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RESEARCH ARTICLE

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The encapsulation and controlled release of proteins from "meltable" chemically cross-linked hydrogels

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Abstract

Chemically crosslinked hydrogels can be used for the controlled delivery of therapeutic proteins, however, cargo loading can be challenging. A common approach involves performing the hydrogel crosslinking step in the presence of the cargo proteins, however, this can require the utilization of selective chemistries to ensure the cargos do not chemically react with the forming hydrogel network. We demonstrate that chemically crosslinked hydrogels based upon the protein polymer Capsular antigen fragment 1 (Caf1) can be used to encapsulate proteins within a chemically crosslinked hydrogel network. This encapsulation exploits the "meltable" feature of Caf1 hydrogels, where upon heating the Caf1 protein unfolds with concomitant depolymerization of the hydrogel network. Protein cargos can be loaded into the "molten" form of the hydrogel which cools with protein refolding and concomitant reformation of the hydrogel network to entrap the cargos, ensuring a more homogenous dispersion of the cargo. Analysis of release profiles indicated lower burst release in comparison to release from identical hydrogels loaded by an absorption method. This work is significant because it suggest that the "meltable" feature of Caf1 hydrogels may present an improved method of loading protein cargos into crosslinked hydrogel networks whilst avoiding the need for crosslinking chemistries.

KEYWORDS

biomaterials, biopolymers and renewable polymers, drug delivery systems, proteins

1 | INTRODUCTION

Therapeutic proteins such as growth factors, cytokines, recombinant proteins, and monoclonal antibodies can be found today in many pharmaceutical applications,[1] and protein-based therapeutics are anticipated to grow significantly in their importance in coming years.[1–3] It is also anticipated that with this growth there will be an increasing need to master controlled delivery methods for protein-based therapeutics. These are arguably underdeveloped in comparison to delivery methods for conventional small molecule therapeutics, where decades of work has driven the development of controllable drug delivery[2–5] which take advantage of conventional

polymeric and liposomal delivery agents that afford the virtues of drug protection,[6] site targeting[2] and toxicity reduction.[7] However, on account of the intrinsic properties of proteins such as their large sophisticated three-dimensional shapes presenting electrostatic charges, hydrophilic and hydrophobic surface domains, together with their susceptibility to degradation, it still remains a challenging task to deliver proteins efficiently.[8]

There has been much work done to develop physical hydrogel-based platforms for the delivery of protein cargos.[4–6] Physically crosslinked hydrogels have the general advantage of forming gels without the need for the addition of chemical crosslinking entities, a feature which makes the loading of bioactive cargos straightforward: the protein cargo is simply mixed together with a solution of polymer which gelates around the cargo. A significant disadvantage, however, is that physical hydrogel networks can be prone to dilution and degradation as polymers dissipate away.[6,7] In contrast, covalently cross-linked networks avoid this issue as chemical crosslinking prevents dilution of the hydrogel matrix and diffusion of constituent polymers. Furthermore, the mechanical properties (e.g., storage moduli, toughness, strength) of chemically crosslinked hydrogels are generally superior to those of physically crosslinked hydrogels,[8–10] which can be advantageous in controlled delivery applications.

The method by which protein cargos are loaded into a chemically crosslinked hydrogel is important. Most often the hydrogel crosslinking step is performed in cargo solutions, allowing the hydrogel network to form and crosslink around the proteins. However, this approach ideally requires the application of highly selective biorthogonal or supramolecular chemistries—which can be expensive and/or require expertise in organic chemistry—to ensure that the functional groups upon the protein cargos do not also react with the polymer components of the hydrogel network.[4,11–13] Photopolymerization methods can also be used,[14] however, a possible disadvantage is that the high energy light and free radicals generated during the process might cause deterioration in the encapsulated protein. Absorption approaches are well known with small molecule drug cargos, where it is relatively simple to load a high quantity of drug into a chemically crosslinked hydrogel by simple partitioning from a concentrated aqueous drug solution, allowing the cargo to diffuse through the pores of the hydrogel network to afford a hydrogel containing a homogenous distribution of small molecule cargo.[7] However, this absorption process is relatively inefficient in the case of large macromolecular drugs which have diffusive limitations to their partitioning into a hydrogel phase,[15] which can result in highly non-homogenous distributions of the cargo throughout the hydrogel network, leading to unpredictable release kinetics.[16]

There is a need for methods that allow a homogenous encapsulation of protein cargos within chemically crosslinked hydrogel networks in a manner which is as experimentally straightforward as cargo encapsulation within physical hydrogels. To address this challenge, we hypothesized that hydrogels composed of the protein polymer Capsular antigen fragment 1 (Caf1)[17] could be utilized as a vehicle to encapsulate model protein cargos within chemically crosslinked networks, avoiding the need for crosslinking chemistries and leading to a homogenous dispersion of cargo. The Caf1 polymer (Figure 1a) is formed from monomeric 15 kDa protein subunits that donate a single N-terminal donor strand to the next monomer in the chain, linking together the subunits through exceptionally strong and kinetically inert noncovalent interactions.[18,19] Caf1 polymers are stable to proteases[20] and present a "non-stick" nature,[21] which makes them an ideal candidate for a drug delivery hydrogel as protein cargos are unlikely to non-specifically "stick" to the Caf1 fibers of the hydrogel network. Caf1 hydrogels have also been successfully used in cell culture applications, demonstrating excellent levels of biocompatibility.[22,23] Importantly, it was also discovered[24] that the Caf1 polymer can be repeatedly recycled between its polymeric and monomeric states by changes in temperature (Figure 1b). When heated at around the protein melting temperature ($T_m \sim 86°C$), the Caf1 subunits unfold, losing their tertiary structure with concomitant loss of the complex with the N-terminal donor strand of the adjacent subunit, leading to depolymerization. Surprisingly, this process is reversible, and when cooled the subunits refold with concomitant recomplexation of the adjacent donor strand, driving Caf1 repolymerization. This behavior is maintained by the Caf1 polymer even when crosslinked within hydrogel networks, endowing the hydrogels with a reversible "meltable" feature (Figure 1c). The Caf1 component of the hydrogel network depolymerizes when heated above the Caf1 protein melting temperature, driving a gel–sol transition. When cooled the protein refolds with concomitant repolymerization and reformation of the hydrogel network, regelating over several hours at room temperature. The kinetics of gelation are convenient to allow a suitable time window for the encapsulation of cargos, which we envisaged would then be entrapped homogenously inside the reformed crosslinked hydrogel matrix, thus eliminating the need for chemical crosslinkers during the cargo encapsulation step. This "meltable" feature relies[24] upon the highly specific nature of the Caf1-Caf1 interaction and the non-aggregating nature of the unfolded acceptor domain of each Caf1 subunit, both of which ensures there are no unwanted interactions between the cargo and the hydrogel matrix during the regelation step.

It is important to note that the thermoresponsive behavior of the Caf1 protein polymer is fundamentally different to that of other well-known protein polymers.

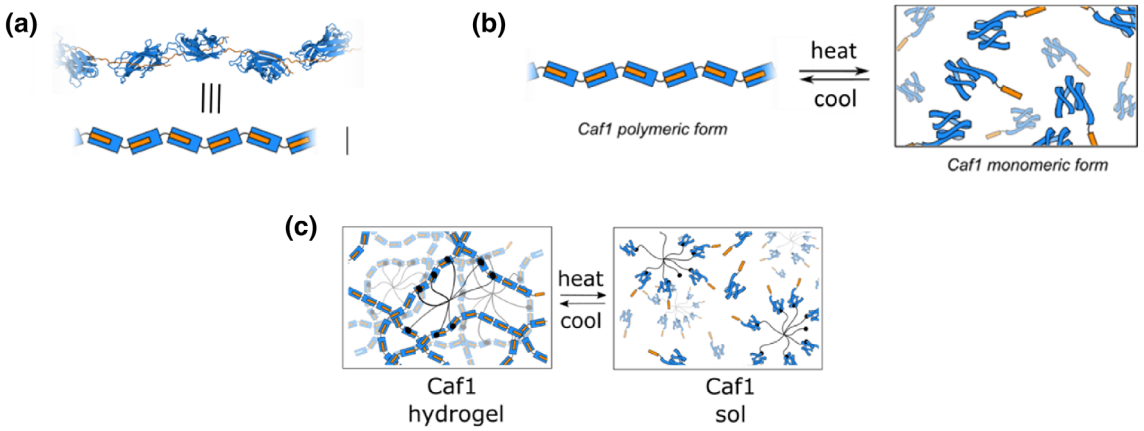

FIGURE 1 (a) Model of a Caf1 polymer (generated from PDB entry 1P5U). The N-terminal donor strands (colored orange) are complexed by the acceptor clefts (colored blue) of adjacent sub-units. (b) The reversible thermal unfolding of Caf1 polymers. When Caf1 is melted, it unfolds with concomitant depolymerisation. When cooled, the monomer subunits refold with concomitant polymerization. Caf1 can be repeatedly cycled between its polymeric and monomeric forms. (c) When Caf1 hydrogel is heated at the protein melting temperature (~86°C), the Caf1 protein transforms into its unfolded monomeric form, resulting in a gel–sol transition. When cooled to room temperature, the Caf1 monomers refold with concomitant repolymerization/gelation to form a refolded-Caf1 hydrogel. [Color figure can be viewed at wileyonlinelibrary.com]

For example, when gelatin—which adopts a strong triple-helical fibrillar structure—is heated its polypeptide strands separate into individual strands. At lower temperature, some strands are able to reform their triple-helical structure, driving gelation.[25,26] Similarly, elastin-like peptides (ELPs) consisting of the repeating (Valine-Proline-Glycine-X-Glycine)$_n$ sequence (where X denotes a variable amino acid) are able to transition from random coil to β-spiral structures above their transition temperature, leading to aggregation and phase separation.[27,28] Caf1, by contrast, repolymerizes on cooling by the precise self-association of folded proteins.

Here, we exploit the "meltable" feature of Caf1 hydrogels to encapsulate a selection of model protein cargos within crosslinked networks, and demonstrate that this approach leads to a more controlled cargo release in comparison to when the same cargos are loaded into the hydrogels through a conventional absorption process. We anticipate that this new approach to the loading of proteins within hydrogel networks will lead to new possibilities in the controlled release of proteins from chemically crosslinked hydrogels.

2 | EXPERIMENTAL

2.1 | Materials

Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich. 4-arm Succinimidyl glutarate PEG (4-arm PEG) (20 kDa) was purchased from Creative PEGWorks. Sodium bicarbonate solution (0.05 M, pH 8.2) was prepared by dissolving sodium bicarbonate (4.2 g) (Acros) in deionized water (1 L). The pH was adjusted to 8.2 with HCl. Phosphate buffered saline PBS was prepared at 100 mM sodium hypophosphite, 100 mM monosodium dihydrogen orthophosphate, 137 mM sodium chloride, 27 mM potassium chloride (from Sigma-Aldrich) in deionized water (100 mL). The pH was adjusted to 7.4 with HCl. BSA and DNase I were purchased from Merck.

2.2 | Preparation of Caf1 protein

Caf1 polymers were produced as described previously.[29] Briefly, BL21 (DE3) *E. coli* cells (New England Biolabs) were transformed with pT7-COP and pT7-COPRGDS plasmids, and single colonies used to inoculate Terrific Broth media. The cultures were grown at 35°C for 22 h before cells were harvested by centrifugation. The supernatant, containing the exported Caf1 polymers, was then passed through a Vivaflow 200, 100,000 MWCO PES tangential flow filtration device (Sartorius) and the polymers further purified by gel-filtration using a Capto Core 700 column (Cytiva). To ensure the absence of bacterial contamination, the Caf1 polymers were then sterilized by heating at 65°C for 15 min in a water bath. To confirm successful sterilization, a sample of sterilized Caf1 material was spread onto an LB agar plate and incubated overnight at 37°C. No bacterial growth was observed, indicating the success of the sterilization procedure. To quantify endotoxin levels, a Caf1 sample was screened

using the PyroGene Recombinant Factor C Endpoint Fluorescent Assay (Lonza) following the manufacturer's instructions. Briefly, Caf1 (100 μg mL^{-1}) was incubated with assay buffer, enzyme and chromogenic substrate at 37°C for 60 min. The fluorescence was measured at 0 and 60 min using Ex/Em wavelengths 380/440 nm and compared to an endotoxin standard curve to obtain a value of 2.90 ± 0.89 EU mL^{-1}.

2.3 | Preparation of Caf1 hydrogel

Caf1 protein polymer (10 mg) was dissolved in PBS (200 μL, pH 7.4) at 37°C for 3 min. 4-arm PEG (10 mg or 20 mg) was dissolved in sodium bicarbonate solution (0.05 M, 200 μL) with some sonication (2 min). The solutions were then mixed by aspiration for approximately 1 s before the onset of hydrogel formation.[30]

2.4 | Preparation of model protein cargos with fluorescent label functionalized

Aqueous solutions of BSA or DNAase (2 mg mL^{-1}) were mixed with fluorescein isothiocyanate in a sodium carbonate buffer (0.05 M) pH 9.0 at a 20:1 volume ratio then incubated in the dark at 4°C overnight. NH_4Cl was added to a final concentration of 50 mM and the solution incubated for a further 3 h. The solution was then dialysed in water to remove unconjugated label, and the solution freeze-dried to obtain the fluorescently-labeled proteins.

2.5 | Protein loading of Caf1 hydrogels by the "melt" approach

Caf1 hydrogels (400 μL) were melted by heating at 100°C for 2 min then mixed with aqueous solutions of BSA or DNAase (20 μL of a 608 μM solution). The resulting solution was allowed to cool in an ice bath for 30 min then allowed to stand at 8°C overnight to afford the protein-loaded hydrogel was obtained (*melt* Caf1).

2.6 | Rheology tests

Rheological measurements were performed with an HR-2 Discovery Hybrid Rheometer (TA Instruments) with standard steel parallel-plate geometry of 20 mm diameter with a gap of 1 mm. The strain and the frequency were set to 1% and 1 Hz, respectively.

2.7 | Protein loading of Caf1 hydrogels by the "absorption" approach

Caf1 hydrogels (400 μL) were melted at 100°C for 2 min. The solution was allowed to cool in an ice bath (30 min) and allowed to stand at 8°C overnight. The obtained *refolded*-hydrogel was freeze-dried to afford a white powder and then rehydrated by adding aqueous solutions of BSA or DNAase (420 μL of a 28.95 μM solution) to obtain the protein loaded hydrogel.

2.8 | Protein release studies from Caf1 hydrogels

Protein loaded Caf1 hydrogels were prepared as described above. PBS (1.0 mL) was then added on top of the hydrogel. The aqueous solutions were carefully removed for spectroscopic analysis and replaced with fresh buffer at predetermined time intervals. The experiments were performed in triplicate. The collected aqueous solutions were analyzed for protein concentration and related by measuring absorbance at 498 nm based on a calibration curve prepared using FITC-BSA/DNAase.

2.9 | Swelling and degradation studies

The %swelling and/or %degradation studies of Caf1 hydrogel melted and freeze-dried was determined in PBS buffer (pH 7.4). The swelling and/or degradation was calculated by the following equation:

$$\%\text{Swelling and Degradation} = [(W_t - W_i)/W_i] \times 100 \quad (1)$$

where W_i is the initial weight and W_t is the weight of the hydrogel at a predetermined time. No significant change in swelling or degradation was observed over the first 24 h (data not shown).

3 | RESULTS AND DISCUSSION

3.1 | Experimental design

Our study was designed to determine the release kinetics of model protein cargos from Caf1 hydrogels whose cargos have been loaded by what we term the "melt" approach, where aqueous solutions of protein cargo are mixed into the hydrogel in its "molten" sol form, which then regelates to entrap the cargo. The release kinetics were then compared to those obtained by samples prepared by what we term the "absorption" approach,

TABLE 1 Properties of model protein cargos used in study.

Protein cargo	Molecular weight (kDa)	Hydrodynamic diameter (nm)	Isoelectronic point
BSA	65	7.1	4.8–5.4
DNAse I	30	4.5	4.9–5.0

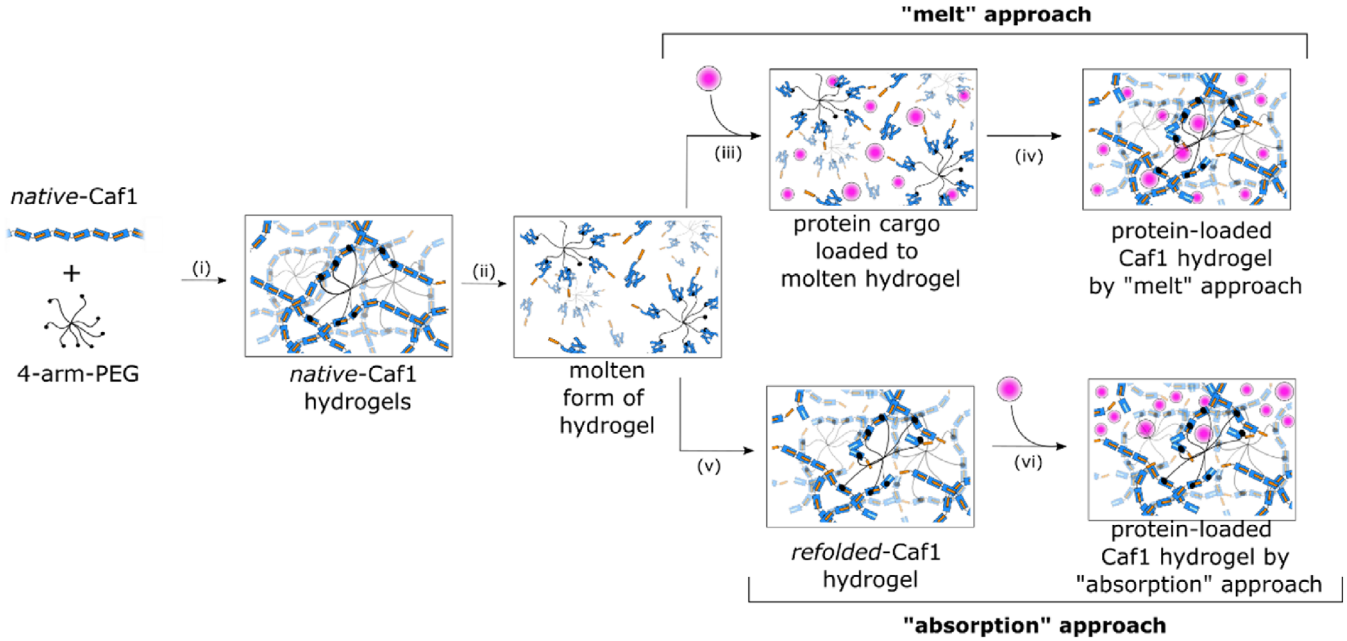

FIGURE 2 Summary of the "melt" and "absorption" approaches to loading protein cargos into Caf1 hydrogels. Step (i). The crosslinking of *native*-Caf1 with a commercially-available 20 kDa *N*-hydroxysuccinic acid-terminated 4-arm-PEG crosslinker to form *native*-Caf1 hydrogels. Hydrogels were prepared at 5% w/v Caf1 and either 5% w/v 4-arm-PEG or 10% w/v 4-arm-PEG to afford hydrogels at two different crosslinking densities. Step (ii). The *native*-Caf1 hydrogels were melted through the application of heat to afford the molten form of the hydrogels. In the "melt" approach to cargo loading, protein cargo (purple spheres) is immediately added to the molten form (step (iii)). Subsequent cooling (step (iv)) afforded the protein-loaded hydrogels. Step (v). The molten forms were immediately cooled and the resulting *refolded*-hydrogels then lyophilized to eliminate water. Step (vi). Aqueous solutions of protein cargos were added and the cargo allowed to absorb into the hydrogel networks to afford the protein-loaded hydrogels by the "absorption" approach. [Color figure can be viewed at wileyonlinelibrary.com]

whereby aqueous solutions of protein cargo are simply absorbed into the intact hydrogel networks. Model protein cargos Bovine Serum Albumin (BSA) or Deoxyribonuclease I (DNase I) were chosen as they are readily-available and allow the effects of different protein molecular weights to be explored (Table 1).

3.2 | Preparation of Caf1 hydrogels and loading of model protein cargos

Native-Caf1 is the term used[17] to describe bacteria-synthesized Caf1, which is the building block for hydrogel preparation. *Native*-Caf1 hydrogels were prepared[30] (Figure 2, step (i)) through the reaction of *native*-Caf1 with two different concentrations of a commercially-available 20 kDa *N*-hydroxysuccinic acid-terminated 4-arm PEG crosslinker, which forms amide bonds with lysine residues upon the Caf1 subunits. By altering the concentrations and ratios of *native*-Caf1: PEG crosslinker, it is possible to tune the stiffness of the resulting Caf1 hydrogels and hence network pore sizes. *Native*-Caf1 protein polymer (5% w/v) was dissolved in PBS buffer with mild heating and mixed with 4-Arm PEG (5 or 10% w/v) to afford 5%-*native*-Caf1 or 10%-*native*-Caf1 hydrogels, where the prefix reports the concentration of 4-Arm PEG used in the formula. Although a wide variety of hydrogel formulations are accessible, in this study it was decided to use these particular formulations as exploratory work indicated they were particularly efficient at regelation after melting. Previous work[30] by us has demonstrated that Caf1 hydrogels are very stable in common buffer solutions, even with very testing conditions (1 M HCl or 7 M Gd.HCl or urea), with no significant dissolution

or change in hydrogel volume occurring during emersion in solutions at 37°C over a 7 day period, which provides confidence in the stabilities of our Caf1 hydrogels with no significant change in the hydrogel network over the duration of release studies.

To load cargo into Caf1 hydrogels by the "melt" approach, aliquots of 5% and 10%-*native*-Caf1 hydrogels were melted by heating at 100°C for 2 min to afford sols (Figure 2, step (ii)). Solutions of protein cargo in PBS were then added immediately (Figure 2, step (iii)) and the solutions gently agitated to ensure mixing; the sample was then put on ice to drive regelation (Figure 2, step (iv)), affording the protein-loaded *refolded*-Caf1 hydrogel. The term *refolded* simply refers to a Caf1 hydrogel that has been subject to one complete cycle of melting and regelation. The absence of a layer of liquid above the hydrogel suggested complete encapsulation of the protein cargos with the hydrogel network. To load model protein cargos into Caf1 hydrogels by the "absorption" approach, aliquots of 5% and 10%-*refolded*-Caf1 hydrogels (prepared from *native*-Caf1 hydrogels as shown in Figure 2, steps (ii) and (v)) were lyophilised. Solutions of protein cargo in PBS were then added and the hydrogel left to absorb the protein solution into its matrix over 168 h (Figure 2, step (vi)), affording the cargo-loaded Caf1 hydrogels. Again, the absence of a distinctive layer of water above the hydrogel suggests all protein cargo was absorbed into the hydrogel network.

Previous work[23,24] by us involving extensive rheological investigations has shown that *refolded*-Caf1 hydrogels possess different rheological properties (slightly lower stiffness and increased critical strain) in comparison to the original *native*-Caf1 hydrogel. The storage modulus (G') of *refolded*-hydrogels was found to be only about 50% of that of the original *native*-hydrogels, observations suggesting that the *refolded*-hydrogel networks were different from the originals. A frequency sweep of the *native*- and *refolded*-hydrogels showed that G' was almost independent across the range of frequencies in both cases, suggesting that both possess chemically crosslinked networks. Strain sweep experiments revealed the *refolded*-hydrogels possessed a larger critical strain (defined as the point where $G' = G''$) than the original *native*-hydrogels, indicating that the *refolded*-hydrogels were more flexible than the original *native*-hydrogels. Furthermore, *refolded*-hydrogels were shown to relax stress considerably faster than *native*-hydrogels. Taken together, these rheological differences led us to speculate that there are differences in network topologies, with the *refolded*-Caf1 network composed of shorter lengths of Caf1 chains and also having more dangling ends than the original *native*-Caf1 network. The refolded Caf1 hydrogels are relatively more porous than their *native*-counterparts, and there will most likely be differences in protein release behavior between *refolded*-Caf1 and *native*-Caf1 hydrogels. To ensure this difference is accounted for, *refolded*-Caf1 hydrogels were also used in the absorption element of the study, and thus irrespective of the cargo loading approach, the hydrogel networks will have near-identical properties and pore sizes. To ensure consistency in the *refolded*-Caf1 hydrogels, the storage and loss moduli of all *refolded*-Caf hydrogels were evaluated after resetting. Typically, the storage moduli were measured to be ~650 Pa ± 5% (10%-hydrogels) and ~500 Pa ± 5% (5%-hydrogels), differences which are very consistent[21,28,29] with previous observations. These values support the idea that all hydrogels likely have similar networks. The presence of protein cargos had negligible effect upon the measured moduli.

To distinguish between the different cargo-loaded hydrogels we use the notation protein-*melt/abs*-Caf1-5/10%, which provides the name of the protein cargo (BSA or DNAase), the method of cargo loading (by "melt" or "absorption" approaches), and the 4-arm-PEG concentration within the final hydrogel formulation (either 5 or 10%). A summary of the cargo-loaded hydrogels prepared is shown in Table 2.

3.3 | Release kinetics

Release kinetics were determined at 37°C (see experimental for details). Cumulative release profiles are shown in Figure 3a (BSA) and Figure 3c (DNAase). Both sets of profiles show an initial burst release over the first ~5 h, then release becomes considerably slower for the remainder of the experiment (~5–100 h), observations consistent with biphasic release. The biphasic nature of the release becomes more clear when cumulative release was plotted against the square root of time (Figure 3b,d), with both the initial and later phases scaling linearly. This observation is consistent with first order release kinetics (which are typically diffusion controlled) operating in both phases.[31] There was no significant swelling or degradation of the hydrogels observed in the first 24 h of our experiments, and thus the sudden change in release kinetics at ~5 h cannot arise on account of these phenomena.

Biphasic or "burst" release kinetics for hydrogel encapsulated proteins are well-known.[32–34] Rethwisch and coworkers proposed[34] that biphasic release most likely arises on account of heterogeneity within hydrogel networks. Crosslinked hydrogels are known[35] to possess heterogeneous structures which arise as a consequence of how the hydrogel network forms during polymerization. Microgels are formed during the initial stages of

TABLE 2 Summary of all cargo-loaded Caf1 hydrogels prepared in our study.

Cargo-encapulated hydrogel	Protein cargo	Method of protein cargo loading	Concentration of *native*-Caf1 used in formulation	Concentration of 4-arm PEG used in formulation
BSA-*melt*-5%-*refolded*-Caf1	BSA	"melt" approach	5% w/v	5% w/v
BSA-*melt*-10%-*refolded*-Caf1	BSA	"melt" approach	5% w/v	10% w/v
BSA-*abs*-5%-*refolded*-Caf1	BSA	"absorption" approach	5% w/v	5% w/v
BSA-*abs*-10%-*refolded*-Caf1	BSA	"absorption" approach	5% w/v	10% w/v
DNA-*melt*-5%-*refolded*-Caf1	DNAase	"melt" approach	5% w/v	5% w/v
DNA-*melt*-10%-*refolded*-Caf1	DNAase	"melt" approach	5% w/v	10% w/v
DNA-*abs*-5%-*refolded*-Caf1	DNAase	"absorption" approach	5% w/v	5% w/v
DNA-*abs*-10%-*refolded*-Caf1	DNAase	"absorption" approach	5% w/v	10% w/v

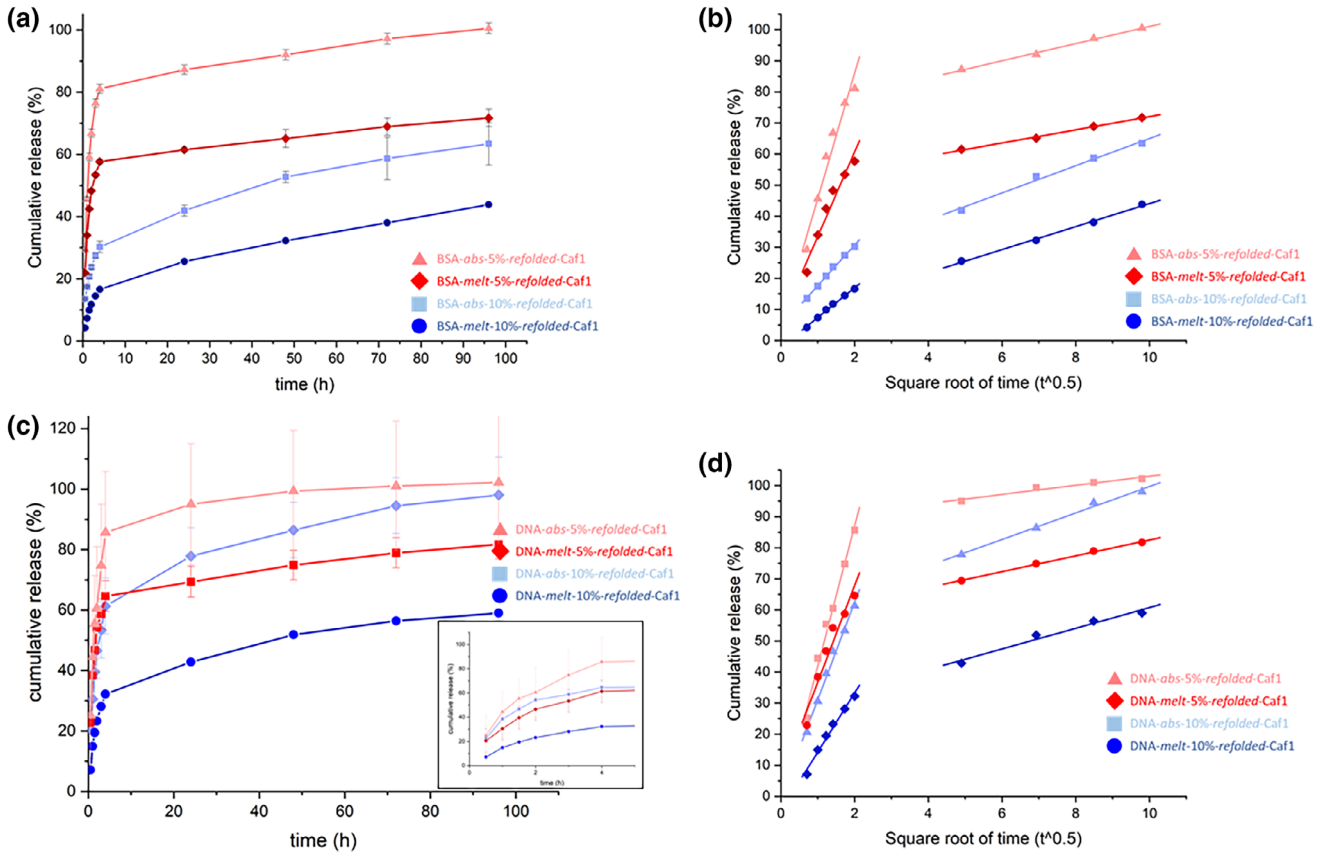

FIGURE 3 (a) Cumulative release profile for BSA as a function of time. (b) Cumulative release profile for BSA as a function of the square root of time. (c) Cumulative release profile for DNAase as a function of time. Inset: Expansion of the cumulative release over 0–5 h. (d) Cumulative release profile for DNAase as a function of the square root of time. For improved clarity, the error bars have been omitted in (b) and (d). [Color figure can be viewed at wileyonlinelibrary.com]

polymerization which possess relatively high monomer concentrations. As network formation continues, the monomer concentration is depleted and the microgels become connected by regions of lower polymer concentrations. The resulting hydrogels thus consist of regions of relatively high-density microgel domains embedded within a continuous lower density matrix. The release of protein from the low density domains is responsible for the initial fast phase of release. Once this pool of proteins is depleted, release is dominated by the slower second

phase where proteins are released from the higher density domains. It is reasonable to assume that *refolded*-Caf1 networks also possess a heterogeneous nature, and that this is the likely source of the biphasic release kinetics observed.

Why is the initial phase of release more significant for cargos loaded by the absorption approach in comparison to the melt approach? We presume that the absorption approach leads to significant quantities of protein cargos located close to the surface of the hydrogel network as it is relatively difficult for large proteins to diffuse into the hydrogel network. Consequently, these cargos have a relatively short path distance to escape the network. Protein cargos loaded by the "melt" approach, on the other hand, are more homogenously distributed throughout the hydrogel matrix and have longer path lengths, and thus their rates of release are relatively slower.

Other observations from the release profiles are to be anticipated. The release rate of the initial phase is faster from the 5% hydrogels in comparison to the 10% hydrogels simply because protein cargo can diffuse faster through the less densely crosslinked network. DNAase release is faster than BSA release—irrespective of the method of encapsulation—simply because DNAase is a relatively smaller protein and thus diffuses more quickly.

Of particular importance, our data show that a lower fraction of protein cargo is released in the initial phase when cargos are loaded by the "melt" approach in comparison to loading by the "absorption" approach. For example, the release profile for DNA-*melt*-10%-refolded-Caf1 shows that 58% of DNAase cargo is released in the initial burst phase, whereas 81% of the cargo was released in the initial phase for DNA-*abs*-10%-refolded-Caf1. More strikingly, the release profile for BSA-*melt*-10%-refolded-Caf1 shows that only 17% of cargo is released in the initial burst phase; in comparison, 30% of the cargo was released in the initial phase for BSA-*abs*-10%-refolded-Caf1. It is clear that with both proteins, the initial burst release phase was reduced simply by increasing the hydrogel crosslinking density (from 5% w/v to 10% w/v 4-arm PEG). It is very likely that the contribution from the initial burst phase could be further minimized simply by increasing the crosslinking densities of the hydrogels—a proven approach to minimizing burst release of protein cargos.

4 | CONCLUSIONS

We have demonstrated how the "meltable" feature of Caf1 hydrogels can be exploited to encapsulate protein cargos inside hydrogel networks. This feature is driven by the seemingly reversible nature of Caf1 unfolding, and thus presents an alternative to other established methods for preparing protein encapsulated hydrogels involving biorthogonal, supramolecular or photochemical crosslinking. Our results also show that our "melt" approach to protein encapsulation leads to lower initial burst release than the "absorption" approach. We believe that this difference most likely arises because the protein cargos are more homogenously dispersed through the hydrogel network in comparison to the "absorption" approach, where we speculate that a greater fraction of the cargo does not penetrate deeply into the hydrogel network. This work suggests that the "melt" approach to cargo loading leads to a reduction in the initial burst phase of release. Consequently, the "melt" approach leads to a slower and more controlled release of the model protein cargos from the hydrogels.

Other proteins have previously been used as the basis of a carrier matrix for controlled delivery applications[5] including silk-like proteins,[36] elastin-like proteins,[37] collagen[38] and gelatin.[39] On account of their amphiphilicities, biocompatibilities, biodegradabilities, favorable mechanical properties and low toxicities, protein-based carriers display considerable promise, however, challenges still remain around cross-linking approaches which can potentially damage cargos, and relatively high initial burst release (>20%).[5] Furthermore, collagen and gelatin are animal-derived materials which can suffer batch-to-batch variation. Being recombinantly prepared in *E. coli*, Caf1 polymers are potentially appealing alternatives, and results presented here suggest they are worthy of further development.

Our work is significant as it presents a novel approach to the loading of cargo proteins into hydrogel networks which avoids the need for absorption approaches to cargo loading. This makes a potentially appealing alternative to biorthogonal/supramolecular crosslinking chemistries, which can be difficult and/or expensive to apply, or photochemical approaches which might incur damage to the protein cargo.

AUTHOR CONTRIBUTIONS

Cheney C. H. Leung: Conceptualization (equal); data curation (lead); investigation (lead); writing – original draft (equal). **Gema Dura:** Conceptualization (equal); supervision (supporting); writing – review and editing (supporting). **Helen Waller:** Investigation (equal). **Jeremy H. Lakey:** Funding acquisition (lead); supervision (supporting); writing – review and editing (supporting). **David A. Fulton:** Formal analysis (lead); funding acquisition (equal); supervision (lead); writing – original draft (lead); writing – review and editing (lead).

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DATA AVAILABILITY STATEMENT

Data is available from the corresponding author upon reasonable request.

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REFERENCES

[1] A. C. Anselmo, Y. Gokarn, S. Mitragotri, *Nat. Rev. Drug Discovery* **2019**, *18*, 19.
[2] T. T. Hansel, H. Kropshofer, T. Singer, J. A. Mitchell, A. J. T. George, *Nat. Rev. Drug Discovery* **2010**, *9*, 325.
[3] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug Discovery* **2008**, *7*, 21.
[4] K. H. Bae, M. Kurisawa, *Biomater. Sci.* **2016**, *4*, 1184.
[5] T. Nie, W. Wang, X. Liu, Y. Wang, K. Li, X. Song, J. Zhang, L. Yu, Z. He, *Biomacromolecules* **2021**, *22*, 2299.
[6] T. Vermonden, R. Censi, W. E. Hennink, *Chem. Rev.* **2012**, *112*, 2853.
[7] T. R. Hoare, D. S. Kohane, *Polymer* **2008**, *49*, 1993.
[8] J. L. Vanderhooft, M. Alcoutlabi, J. J. Magda, G. D. Prestwich, *Macromol. Biosci.* **2009**, *9*, 20.
[9] G. Paradossi, I. Finelli, B. Cerroni, E. Chiessi, *Molecules* **2009**, *14*, 3662.
[10] W. E. Hennink, C. F. van Nostrum, *Adv. Drug Delivery Rev.* **2002**, *54*, 13.
[11] E. A. Appel, X. J. Loh, S. T. Jones, C. A. Dreiss, O. A. Scherman, *Biomaterials* **2012**, *33*, 4646.
[12] F. van de Manakker, K. Braeckmans, N. E. Morabit, S. C. De Smedt, C. F. van Nostrum, W. E. Hennink, *Adv. Funct. Mater.* **2009**, *19*, 2992.
[13] S. P. Zustiak, J. B. Leach, *Biotechnol. Bioeng.* **2011**, *108*, 197.
[14] R. Censi, T. Vermonden, M. J. van Steenbergen, H. Deschout, K. Braeckmans, S. C. De Smedt, C. F. van Nostrum, P. di Martino, W. E. Hennink, *J. Controlled Release* **2009**, *140*, 230.
[15] B. J. Peret, W. L. Murphy, *Adv. Funct. Mater.* **2008**, *18*, 3410.
[16] T. Bal, B. Kepsutlu, S. Kizilel, *J. Biomed. Mater. Res. A* **2014**, *102*, 487.
[17] D. A. Fulton, G. Dura, D. T. Peters, *Biomater. Sci.* **2023**, *11*, 7229.
[18] A. V. Zavialov, J. Berglund, A. F. Pudney, L. J. Fooks, T. M. Ibrahim, S. MacIntyre, S. D. Knight, *Cell* **2003**, *113*, 587.
[19] A. Soliakov, J. R. Harris, A. Watkinson, J. H. Lakey, *Vaccine* **2010**, *28*, 5746.
[20] J. Miller, E. D. Williamson, J. H. Lakey, M. J. Pearce, S. M. Jones, R. W. Titball, *FEMS Immunol. Med. Microbiol.* **1998**, *21*, 213.
[21] A. I. Roque, A. Soliakov, M. A. Birch, S. R. Philips, D. S. Shah, J. H. Lakey, *Adv. Mater.* **2014**, *26*, 2704 2616.
[22] G. Dura, M. Crespo-Cuadrado, H. Waller, D. T. Peters, A. M. Ferreira, J. H. Lakey, D. A. Fulton, *Biomater. Sci.* **2021**, *9*, 2542.
[23] G. Dura, M. Crespo-Cuadrado, H. Waller, D. T. Peters, A. Ferreira-Duarte, J. H. Lakey, D. A. Fulton, *Macromol. Biosci.* **2022**, *22*, 2200134.
[24] G. Dura, D. T. Peters, H. Waller, A. I. Yemm, N. D. Perkins, A. M. Ferreira, M. Crespo-Cuadrado, J. H. Lakey, D. A. Fulton, *Chem* **2020**, *6*, 3132.
[25] K. te Nijenhuis, *Polym. Bull.* **2007**, *58*, 27.
[26] F. Tanaka, *Polym. J.* **2002**, *34*, 479.
[27] F. C. M. Smits, B. C. Buddingh, M. B. van Eldijk, J. C. M. van Hest, *Macromol. Biosci.* **2015**, *15*, 36.
[28] C. D. Spicer, C. Jumeaux, B. Gupta, M. M. Stevens, *Chem. Soc. Rev.* **2018**, *47*, 3574.
[29] D. T. Peters, H. Waller, M. A. Birch, J. H. Lakey, *J. Biol. Eng.* **2019**, *13*, 54.
[30] G. Dura, H. Waller, P. Gentile, J. H. Lakey, D. A. Fulton, *Mater. Sci. Eng. C-Mater. Biol. Appl.* **2018**, *93*, 88.
[31] B. S. Joseph, P. S. Anthony, I. H. William, *J. Pharm. Sci.* **1968**, *57*, 274.
[32] R. Censi, T. Vermonden, H. Deschout, K. Braeckmans, P. di Martino, S. C. De Smedt, C. F. van Nostrum, W. E. Hennink, *Biomacromolecules* **2010**, *11*, 2143.
[33] S. Koutsopoulos, L. D. Unsworth, Y. Nagai, S. Zhang, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4623.
[34] N. S. Patil, J. S. Dordick, D. G. Rethwisch, *Biomaterials* **1996**, *17*, 2343.
[35] J. Bastide, L. Leibler, *Macromolecules* **1988**, *21*, 2647.
[36] L. Chambre, Z. Martin-Moldes, R. N. Parker, D. L. Kaplan, *Adv. Drug Delivery Rev.* **2020**, *160*, 186.
[37] D. Asai, D. Xu, W. Liu, F. Garcia Quiroz, D. J. Callahan, M. R. Zalutsky, S. L. Craig, A. Chilkoti, *Biomaterials* **2012**, *33*, 5451.
[38] C. Dong, Y. Lv, *Polymers (Basel)* **2016**, *8*, 42.
[39] M. Chen, Y. Zhang, W. Zhang, J. Li, *ACS Appl. Mater. Interfaces* **2020**, *12*, 22410.

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