cycle programme	Polymerisation medium (1 : 1, v/v)
PNI-BA0.03 **4**	3.90	29	**B**	Acetone : H_2O
PNI-BA0.3 **5**	3.50	28	**B**	Acetone : H_2O
PNI-BA3.0 **6**	3.17	34	**A**	Acetone : H_2O
MaPNI-BA36.6 **7**	0.69	34	**A**	Acetone : H_2O
PHE10-BA0.03 **8**	3.76	27	**B**	Acetone : H_2O
PHE10-BA0.3 **9**	2.78	30	**B**	Acetone : H_2O
PHE10-BA3.0 **10**	2.33	29	**B**	Acetone : H_2O
	4.88	30	**B**	Ethanol : H_2O
PHE30-BA3.0 **11**	1.78	29	**B**	Ethanol : H_2O



1 2 3



PNI-BAx gel



PHEy-BAx gel

Scheme 1 Molecular structures and naming scheme. Hydrogels are named such that *x* and *y* refer to the percentage of the associated crosslinker or monomer. Hence, *PNI-BA3.0* comprises 3% BAAm and 97% NIPAAm and *PHE10-BA3.0* comprises 3% BAAm, 10% HEMA and 87% NIPAAm.

Initially, NIPAAm **1** only gels were prepared, cross-linked using 0.03, 0.3 or 3.0% of BAAm **3** (*i.e.*, PNI-BA0.03 gel **4**, PNI-BA0.3 gel **5**, or PNI-BA3.0 gel **6** respectively). In all cases, PNI-BA gels **4**–**6** were ineffective but PNI-BA3.0 gel **6** data is shown as representative of this class of thermo-responsive hydrogels (Fig. 3b). The refolding of mMDH was then enabled in the presence of a macroporous gel that was prepared from NIPAAm **1** with 36.6% of BAAm **3** (*i.e.*, MaPNI-BA36.6 gel **7** respectively).[9] However, the gel **7** appeared to be only partially functional with or without thermal cycling. The same was found to be true when NIPAAm-HEMA gels were prepared with 10% HEMA **2** and cross-linked using 0.03, 0.3 or 3.0% of BAAm **3** (*i.e.*, PHE10-BA0.03 gel **8**, PHE10-BA0.3 gel **9**, or PHE10-BA3.0 gel **10** respectively). PHE-BA3.0 gel **10** data is shown.

Complete success was obtained by increasing the HEMA **2** content to 30%. In this case, a PHE30-BA3.0 gel **11** gave the desired result (Fig. 3b) assisting mMDH refolding to yield a near-100% recovery of enzyme activity (compared to native mMDH

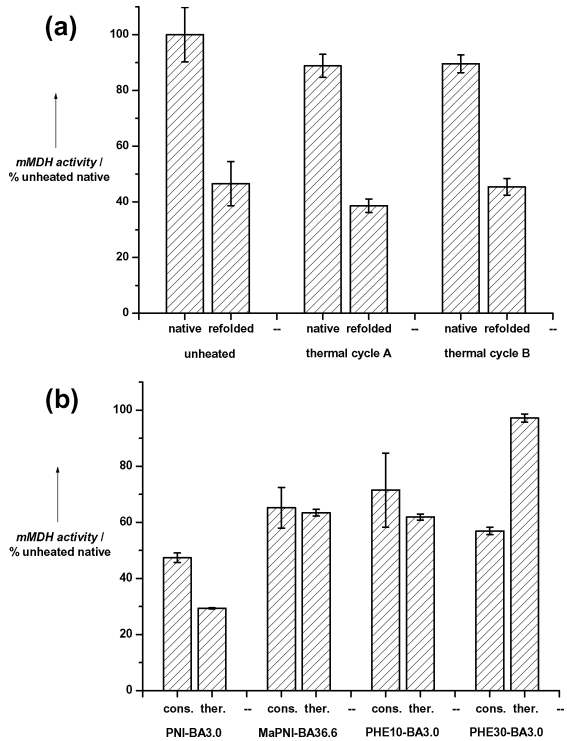


Fig. 3 Refolding of mMDH in the presence and absence of hydrogel, with and without thermal cycling. (**a**) A comparison between native and spontaneously refolded mMDH enzyme activity levels (10 μg ml^{-1}), after 3 h, with and without the use of thermal cycling programmes A and B. (**b**) A comparison between spontaneously refolded mMDH enzyme activity levels (10 μg ml^{-1}), after 3 h, expressed as a percentage of native mMDH controls in the presence of the indicated hydrogels, with (ther.) or without (cons.) the use of a thermal cycling programme. The thermal cycling programme was selected appropriately according to hydrogel LCST (see Table 1). Refolding buffer conditions in all cases were 150 mM sodium phosphate, pH 7.6, containing 20 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 10 mM KCl. mMDH was unfolded prior to use with 3 M-guanidinium chloride for 2 h at room temperature and then diluted (to 10 μg ml^{-1} final concentration) into refolding buffer to initiate spontaneous refolding in the presence of the indicated additives. The efficiency of refolding is judged to be a function of the recovery of mMDH enzyme activity relative to concentration matched, unheated native mMDH enzyme controls, in the absence of hydrogel. All experiments reported were performed in triplicate in order to minimize errors.

controls) with thermal cycling. Measured protein concentrations post gel **11** gel-assisted refolding of mMDH revealed that protein recoveries were essentially constant irrespective of the presence of hydrogel or not. This indicates that the PHE30-BA3.0 gel **11** was not engaging in non-specific scavenging and sequestration of mMDH (data not shown). On closer analysis of gel **11** activities we observed that most of the gel-assisted refolding of mMDH (>80%) took place within the first temperature cycle, but two further cycles were necessary to ensure that the yield of native mMDH reached 100%. As noted above, the first cycle begins with the gel in a hydrophobic (high affinity, pseudo **T**) state for 10 min followed by slow adaptation of the gel to the hydrophilic state (low affinity, pseudo **R**) during which time period hydrophobic interactions may still be possible owing to the slowness of structural adaptation. Hence, PHE30-BA3.0 gel **11** may be able to accommodate multiple repeated hydrophobic associations between mMDH folding intermediates and the gel during this structural adaptation thereby continuously suppressing the free, solution concentration of vulnerable mMDH refolding intermediates without preventing productive refolding from taking place, in direct analogy to the GroEL/GroES mechanism (Fig. 1).

Others have demonstrated the capture of unfolded proteins with thermo-stimulated hydrogels although in those cases release from the polymer and subsequent protein refolding was triggered through the addition of cyclodextrins acting as detergent strippers.[15–21] Our PHE30-BA3.0 gel **11** system presented here requires no osmolytes or small molecule aids to effect refolding while mimicking a capture–release function through temperature cycling. Furthermore, gel **11** shows some interesting differences with the other hydrogels prepared and used here. Firstly, this hydrogel was prepared in a polymerisation medium comprised of ethanol : water (1 : 1, v/v) rather than acetone : water (1 : 1, v/v) more usually favoured for the preparation of the other gels (Table 1). Secondly, when the LCST values and the mass-swelling (q_w) ratios of all the hydrogels used above are analysed (Table 1), gel **11** can be seen to have one of the lowest q_w and one of the lowest LCST values. This indicates that PHE30-BA3.0 gel **11** is one of the densest gels studied with a small level of porosity, but high levels of thermal flexibility. The high ratio of HEMA **2** involved and the extent of BAAm **3** cross-linking have together resulted in a hydrogel with an interesting balance of properties that appears to be useful to assist protein folding/refolding.[22] The fact that this hydrogel was more effective than those corresponding hydrogel polymers that appeared to possess a larger pore volume (*i.e.*, larger values of q_w) with equivalent flexibility suggests that site isolation of folding intermediates within the porous hydrogel network is indeed not necessary to promote correct protein folding/refolding by thermo-responsive hydrogels, as mentioned above.

Experimental

Hydrogel preparation

Hydrogels were prepared as follows. The general procedure was adapted from Sayil and Okay,[9] and was used for all hydrogels synthesised. Both monomer(s) and crosslinker were dissolved in the polymerisation medium [acetone : water (1 : 1, v/v) or ethanol : water (1 : 1, v/v)] and the total monomer concentration adjusted to 20% (w/v) with crosslinker concentration set to 0–36.6% (w/v), all in a total volume of 10 ml. This solution was then purged with N_2 for 20 min. The polymerisation initiator ammonium persulfate (APS) was added (0.35 mM final), along with the accelerator *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (0.16 mM final) and then the polymerisation reaction tube was sealed, immersed in an ice bath and the reaction allowed to proceed for 24 h. Following polymerisation, the gel was immersed in an excess of water (100 ml) to remove unpolymerised monomers, soluble polymers, accelerator and initiator. To facilitate drying, the resulting hydrogels were submerged in a series of baths (100 ml) composed of water and increasing concentrations of acetone or ethanol (20, 40, 60 and 80%) to three final baths of 100% acetone or ethanol. Finally, the hydrogel was lyophilised to a constant mass to remove all traces of solvent.

Mass swelling ratio determination

The mass swelling ratio (q_w) is a measure of the mass of solvent that a hydrogel absorbs and allows comparison of gels of different composition.[9] It is calculated with eqn (1):

$$q_w = m_{sw}/m_d \quad (1)$$

where m_{sw} is swollen mass and m_d is dry mass. The dry mass was determined following lyophilisation in a Heto Drywinner freeze dryer. The dry gel was then immersed in 150 mM sodium phosphate, pH 7.6, containing 20 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 10 mM KCl until equilibration, such that the gel had absorbed a maximum volume of solvent (~4 days). Excess buffer was removed and the gel weighed to give the swollen mass.

LCST determination

The lower critical solution temperature (LCST) is the point at which the hydrogel gel switches from the hydrophilic to the hydrophobic state and *vice versa*. As with q_w, the LCST depends greatly on the composition of the gel and the LCST of each gel was measured. Hydrogels were immersed in 150 mM sodium phosphate, pH 7.6, containing 20 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 10 mM KCl until equilibration, and then placed in a Stuart Scientific block heater at 25 °C. The temperature was raised by 1 °C in 5 min intervals. Hydrogel samples were observed and the LCST recorded as the temperature at which complete clouding and gel shrinkage occurred.

mMDH refolding assays

The hydrogel mMDH assay is an adaptation of the mMDH assay used previously to determine the activity of GroEL and GroES.[2,3] In short, mMDH concentration was determined spectroscopically according to the absorbance $A_{280}^{1\%} = 2.5$[23] and a monomer molecular weight M_r of 35kDa. All mMDH concentrations stated refer to the homo-dimer. Hydrogels were soaked from dry in 150 mM sodium phosphate, pH 7.6, containing 2 mM β-mercaptoethanol, 10 mM EDTA, and 3 M guanidinium chloride to equilibration (~4 days). When hydrogels were ready, mMDH protein was then dialysed in 150 mM sodium phosphate, pH 7.6, containing 2 mM β-mercaptoethanol, 10 mM EDTA (3 × of 500 ml) overnight to give native mMDH, then in 150 mM sodium phosphate, pH 7.6, containing 2 mM β-mercaptoethanol, 10 mM EDTA, and 3 M guanidinium chloride for 2 h at room temperature to give unfolded mMDH. Refolding was initiated by dilution of unfolded mMDH (to 10 μg ml^{-1}, 143 nM final concentration) in refolding buffer (500 μl) of 150 mM sodium phosphate, pH 7.6, containing 20 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 10 mM KCl. Refolding experiments were performed either without further additives (spontaneous refolding) or in the presence of different hydrogels with or without thermal cycling. Thermal cycling was introduced with either **programme A** or **programme B** that are described in the main text. Programmes were selected appropriately according to hydrogel LCST, also as described in the main text (see Table 1). For each refolding experiment, the efficiency of refolding was quantified by measuring the level of mMDH enzyme activity in solution aliquots (20 μl) at the end of each refolding experiment. All artificial polymer molecular chaperone experiments were performed in triplicate.

In brief, enzyme activities were measured by introducing these solution aliquots (20 μl) into assay buffer (980 μl) comprised of 150 mM sodium phosphate, pH 7.6, with 2 mM β-mercaptoethanol, 0.5 mM oxaloacetate and 0.2 mM NADH, preheated in a UV-transparent cuvette at 30 °C in the Pharmacia Ultraspec III spectrophotometer. The initial gradient of $-dA_{360}/dt$ was determined as a measure of mMDH activity in the cuvette under substrate saturating k_{cat} conditions, and normalized as a percentage of the gradient produced by concentration matched, unheated native mMDH enzyme controls, kept under equivalent pH and buffer conditions in the absence of hydrogel. Final protein concentrations at the end of each refolding experiment were determined by Pierce BCA protein assay kit according to the manufacturer's instructions.

Conclusion

Our data suggest that by taking inspiration from the mechanism of the GroEL/GroES molecular chaperone machine, we have been able to discover a simple, robust hydrogel that assists refolding of a stringent, unfolded protein substrate. We suggest that the PHE30-BA3.0 gel **11** may assist protein folding/refolding in a manner that mimics the GroEL/GroES mechanism (at least in part), but at this stage this suggestion is only hypothetical without additional evidence. We suppose that our artificial hydrogel molecular chaperone could also be a useful tool in biotechnology to assist the correct folding of troublesome recombinant proteins as part of a folding bioreactor,[5,6] although we would expect that key parameters such as cycle temperatures and buffer conditions would need to be optimized for each different unfolded substrate protein concerned. Furthermore, additional variations in polymer composition may be necessary to allow for shorter, more responsive cycle times.

Acknowledgements

We thank the Mitsubishi Chemical Corporation and IC-Vec Ltd for their support of the Imperial College Genetic Therapies Centre. JD would like to thank the EPSRC for personal support.

References

1 F. U. Hartl and M. Hayer-Hartl, *Science*, 2002, **295**, 1852.
2 A. D. Miller, K. Maghlaoui, G. Albanese, D. A. Kleinjan and C. Smith, *Biochem. J.*, 1993, **291**, 139.
3 J. P. Hutchinson, T. S. H. El-Thaher and A. D. Miller, *Biochem. J.*, 1994, **302**, 405.
4 C. M. Smith, R. J. Kohler, E. Barho, T. S. H. El-Thaher, M. Preuss and A. D. Miller, *J. Chem. Soc., Perkin Trans. 2*, 1999, 1537.
5 R. J. Kohler, M. Preuss, H. Jones and A. D. Miller, in *Gene cloning and expression technologies*, ed. M. P. Weiner and Q. Lu, Eaton Publishing, Westborough, MA, 2002, pp. 439.
6 R. J. Kohler, M. Preuss and A. D. Miller, *Biotechnol. Prog.*, 2000, **16**, 671.
7 M. Preuss, J. P. Hutchinson and A. D. Miller, *Biochemistry*, 1999, **38**, 10272.
8 H. Jones, M. Preuss, M. Wright and A. D. Miller, *Org. Biomol. Chem.*, 2006, **4**(7), 1223.
9 C. Sayil and O. Okay, *Polymer*, 2001, **42**, 7639.
10 W. F. Lee and Y. L. Huang, *J. Appl. Polym. Sci.*, 2000, **77**, 1769.
11 T. K. Chaudhuri, G. W. Farr, W. A. Fenton, S. Rospert and A. L. Horwich, *Cell*, 2001, **107**, 235.
12 G. W. Farr, W. A. Fenton, T. K. Chaudhuri, D. K. Clare, H. R. Saibil and A. L. Horwich, *EMBO J.*, 2003, **22**, 3220.

13 M. M. Ozmen and O. Okay, *Polymer*, 2005, **46**, 8119.
14 J. T. Zhang, S. X. Cheng, S. W. Huang and R. X. Zhuo, *Macromol. Rapid Commun.*, 2003, **24**, 447.
15 D. Lu, K. Zhang and Z. Liu, *Biochem. Eng. J.*, 2005, **25**, 141.
16 D. Lu, Z. Liu, M. Zhang, X. Wang and Z. Liu, *Biochem. Eng. J.*, 2005, **27**, 336.
17 S. Yamaguchi, C. Hong, T. Mannen, S. Tsukiji and T. Nagamune, *Biotech. Lett.*, 2004, **26**, 1787.
18 Z. F. Cui, Y. X. Guan, J. L. Chen and S. J. Yao, *J. Appl. Polym. Sci.*, 2005, **96**, 1734.
19 Z. F. Cui, Y. X. Guan and S. J. Yao, *Chin. J. Chem. Eng.*, 2004, **12**, 556.
20 Y. J. Chen, L. W. Huang, H. C. Chiu and S. C. Lin, *Enzyme Microb. Technol.*, 2003, 120.
21 Y. Nomura, Y. Sasaki, M. Takagi, T. Narita, Y. Aoyama and K. Akiyoshi, *Biomacromolecules*, 2005, **6**, 447.
22 D. Lu, Z. X. Liu, M. L. Zhang, Z. Liu and H. M. Zhou, *Biochem. Eng. J.*, 2005, **24**, 55.
23 E. M. Gregory, F. J. Yost, Jr., M. S. Rohrbach and J. H. Harrison, *J. Biol. Chem.*, 1971, **246**, 5491.
24 Z. Xu, A. L. Horwich and P. B. Sigler, *Nature*, 1997, **388**, 741.
25 M. Preuss and A. D. Miller, *FEBS Lett.*, 2000, **466**, 75.
26 G. Lorimer, *Nature*, 1997, **388**, 720.
27 Z. H. Xu and P. B. Sigler, *J. Struct. Biol.*, 1998, **124**, 129.

