Recovery of Intact Homogenous Proteins Stored within Mechanically Releasable Supramolecular Gels

Simona Bianco,[1]† Muhammad Hasan,[2]† Ashfaq Ahmad,[1] Sarah-Jane Richards,[3,4] Bart Dietrich,[1] Matthew Wallace,[5] Qiao Tang,[3] Andrew J. Smith,[6] Matthew I. Gibson,[2,3,4,7]* Dave J. Adams[1]*

[1]Department of Chemistry, University of Glasgow, Glasgow, G12 8QQ, UK

[2]Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry, UK

[3]Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK

[4]Department of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL, UK

[5]School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

[6]Diamond Light Source Ltd., Diamond House, Harwell Science and Innovation Campus, Didcot, Oxfordshire OX11 0DE, UK

[7]Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK

† Authors contributed equally

*Corresponding author. Email: matt.gibson@manchester.ac.uk; dave.adams@glasgow.ac.uk

Abstract:

Advanced biologic therapies are distributed cold, using significant energy, limiting equitable distribution in low-resource countries, and placing responsibility on the user for correct storage and handling. Here, we designed a stiff hydrogel which stabilizes proteins against thermal denaturation even at 50°C, combined (unlike current technologies) with the unprecedented function of delivering pure, excipient-free protein upon mechanical release from a syringe. Macromolecules can be loaded at up to 10 wt % without impacting the mechanism of release. This unique stabilization and excipient-free release synergy offers a practical, scalable and versatile solution to enable the low cost, cold chain-free and equitable delivery of advanced therapies to all corners of the planet.

A long-standing challenge is how to formulate proteins and vaccines to retain function during storage and transport and to remove the burdens of cold-chain management. Any solution must be practical to use, with the protein being released/applied using clinically relevant triggers. Cold chain management is the current best solution for protein transport but requires significant infrastructure and energy. For example, in research labs, a single freezer at -80°C can consume as much energy per day as a small household.[1] This is especially challenging in low or middle income (LMIC) countries where the energy requirements exclude equitable sharing of advanced medicines. 75% of biological (protein/cell) therapies and all vaccines require cold-chain

management; the cost of cold-chain management in clinical trials has increased by ~ 20% since 2015, reflecting this complexity. Bespoke formulations and excipients are currently required, with trehalose,[2] sucrose or polymers[3] widely used which stabilize proteins by replacing surface water molecules making denaturation thermodynamically less likely; this has enabled both freeze-dried proteins and frozen proteins. For example, the HPV (human papilloma virus) vaccine requires aluminum salt adjuvants to function, but these render it unstable against freeze/thaw,[4] leading to a complex and expensive supply chain. mRNA vaccines are transported and stored at -80°C, limiting their geographical distribution. Other ideas involve ensilication[5] and chemical modification of proteins.[6] In short, protein stabilization is major challenge with no universal solution.[7,8]

Consideration of the pathways of thermal protein deactivation shows that irreversible aggregation, rather than chemical degradation or unfolding is the primary mechanism for loss of activity. For example, simple mechanical stimulation (shaking) of insulin leads to aggregation into amyloid-type fibers and loss of efficacy and bioavailability and the gene therapy Zolgensma™ has just a 14-day shelf life, cannot be agitated, and must be retained between 2 and 8°C. Clearly, this is a barrier to wider use. Aggregation of recombinant human interferon beta (rhIFN-β) leads to immunogenicity for patients with multiple sclerosis.[9] Emerging antibody-based therapies have a major problem with aggregation at all stages of their life cycle, necessitating complex formulation processes.

Maynard et al. have developed synthetic polymers with trehalose side chains for protein stabilization, enabling both freeze-drying and heating stabilization, but the conjugation reduces activity.[10] However, for many therapeutic applications, covalent conjugation of a polymer using multistep synthetic chemistry and purification is not practical and has the requirement to seek regulatory approvals for each material, significantly increasing the barrier to clinical translation. This is a broad issue that means any stored protein is eventually delivered mixed with its stabilization agents, rather than the desirable solution of protein in buffer. Fully reversible hydrogels based on PEG (polyethylene glycol) have emerged to protect diverse proteins against thermal stress[11,12] and mRNA formulations have been stabilized in a dissolvable matrix.[13] Similarly, frozen formulations to prevent ice crystal growth suppress protein aggregation.[14] However, in each case these gels are based upon new chemical entities (and hence a significant barrier to human use), may require a chemical stimulus (pH, sugars) to release the protein, which in practice must be dosed by the user, and take more than 1 hour to release the protein. Whilst potent, these strategies result in protein mixed with gel-forming components, which require separate approval and rigorous evaluation of their safety profile before being suitable for human use. Even materials based on PEG have immunological concerns for frequent exposure.[12,15,16]

An ideal biologic storage/transport solution would i) remove/reduce cold chain requirements; ii) be broadly applicable; iii) have zero or minimal excipients in the delivered protein solution; iv) not require complex chemical triggers to release; v) tolerate high loadings up to 100 mg/mL. Here we solve each of these points using a simple, low resource strategy. We show that low molecular weight supramolecular gel networks can physically entrap proteins preventing irreversible aggregation, and hence retain function at temperatures as high as 50°C for at least 4 weeks. Upon pushing out of the syringe through a filter, pure non-aggregated homogeneous and functional protein is released with all excipients trapped in the filter, ensuring only protein and buffer are delivered. This differs fundamentally from previous approaches[16] which require a chemical trigger or dilution[17] and do not therefore provide pure protein.

Results and discussion

Gels can be formed by the self-assembly of low molecular weight gelators (LMWG).[18,19] These are small molecules that self-assemble to give long fibrous structures that entangle to form a 3-dimensional network. These gels tend to be very stiff but break at low strain. This breaking at low strain has been widely described as a drawback of these systems.[20,21] However, here we use this perceived failing as a unique benefit enabling a mechanical trigger for homogeneous protein delivery that is not possible with conventional gels.

We formed gels from a range of LMWG (Fig. S1). Based on gelation at physiological conditions, we focused on one system (Figure 1A). The advantage of this system is that gelation can be induced by the addition of a TRIS buffer solution to a concentrated solution of the LMWG at pH 8 to give a final self-supporting material at pH 6.8. The mechanical properties can be tuned by the addition of a calcium salt (Figure 1B). In the absence of the calcium salt the system has relatively low storage (G′) and loss (G″) moduli, with the material breaking at low strain (<1%). The materials are not true gels in that there is some frequency dependence on G′ and G″, and at low frequency G′ and G″ are essentially the same (Fig. S3e). In the presence of the calcium salt, the moduli are improved, and the samples are less frequency dependent (Fig. S3e), but crucially the material still breaks at low strain (Figure 1C, Fig. S2, S3 and S4). Using small angle X-ray scattering (SAXS, Fig. S5, Table S1) to probe the sample without salt, the scattering data can be fit simply to a power law (Fig. 1D, red), suggesting the presence of large heterogeneities in the network or irregular aggregates in the gel. The data for the gel prepared in presence of $CaCl_2$ could be fit to an elliptical cylinder model combined with a power law (Fig. 1D, black; Fig. S6, Table S2). The data further presents a peak at q = 0.154 Å (d = 40.8 Å), which suggests that the gelator fibers are part of a periodic arrangement in the structure with a crystalline character. Circular dichroism (CD) indicates a different arrangement in the secondary structures of the systems. The CD spectrum of the sample without any salt exhibits a negative band with a shoulder in the range of 200-240 nm (Fig. 1E, red and Fig. S7). The sample with $CaCl_2$ shows a negative band around 235 nm and a positive band occurring near 190 nm, which could be indicative of a β-sheet-like arrangement (Fig. 1E, black and Fig. S7).

Encapsulating additives including proteins in gels formed by LMWG is trivial.[22,23] The proteins are trapped in the space between the fibers; if the gel network is sufficiently permanent and the pores between fibers sufficiently small, the protein is unable to easily diffuse within the gel and hence aggregate.[24] Indeed, restricted diffusion has been shown to occur in LWMG via controlled release experiments.[25,26] Combining this concept with the potential for these gels to break at low strain means we can form gels within a syringe for long term room temperature storage, followed by release through a syringe filter; the essentially insoluble extended supramolecular network will be trapped within the syringe filter, meaning that only the pure protein is released (Fig. 1F and 1G).

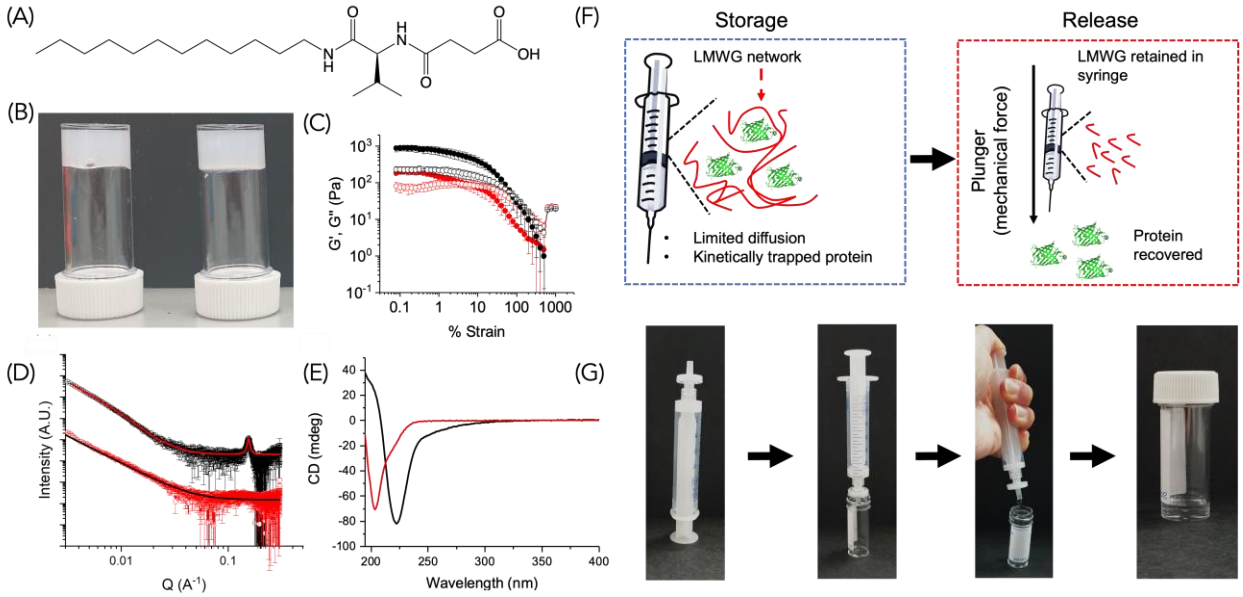

Fig. 1. Initial gel studies. (**A**) Chemical structure of the gelator used here. (**B**) Photographs of example gels formed (left) at pH 6.8 and (right) at pH 6.8 in the presence of a calcium salt. (**C**) Typical strain sweep for gels formed in the absence (red) and (black) presence of a calcium salt, showing a high stiffness (G′, filled circles) but a low breakage strain. (**D**) SAXS patterns of gels made in presence of (black) calcium chloride and (red) without $CaCl_2$. The fits obtained through model fitting are overlayed on each spectrum (details in Supporting Information). (**E**) CD spectra of gels in in presence of (black) calcium chloride and (red) without $CaCl_2$. (**F**) Cartoon showing the concept of this work. (G) Images exemplifying the syringe filter release protocol for gels. The gel is first loaded in a syringe fitted with a 0.22 μm filter. The gel is passed through the filter by gentle extrusion, releasing a clear solution.

A model macromolecular cargo was loaded into gels to probe the impact on gel structure, function, and release. Dextran (M_r 6000 Da, hydrodynamic radius = 1.6 nm[27]) was chosen as a non-interacting additive with a similar size to insulin.[28] Dextran was added at concentrations up to 10 wt% (Fig. 2A, a value far above the loadings required for real-world drug storage and in line with recent moves towards higher concentration biologic formulations; see also Fig. S8), whilst keeping all other parameters constant. Our gelation approach is almost instantaneous: in comparison, chemically triggered hydrogel storage solutions require addition of dosed release agents, such as sugars, and require up to an hour to gel.[16] The gel properties were retained over all loadings, which is essential for a broadly applicable storage solution (Fig. 2B). Extended aging experiments showed that these properties did not change significantly over at least 136 days (Fig. 2C). The gels can be formed *in situ* inside a syringe. The added dextran does not affect the gelator's packing (Fig. S8 and Table S2). As initial proof-of-concept, we entrapped a fluorescent dextran within a gel and then extruded through a syringe filter. Pure dextran was collected directly (Fig. 2D), with 92±2 % of the expected amount collected showing that there is little entrapped permanently within the network (Fig. S9). No gelator could be detected in the extruded liquid by ^{1}H NMR spectroscopy (Fig. 2E), showing that the gel network is trapped within the syringe filter and our simple approach leads to excipient-free homogeneous protein on demand. The gelator is trapped in the filter as shown by FT-IR data (Fig. S10). To add further quantification, we used LMWG which incorporate

an inherently fluorescent group. After extrusion, very low fluorescence could be detected in the extrudates (< 5 ppm; Fig. S11), showing the power of this approach (Fig. S12-S16).

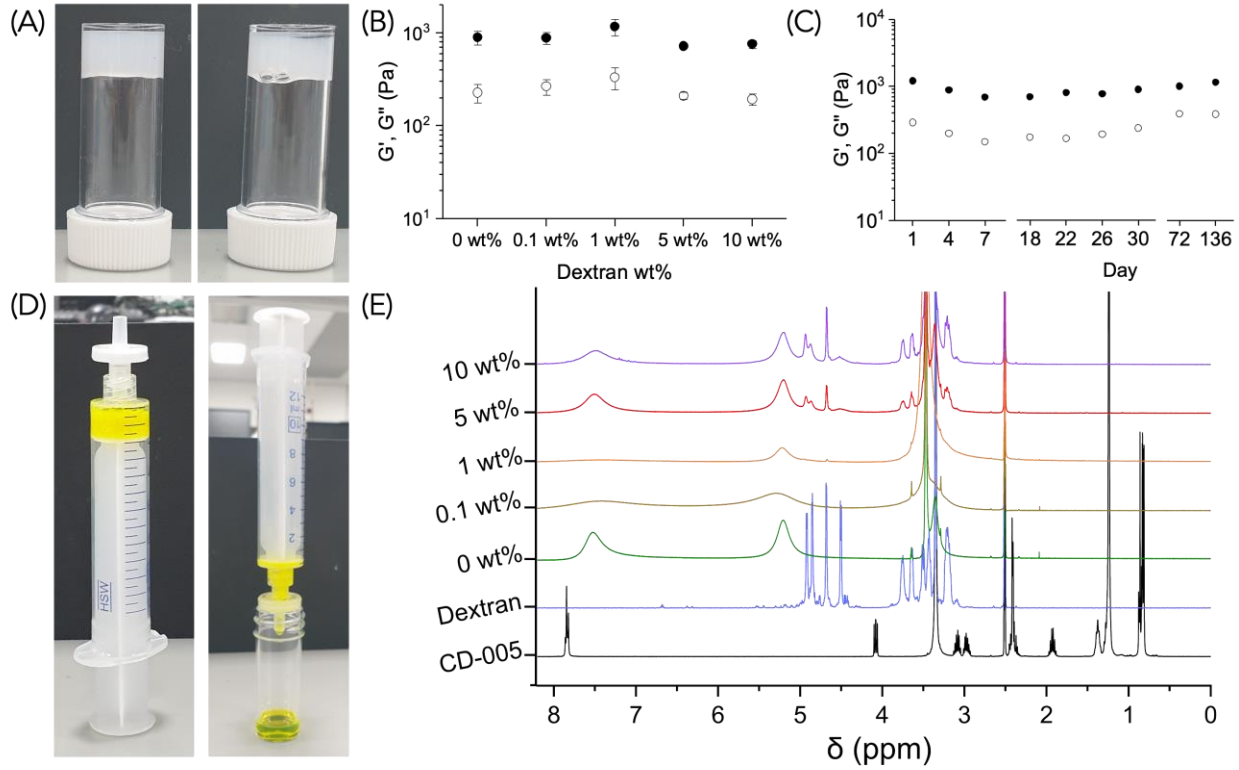

Fig. 2. Preliminary studies. (**A**) Gels formed using $CaCl_2$ encapsulating dextran (left, 1 wt%; right, 10 wt%). (**B**) Rheological data for gels formed encapsulating dextran. (**C**) Rheological data for gels formed encapsulating 1 wt% dextran over time. (**D**) Extrusion through a syringe filter releases the entrapped material. (**E**) NMR evidence showing that no gelator is released during extrusion. The peaks at around 5.0 and 7.6 ppm are from the buffer.

With confirmation that macromolecules can be encapsulated and selectively released by mechanical trigger, protein storage was undertaken to demonstrate recovery and the mode of action (preventing aggregation). As a first challenge, we chose to encapsulate (bovine) insulin as a model therapeutic protein (Fig. 3A). Insulin is one of the most widely used protein drugs in the world but must be stored cool or freeze-dried requiring the user to self-prepare the solution prior to injection.[29] The instructions for insulin are very specific not to shake, as it is prone to aggregate into extended amyloid fibers losing efficacy and bioavailability. After initial screening using dynamic light scattering, insulin was loaded into gels (with no Ca^{2+} in this case), which were then incubated at 25°C and shaken at 600 rpm: this is very aggressive agitation far beyond a real-world stress. After this, pure excipient-free protein was recovered by action of the syringe and any aggregation to amyloid was determined by the ThT (thioflavin T) assay, Figure 3A. It should be noted, all samples were treated identically, and the solution-only samples were passed through the syringe filter, so there was no bias due to filter/exclusion of aggregates. Limited aggregation was detected in the gel loaded samples, but extensive aggregation occurred in the absence of the gel. Saturation transfer difference (STD) NMR spectroscopy strongly implies that that the insulin is not adsorbed onto the fibers of the gel whilst encapsulated (Figure S17). We note that the rheological properties of the gels are somewhat affected by the addition of insulin (Fig. S18), but

the overall process is still effective; in no case was gelator detected in the solutions extruded through the syringe filter (Fig. S19 and S20), although we highlight overlapping of peaks makes the analysis more difficult than in the dextran case. Complete recovery of the expected volume of insulin (100% taking into account the volume of the syringe filter used to collect the gelator) was recovered in the extruded sample (Fig. S21). Mass spectrometry before and after the encapsulation and release demonstrate the chemical stability of the insulin under these conditions (Fig. S22). Additionally, SAXS shows that the scattering from a solution of insulin before encapsulation and from a solution after encapsulation and release is very similar, demonstrating a lack of aggregation (Fig. S23). Hence, our approach provides dual protection against aggregation and allows protein release in a pure form. Indeed, we can encapsulate insulin at a concentration of 3.2 mg/mL, a concentration around that of commercial formulations (U100), with complete release of excipient free insulin (Fig. S19 and S20). We also tested two structurally related LMWG (Fig. S24) which also protected insulin against aggregation, confirming our mode of action linked to physical immobilization, not a specific interaction. Finally, a cell-based assay confirmed that released (human) insulin after 24hrs in-gel agitation retained CD220 binding (EC_{50} fresh/post-gel = 237 to 337 ng/mL) demonstrating correct folding and retention of biological activity (Fig. 3D) after extrusion albeit with some loss. We note 24 hrs shaking at 600 rpm is rather exaggerated and intended to challenge the gel which retained its structure throughout.

As a further test, beta galactosidase (β-gal) was encapsulated (with added Ca^{2+}). β-Gal catalyzes the decomposition of *o*-nitrophenyl-galactoside allowing colorimetric evaluation of activity. The gels were placed in an incubator at 50°C (a stringent test beyond feasible transport conditions) for 7 days. We note that the rheological properties of samples at this temperature are still gel-like as long as the calcium salt is present (Fig. S25), but that calcium does reduce the recovered protein yield. After this time, protein was recovered by simple syringe action. Compared to fresh, β-gal in buffer alone retained just 26.1 ± 0.4 % of its function but across 15 repeats (3 technical, 5 biological) the gel-stored protein recovered 84.4 – 108.1 % of function (per protein mass), with a mean of 97.1 ± 2.8 %. This is equal performance to a multi-armed boronic-acid function PEG hydrogel,[11] but our technology functions at biomedically relevant volumes (0.5 mL versus 50 μL), does not require addition of any triggers such as sugars or acid, and the resulting protein was excipient-free. As an even more challenging test, gels were stored at 50°C but for 4 weeks. This removed all activity from the solution-stored β-gal, but the gel-stored protein retained up to 20 % of its function: this is a remarkable level of recovery from a harsh storage condition at conditions that even the hottest locations on earth rarely face. To show the gels are robust, samples were mailed through the UK postal service to experience a realistic distribution challenge (shaking, dropping, temperature range; Fig. S26). Over the 2-day delivery, the gels were recovered intact and ~ 100% activity was retained, showing that this method could conceptually be used for the postal delivery of active proteins for healthcare (Fig. S27).

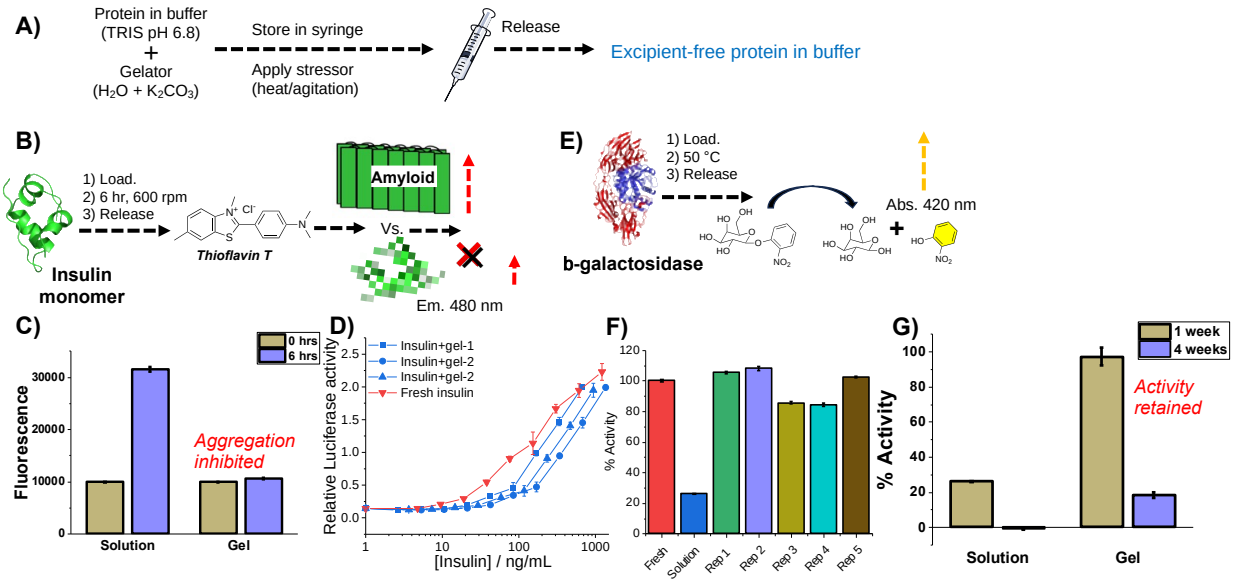

Fig. 3. Retention of protein function in hydrogels.; A) General preparation process for gel; B) Probe for aggregation inhibition using Insulin with THT assay; C) Quantification of THT assay; D) Cell-based assay for CD220 recognition by insulin following recovery from gel with shaking @600rpm for 24 hrs, compared to fresh; E) β -galactosidase assay for post-recovery function; F) Activity recovery after 7 days at 50 C for independent repeats; G) Comparison of 1 week and 4 week storage and recovery of β-galactosidase.

Conclusions

We have demonstrated that protein function is retained in stiff low molecular weight gels when stored at temperatures as high as 50°C for up to 4 weeks by preventing irreversible aggregation. Due to the unique mechanical properties of the low molecular weight gels, they break when passed through an in-line syringe filter releasing the fully functional protein cargo with no need for any chemical triggers and the effect is instantaneous. Due to the extended supramolecular network formed by the gelators, all the gel components (e.g., excipients) are retained in the filter, meaning only protein and buffer is released. To be very clear, this means only pure protein in buffer is delivered, unlike all competing technologies which lead to protein containing novel, often untested, excipients which would then be delivered to a patient, and hence face regulatory and safety barriers to use. For example, Meis et al. have described a system encapsulating a therapeutic to maintain function. In their case, release occurs on dilution meaning the polymer gel components are still present.[17] Similarly, encapsulation and stability has been shown within a multiarm PEG gel.[16] A release solution is required to break the gel, meaning that delivery would contain the gel components and release solution. Hence, our excipient-free approach is not just an iteration, but a major step forward. This is also a benefit over freeze-dried or frozen formulations requiring no on-use reconstitution, nor energy-intensive ultra-low freezers, respectively. We have therefore provided a new scalable means of storing, transporting, and safely using biologics, affording a simple and effective strategy to deliver biologic therapies to low resource settings where they are needed most.

Acknowledgments. This work has been supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 814236, European Research council grant 866056, Royal Society for an Industry Fellowship (MIG) 191037, Engineering and Physical Sciences Research Council (EP/L021978/2) and the University of Glasgow. MW thanks UKRI for a Future Leaders Fellowship (MR/T044020/1). This work was carried out with the support of Diamond Light Source, instrument I22 (proposal SM27906). We thank Dr. Nathan Cowieson (Diamond Light Source) for help collecting the SAXS data at B21 (SM37100). This work benefitted from the SasView software, originally developed by the DANSE project under NSF award DMR-0520547. We would like to that Dr. Jessica R. Bame and Dr. Graeme J. Anderson (University of Strathclyde) for collecting the HRMS data.

Author contributions. Conceptualization: MIG, DJA; Data Curation: SB, QT, SJR, MH, AA, AS, MW; Formal Analysis: SB, MH, MIG, DJA; Investigation: SB, QT, MH, SJR, AA, MW, AS; Methodology: SB, MH, AS, MW, MIG, DJA; Visualization: SB, MH, MIG; Funding acquisition: MIG, DJA; Project administration: MIG, DJA; Supervision: MIG, DJA; Writing – original draft: MIG, DJA; Writing – review & editing: SB, MH, MIG, DJA, SJR, AA, MW, QT

Competing interests. A subset of the authors are named inventors on a patent application relating to this work.

Data and materials availability. All data are available in the main text or the supplementary materials or available on reasonable request.

References

1 Hafer, M. Quantity and electricity consumption of plug load equipment on a university campus. *Energy Efficiency* **10**, 1013-1039, doi:10.1007/s12053-016-9503-2 (2017).

2 Kaushik, J. K. & Bhat, R. Why Is Trehalose an Exceptional Protein Stabilizer?: AN ANALYSIS OF THE THERMAL STABILITY OF PROTEINS IN THE PRESENCE OF THE COMPATIBLE OSMOLYTE TREHALOSE*. *Journal of Biological Chemistry* **278**, 26458-26465, doi:https://doi.org/10.1074/jbc.M300815200 (2003).

3 Anchordoquy, T. J. & Carpenter, J. F. Polymers Protect Lactate Dehydrogenase during Freeze-Drying by Inhibiting Dissociation in the Frozen State. *Archives of Biochemistry and Biophysics* **332**, 231-238, doi:https://doi.org/10.1006/abbi.1996.0337 (1996).

4 Jain, N. K. *et al.* Formulation and stabilization of recombinant protein based virus-like particle vaccines. *Advanced Drug Delivery Reviews* **93**, 42-55, doi:https://doi.org/10.1016/j.addr.2014.10.023 (2015).

5 Chen, Y.-C. *et al.* Thermal stability, storage and release of proteins with tailored fit in silica. *Scientific Reports* **7**, 46568, doi:10.1038/srep46568 (2017).

6 Brogan, A. P. S. Preparation and application of solvent-free liquid proteins with enhanced thermal and anhydrous stabilities. *New Journal of Chemistry* **45**, 6577-6585, doi:10.1039/D1NJ00467K (2021).

7 Crommelin, D. J. A., Anchordoquy, T. J., Volkin, D. B., Jiskoot, W. & Mastrobattista, E. Addressing the Cold Reality of mRNA Vaccine Stability. *Journal of Pharmaceutical Sciences* **110**, 997-1001, doi:10.1016/j.xphs.2020.12.006 (2021).
8 Kumru, O. S. *et al.* Vaccine instability in the cold chain: Mechanisms, analysis and formulation strategies. *Biologicals* **42**, 237-259, doi:https://doi.org/10.1016/j.biologicals.2014.05.007 (2014).
9 van Beers, M. M. C., Jiskoot, W. & Schellekens, H. On the Role of Aggregates in the Immunogenicity of Recombinant Human Interferon Beta in Patients with Multiple Sclerosis. *Journal of Interferon & Cytokine Research* **30**, 767-775, doi:10.1089/jir.2010.0086 (2010).
10 Lee, J. *et al.* Trehalose Glycopolymers as Excipients for Protein Stabilization. *Biomacromolecules* **14**, 2561-2569, doi:10.1021/bm4003046 (2013).
11 Marco-Dufort, B. *et al.* Thermal stabilization of diverse biologics using reversible hydrogels. *Science Advances* **8**, eabo0502, doi:10.1126/sciadv.abo0502.
12 Sridhar, B. V. *et al.* Thermal Stabilization of Biologics with Photoresponsive Hydrogels. *Biomacromolecules* **19**, 740-747, doi:10.1021/acs.biomac.7b01507 (2018).
13 vander Straeten, A. *et al.* A microneedle vaccine printer for thermostable COVID-19 mRNA vaccines. *Nature Biotechnology*, doi:10.1038/s41587-023-01774-z (2023).
14 Georgiou, P. G., Kontopoulou, I., Congdon, T. R. & Gibson, M. I. Ice recrystallisation inhibiting polymer nano-objects via saline-tolerant polymerisation-induced self-assembly. *Materials Horizons* **7**, 1883-1887, doi:10.1039/D0MH00354A (2020).
15 Chen, B.-M., Cheng, T.-L. & Roffler, S. R. Polyethylene Glycol Immunogenicity: Theoretical, Clinical, and Practical Aspects of Anti-Polyethylene Glycol Antibodies. *ACS Nano* **15**, 14022-14048, doi:10.1021/acsnano.1c05922 (2021).
16 Marco-Dufort, B. *et al.* Thermal stabilization of diverse biologics using reversible hydrogels. *Science Advances* **8**, eabo0502, doi:doi:10.1126/sciadv.abo0502 (2022).
17 Meis, C. M. *et al.* Self-Assembled, Dilution-Responsive Hydrogels for Enhanced Thermal Stability of Insulin Biopharmaceuticals. *ACS Biomaterials Science & Engineering* **7**, 4221-4229, doi:10.1021/acsbiomaterials.0c01306 (2021).
18 Terech, P. & Weiss, R. G. Low Molecular Mass Gelators of Organic Liquids and the Properties of Their Gels. *Chemical Reviews* **97**, 3133-3160, doi:10.1021/cr9700282 (1997).
19 Draper, E. R. & Adams, D. J. Low-Molecular-Weight Gels: The State of the Art. *Chem* **3**, 390-410, doi:https://doi.org/10.1016/j.chempr.2017.07.012 (2017).
20 Piras, C. C., Genever, P. G. & Smith, D. K. Combining gellan gum with a functional low-molecular-weight gelator to assemble stiff shaped hybrid hydrogels for stem cell growth. *Materials Advances* **3**, 7966-7975, doi:10.1039/D2MA00565D (2022).
21 Wang, J. *et al.* Incorporation of supramolecular hydrogels into agarose hydrogels—a potential drug delivery carrier. *Journal of Materials Chemistry* **19**, 7892-7896, doi:10.1039/B913158B (2009).
22 Altunbas, A., Lee, S. J., Rajasekaran, S. A., Schneider, J. P. & Pochan, D. J. Encapsulation of curcumin in self-assembling peptide hydrogels as injectable drug delivery vehicles. *Biomaterials* **32**, 5906-5914, doi:https://doi.org/10.1016/j.biomaterials.2011.04.069 (2011).
23 Raymond, D. M. *et al.* Low-Molecular-Weight Supramolecular Hydrogels for Sustained and Localized in Vivo Drug Delivery. *ACS Applied Bio Materials* **2**, 2116-2124, doi:10.1021/acsabm.9b00125 (2019).

24 Faidra Angelerou, M. G. *et al.* Mechanistic investigations into the encapsulation and release of small molecules and proteins from a supramolecular nucleoside gel in vitro and in vivo. *Journal of Controlled Release* **317**, 118-129, doi:https://doi.org/10.1016/j.jconrel.2019.10.011 (2020).
25 Sutton, S. *et al.* Controlled Release from Modified Amino Acid Hydrogels Governed by Molecular Size or Network Dynamics. *Langmuir* **25**, 10285-10291, doi:10.1021/la9011058 (2009).
26 Jagrosse, M. L., Agredo, P., Abraham, B. L., Toriki, E. S. & Nilsson, B. L. Supramolecular Phenylalanine-Derived Hydrogels for the Sustained Release of Functional Proteins. *ACS Biomaterials Science & Engineering* **9**, 784-796, doi:10.1021/acsbiomaterials.2c01299 (2023).
27 Wallace, M., Adams, D. J. & Iggo, J. A. Analysis of the mesh size in a supramolecular hydrogel by PFG-NMR spectroscopy. *Soft Matter* **9**, 5483-5491, doi:10.1039/C3SM27793C (2013).
28 Smith, M. I., Foderà, V., Sharp, J. S., Roberts, C. J. & Donald, A. M. Factors affecting the formation of insulin amyloid spherulites. *Colloids and Surfaces B: Biointerfaces* **89**, 216-222, doi:https://doi.org/10.1016/j.colsurfb.2011.09.018 (2012).
29 Qafary, M., Rashno, F., Khajeh, K., Khaledi, M. & Moosavi-Movahedi, A. A. Insulin fibrillation: Strategies for inhibition. *Progress in Biophysics and Molecular Biology* **175**, 49-62, doi:https://doi.org/10.1016/j.pbiomolbio.2022.09.001 (2022).