Solvent-free liquid avidin as a step toward cold chain elimination

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Abstract
The temperature sensitivity of vaccines and therapeutic proteins forces the distribution of life-saving treatments to rely heavily on the temperature-controlled (usually 2–8°C) supply and distribution network known as the cold chain. Here, using avidin as a model, we demonstrate how surface engineering could significantly increase the thermal stability of therapeutic proteins. A combination of spectroscopic (Fourier transform infrared, circular dichroism, and ultraviolet-visible) and scattering techniques (dynamic light scattering, small-angle, and wide-angle X-ray scattering) were deployed to probe the activity, structure, and stability of the model protein. Temperature-dependent synchrotron radiation circular dichroism spectroscopy was used to demonstrate a significant increase in thermal stability, with a half denaturation temperature of 139.0°C and reversible unfolding with modified avidin returning to a 90% folded state when heated to temperatures below 100°C. Accelerated aging studies revealed that modified avidin retained its secondary structure after storage at 40°C for 56 days, equivalent to 160 days at 25°C. Furthermore, binding studies with multiple ligands revealed that the binding site remained functional after modification. As a result, this approach has potential as a storage technology for therapeutic proteins and the elimination of the cold chain, enabling the dissemination of life-saving vaccines worldwide.

KEYWORDS
cold-chain, solvent-free biofluid, surface engineering, thermal stability, vaccine storage

1 | INTRODUCTION

Infectious diseases kill more than 17 million people every year, accounting for over half of the deaths in resource-limited areas (D. Chen et al., 2009; X. Chen et al., 2011). Protein-based treatments—vaccines and therapeutic antibodies—are commonly deployed to prevent and treat infectious diseases. Vaccines are currently the most well-known and effective method to control infectious diseases and consist of either attenuated viruses, a weakened form of a virus, or harmless pathogenic antigens. As an alternative, therapeutic monoclonal antibodies have become one of the fastest-growing groups of biotechnology-derived molecules, doubling in market size between 2013 and 2017, with a predicted global market of US$130–200 billion by 2022 (Grilo & Mantalaris, 2019). However, a key challenge during both clinical development and commercial distribution of both vaccines and therapeutic antibodies is their instability towards ambient conditions (Kumru et al., 2014). Protein-based therapeutics are prone to aggregation and degradation at room temperature, hindering the bioavailability and safety of these products. The prevention of aggregation is a common
challenge in the processing and handling of therapeutics: a small percentage of aggregated molecules can elicit undesired immunogenicity (Bhatnagar et al., 2007). To prevent aggregation, these biomolecules are refrigerated (2–8°C) or frozen (−20°C or −50°C), in the temperature-controlled supply chain from manufacturing to administration known as the “cold chain.” Several challenges must be overcome for an effective cold chain, these include: poor temperature control and maintenance leading to the reduced potency of vaccines (Ashok et al., 2016), and the considerable cost of maintaining control temperatures, which can account for up to 80% of vaccination program costs (Pelliccia et al., 2016). As such, developing countries and resource-limited areas, with little infrastructure or refrigeration facilities, are unable to benefit fully from advances in the production of therapeutic antibodies and vaccines.

Many strategies to overcome the limitations of the cold chain have been developed to produce more thermally stable vaccines. These include freeze-drying (Braun et al., 2009), immobilization of viral particles onto carbohydrate glass (Alcock et al., 2010), the addition of sugars to vaccine formulations (Alcock et al., 2010; Croyle et al., 1998; Evans et al., 2004; Gupta et al., 1996; Rexroad et al., 2003; Stewart et al., 2014), and encapsulation in silica (Y.-C. Chen et al., 2017). Despite the variety of strategies proposed, there are still significant hurdles as these technologies frequently do not raise the thermal stability sufficiently, and therefore still require storage between 2°C and 8°C (Rexroad et al., 2002), or come with undesirably complex reconstitution processes. In addition, when vaccines are exposed to freezing temperatures, the adjuvants in current formulations used to increase the efficacy of vaccines forms aggregates that can produce an unwanted immunological response (Hanson et al., 2017). Consequently, there is a necessity to develop new stabilization strategies.

Solvent-free biofluids are a new class of biomaterial involving the engineering of protein surfaces with a protective polymer-surfactant corona (A. P. S. Brogan et al., 2014a, 2014b, 2012; Gallat et al., 2012; Perriman et al., 2010). These biofluids have been shown to retain both biomolecule three-dimensional structure and activity, and can be used as a solvent for the dissolution of anhydrous solutes (A. P. S. Brogan et al., 2018; A. P. S. Brogan & Hallett, 2016; A. P. S. Brogan et al., 2013, 2012; Mukhopadhyay et al., 2018; K. Sharma et al., 2020). In addition, the biofluid conferred extremely high thermal stability on the protein, allowing for enzyme activity at temperatures as high as 150°C (A. P. S. Brogan et al., 2014b). We now wish to use the advantage of the thermophilic behavior of solvent-free biofluids to extend this technology to the long-term room temperature storage of therapeutic proteins.

Here, we demonstrate the feasibility of therapeutic biofluids using the globular binding protein avidin (Av) as a simplified model for therapeutic proteins (analogous ligand recognition and binding). Antibodies can bind up to two ligands with high binding affinity and typically have a dissociation constant (\(K_d\)) between \(1 \times 10^{-11}\) M and \(2 \times 10^{-10}\) M (Landry et al., 2015). Av is a tetramer that can bind to the vitamin biotin with a dissociation constant \(K_d\) of \(6 \times 10^{-16}\) M (Green, 1990), the highest known noncovalent interaction between a protein and a ligand. Av has four independent binding sites with the same \(K_d\), which can be used to monitor any changes in binding strength due to its high affinity with biotin. We have successfully produced a protein-polymer surfactant nanoconstruct from Av with a melting temperature of 139.0°C and retained secondary structure and binding activity after storage at 40°C for 56 days, equivalent to 160 days at 25°C. Our results show that the robust synthesis procedure provides the blueprint for engineering thermally stable antibodies and viruses for life-saving treatments worldwide, negating the requirement for the costly and flawed cold chain.

2 | MATERIALS AND METHODS

2.1 | Protein surface modification

Chemicals were purchased from Sigma-Aldrich, Merck Millipore, and VWR and used without further purification. The protein surface of Chicken Av (VWR) was cationized (Figure 1) by coupling \(N, N\)-dimethyl-1,3-propanediamine (C; Sigma-Aldrich) with aspartic acid and glutamic acid residues via EDC-mediated carbodiimide activation using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma-Aldrich) under acidic conditions (pH 5.8). The solution was filtered (0.22 µm pore size) and extensively dialyzed in Visking Dialysis Tubing (12,000–14,000 MWCO; Medicell Membranes Ltd.) against Milli-Q ultrapure water (18.2 MΩ cm) for 24 h. The resultant cationized protein solution ([C-Av]) was added to a neutralized solution of anionic surfactant, glycolic acid ethoxylate lauryl ether (\(M_p = 690\); S; Sigma-Aldrich), resulting in complexation with cationic residues (lysines, arginines, and the subsequently cationized aspartic acids and glutamic acids) on the protein surface. The solution was dialyzed against Milli-Q ultrapure water (18.2 MΩ cm) for 24 h to remove any unbound surfactant. The solution was then centrifuged (4,000 rpm, 30 min) and the supernatant was lyophilized for 48 h. The resulting protein-surfactant nanoconjugates ([C-Av][S]) were then heated to 60°C and cooled down to room-temperature forming solvent-free liquid proteins.

3 | RESULTS AND DISCUSSION

Av was successfully surface-engineered using established methods, with slight modifications for optimization of the protocol for the Av system, to produce discrete nanoconjugates of protein and polymer-surfactant (A. P. S. Brogan et al., 2018; A. P. S. Brogan & Hallett, 2016; A. P. S. Brogan et al., 2014b, 2012). This two-step process first involved the cationization of Av (at an efficiency of 63%, Figure S1) to yield C-Av. This was then conjugated with glycolic acid ethoxylate lauryl ether (S) to yield the nanoconjugate ([C-Av][S]), where thermal annealing produced a pale-yellow solvent-free biofluid with a melting point of 23°C and a water content of 1.19 wt% (Figure S2). Circular dichroism (CD) spectroscopy was used to assess the impact of the modification on the secondary structure of avidin (Figure 2a).
Cationization caused little change in the secondary structure of avidin, with C-Av having a predominately β-sheet structure (46%), comparing well to Av (51%; Figure 2a; Table 1). However, after electrostatic complexation of surfactant molecules to the protein surface to produce [C-Av][S], there was a broadening of the negative band at 215 nm. In addition, the positive peak at 230 nm found in the native and cationized form disappeared, and the positive peak at 196 nm blue-shifted to 193 nm (Figure 2a). Deconvolution of the spectra showed that β-sheet content reduced to 37%, which was concomitant with an increase in α-helical structure to 16%, indicating a shift in the global fold of Av (Table 1). Small-angle X-ray scattering

**TABLE 1** Estimated percentage of the secondary structure of aqueous CD spectra (Figures 2a and S3)

| Sample   | α-Helix (%) | β-Sheet (%) | Turns (%) | Unordered (%) | NMRSD   |
|----------|-------------|-------------|-----------|---------------|---------|
| Av       | 1           | 51          | 9         | 37            | 0.051   |
| C-Av     | 2           | 46          | 11        | 40            | 0.045   |
| [C-Av][S]| 16          | 37          | 12        | 34            | 0.021   |

Abbreviations: Av, avidin; CD, circular dichroism; NMRSD, normalised root mean square deviation.
Scattering plots for Av, C-Av, and [C-Av][S] (Figure 2b) in aqueous solution were fitted to determine diameters of 5.2, 5.3, and 6.6 nm, respectively. This showed that there was little change after coupling the amido to the protein surface, an observation in line with the observed secondary structure. The increased diameter for [C-Av][S] of 1.3 nm was consistent with the introduction of the surfactant corona around the protein (A. P. S. Brogan et al., 2018; Perriman et al., 2010; K. P. Sharma et al., 2014). Similarly, the secondary structure of [C-Av][S] in the solvent-free form was evaluated in the absence of water. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was used to probe the amido bands of the solvent-free form and to reveal further information about the secondary structure of the protein (Figure 2c). The FTIR spectra of solvent-free [C-Av][S] showed amide I and amide II bands at 1624/cm and 1537/cm respectively, indicating a predominately β-sheet structure (Jackson & Mantsch, 1995), agreeing well with the structure of lyophilized Av (Figure 2c) and the aqueous structure of Av determined by CD (Figure 2a). SAXS was used to measure the diameter of solvent-free [C-Av][S] to check 3-dimensional fidelity (Figure S4c; Table 2). The SAXS profile had a q-peak at 0.135 Å⁻¹ which corresponded to discrete particles with an internal diameter of 4.7 nm, consistent with a condensed phase of tightly packed [C-Av][S] particles. In addition, no peaks were observed at lower q-values, which indicated an absence of aggregates in solvent-free [C-Av][S]. The WAXS profile of solvent-free [C-Av][S] (Figure S4d) showed a peak at 1.85 Å⁻¹ and a shoulder at 1.44 Å⁻¹ equating to distances of 3.4 and 4.4 Å, respectively. These distances were consistent with surfactant polyethylene glycol (PEG)-PEG interactions and alkyl-alkyl chain interactions observed in the solvent-free biofluid (A. P. S. Brogan et al., 2019). Having determined that [C-Av][S] in the solvent-free biofluid had retained its global architecture, temperature-dependent CD, and SAXS experiments were used to determine the thermal stability of solvent-free [C-Av][S]. Assuming a two-state model of protein denaturation (Pace, 1975), the fraction denatured (fD) for aqueous Av, C-Av, [C-Av][S], and solvent-free [C-Av][S] were determined (Figure 3a). The chemical modifications increased the thermal stability of Av in aqueous solution, with half denaturation temperatures (Tmₜ) of 74.3°C for Av and 81.0°C [C-Av][S] (Figure 3a). In the solvent-free biofluid form, [C-Av][S], the thermal stability increased further with a Tmₜ of 139.0°C (an increase of 71.7°C compared to aqueous Av). Thermodynamic analysis (Table 2) revealed, contrary to previous studies (A. P. S. Brogan et al., 2012), that thermal stability within the biofluid was predominately due to an increase in stabilizing interactions. This is evidenced by an increase in the enthalpy of denaturation (ΔHmₜ) from 168.5 kJ/mol for Av in aqueous conditions to 294.0 kJ/mol for solvent-free [C-Av][S]. Enthalpic stabilization within the biofluid potentially reflected the shift in secondary structure to increased levels of α-helix. High thermal stability of [C-Av][S] was confirmed by temperature-dependent SAXS, which showed minimal changes in [C-Av][S] diameter up to 190°C (Figure 3c; Table S2). Thermal cycling experiments were performed to assess whether thermally induced unfolding of Av, particularly after modifications, was reversible or permanent. Aqueous Av returned to 90% of its folded state when the temperature was cycled from 25°C to temperatures up to 70°C (Figure S7). Conversely, aqueous [C-Av][S] only returned to 90% of its folded state when the temperature was cycled to temperatures up to 58°C. However, once in the solvent-free state, [C-Av][S] returned to at least 90% of its folded state at temperatures up to 100°C (Figure 3b) consistent with previous results (A. P. S. Brogan et al., 2012). Given the high thermal stability of solvent-free [C-Av][S], coupled with the observed refolding after thermal stresses, we wanted to see whether this translated to long-term storage stability. The long-term secondary structure stability was determined by incubating solvent-free [C-Av][S] at 25°C, 40°C, and 60°C and then measuring the CD spectra after reconstitution in water (Figures 3d and S8). The average change in MRE (ΔθMRE; Figure S8c) showed that when solvent-free [C-Av][S] was incubated at 25°C and 40°C for 56 days that there was no structural degradation. The deconvolution of the CD spectra (Figure S9a,b) showed that the proportion of secondary structure remained unchanged after being incubated at 25°C and 40°C, further proof that the secondary structure of [C-Av][S] in the solvent-free form was retained. However, when incubated at 60°C, there was a slight but persistent decrease in ΔθMRE over 56 days (Figure S8c). The deconvolution of the CD spectra (Figure S9c) showed that after incubation at 60°C for 56 days that the α-helix content decreased from 16% to 6% while the β-sheet and unordered secondary structure increased from 37% to 43% and 34% to 37%, respectively. A temperature coefficient (Q10), the rate of change of degradation as a result of an increase in 10°C, is typically between 2 and 3 for most biological reactions (Reyes et al., 2008). Therefore, a Q10 of 2 was assumed to determine the minimum long-term thermal stability at 25°C. Using this, we calculated that 56 days at 40°C is equivalent to storing [C-Av][S] at 25°C for 160 days. This represents a significant improvement in previously reported stabilization formulations with antibodies and viruses. For example, antibodies in the presence of bovine serum albumin maintained the antibody bioactivity over 50% after 63 days at 23°C (Huang et al., 2018), and the use of PEG8000 was able to increase the half-life of adenovirus type 5 to 21 days at

| Sample        | Tm (°C) | ΔHm (kJ/mol) | ΔS (J/K/mol) |
|---------------|---------|--------------|--------------|
| Aqueous Av    | 74.3 ± 0.1 | 168.5 ± 4.1 | 485 ± 12     |
| C-Av          | 75.8 ± 0.1 | 150.0 ± 3.7 | 448 ± 4      |
| [C-Av][S]     | 81.0 ± 0.4 | 100.2 ± 17.9 | 283 ± 51    |
| Solvent-free  |         |              |              |
| [C-Av][S]     | 139.0 ± 0.3 | 294.0 ± 26.3 | 713 ± 64     |

Abbreviations: Av, avidin; CD, circular dichroism; SRCD, synchrotron radiation circular dichroism.
37°C (Pelliccia et al., 2016). This provided evidence that this surface modification strategy could allow for the storage of therapeutic proteins and viruses (A. P. S. Brogan et al., 2019; Patil et al., 2012) at a variety of ambient room temperatures for long enough to remove the necessity for the cold chain.

Having established that the global conformation of avidin could be maintained in the biofluid, and exhibited high thermal stability, we wanted to assess the biotin-binding ability of the protein after modification. We designed a spectrophotometric assay to determine binding site availability and whether high thermal stability came at a cost to binding activity (see Section 2). For this, we directly assessed the integrity of the biotin-binding sites by binding fluorescein isothiocyanate (FITC)-PEG-biotin and tested reversibility of binding using the well-characterized 4’-hydroxyazobenzene-2-carboxylic acid (HABA)-biotin ligand displacement reaction. FITC-PEG-biotin was able to bind to Av, C-Av, and [C-Av][S] as evidenced by the retention of the characteristic peak at 492 nm after washing away the excess (Figure S11). This indicated that both C-Av and [C-Av][S] retained functional binding pockets after modification, with binding activities compared to Av of 133% and 29.6%, respectively, after a HABA to binding ratio of 16 was used (Figure 4a). However, the binding data showed that only one out of four binding sites was active for Av and its conjugates, which could be caused by the PEG chain blocking the other three binding sites. The binding data were normalized and fitted with the Hill equation to compare the ligand binding strength to Av and its conjugates. FITC-PEG-biotin bound to both Av and C-Av (Figure 4b; Table S3) with similar strengths, Kd of 3.64 × 10⁻⁵ M and 4.29 × 10⁻⁵ M, respectively, which was in line with the structure remaining broadly the same after cationization. In addition, the ligand bound to Av and C-Av with similar Hill coefficients (n) of 1.23 and 1.13, respectively. Given the negative charge of FITC-PEG-biotin, the slight increase in activity observed for C-Av was likely due to the rise in surface charge of avidin after cationization, rising from +7.3 to +20.4 mV (Table S4). The normalized binding activity from [C-Av][S]

FIGURE 3  Determining the thermal stability of Av, C-Av, and [C-Av][S] (a) Equilibrium fD as a function of temperature for aqueous Av (black), C-Av (red), [C-Av][S] (blue), and solvent-free [C-Av][S] (green; data from Figure S5). (b) Thermal cycling of solvent-free [C-Av][S] showing the percentage of folded state compared to unfolded state at 30°C after being heated in cycles from 30°C to 220°C in 10°C intervals. (c) Temperature-dependent SAXS (separated for clarity) of [C-Av][S] in the solvent-free form heated from 30°C (blue) to 190°C (red) in 40°C intervals. (d) CD spectra of reconstituted solvent-free [C-Av][S] after being heated at 40°C for up to 56 days. Av, avidin; CD, circular dichroism [Color figure can be viewed at wileyonlinelibrary.com]
showed a slightly reduced $K_d$ of approximately $10^{-4}$ M, which could be attributed to steric repulsion between the bulky FITC-PEG-biotin and the surfactant corona of [C-Av][S]. Additionally, the zeta potential of aqueous [C-Av][S] was determined to be $-26.7$ mV, where increased electrostatic repulsion could also have contributed.

To investigate potential binding site blockage further, we turned to the ligand displacement reaction between HABA and biotin (Figures 4c,d and S13–S15). The reversibility of the binding pocket was investigated by displacing HABA ($\lambda_{\text{max}} = 350$ nm) from the Av-HABA complex ($\lambda_{\text{max}} = 500$ nm) with biotin. The absolute data showed that Av possessed a $K_d$ of $1.12 \times 10^{-5}$ M (Table S6) with a maximum occupancy $O_{\text{max}}$ of 0.99 and Hill coefficient ($n$) of 1.24, indicating that all four binding sites were functional with no cooperativity between binding sites, agreeing well with literature (Delgadillo et al., 2019). The binding was retained in both C-Av and [C-Av][S] as shown by having 63.8% and 6.1% activity after incubation with a HABA to binding site ratio of 32:1 (Figure 4c). The normalized data (Figure 4d) showed that HABA bound to C-Av, similarly to Av, with non-cooperative binding with an $n$ of 0.96 and equal binding strength with a $K_d$ of $1.95 \times 10^{-7}$ M. After the addition of the surfactant, HABA bound to [C-Av][S] with a $K_d$ of the same order of magnitude as Av and therefore the reduction in absolute binding activity was likely a result of the reduced number of available binding sites likely due to steric hindrance of the surfactant corona. The addition of biotin showed displacement of HABA in both Av and C-Av with inhibition constant ($K_i$) of $1.16 \times 10^{-7}$ M and $2.82 \times 10^{-7}$ M, respectively (Figures S14 and S15; Table S8). However, biotin was unable to displace HABA out of the binding pocket of [C-Av][S], suggesting further that the surfactant corona was sterically hindering access. As a result, modifications did not significantly affect binding strength between both FITC-PEG-biotin and HABA with both C-Av and [C-Av][S] compared to Av. However, due to the surfactant corona, there was a reduction in binding site availability for [C-Av][S], indicating a potential trade-off between high thermal stability and binding activity. To increase the activity of solvent-free biofluids, there are possible methodologies emerging from the literature, such...
as the use of cationic and anionic surfactants instead of cationization with a diamine (Zhang et al., 2019), and the use of site-directed mutagenesis (Zhou et al., 2019).

4 | CONCLUSIONS

We have demonstrated for the first time that surface engineering of a ligand-binding protein yields a solvent-free biofluid that maintains its structure and binding ability, thus potentially providing a route to enhance the thermal stability of therapeutic proteins. Temperature-dependent SRCD and SAXS measurements demonstrated high-temperature stability of solvent-free avidin with a T_m (139.0°C), 72°C higher than the native protein in aqueous solution. This was coupled with high reversibility toward thermal stresses, with at least 90% of the folded state maintained at 30°C after incubation at temperatures as high as 100°C. Accelerated aging studies revealed that modified avidin retained structure after storage at 40°C for 56 days, equivalent to 160 days at 25°C. This indicated that solvent-free avidin could potentially be stored on a shelf at room temperature for a significant amount of time without structural degradation. When translated to therapeutic proteins, this method would circumvent the requirement for the cold chain and therefore democratize access to life-saving therapeutics in resource-limited areas that currently lack cold-chain infrastructure. While our binding experiments showed that the biofluid exhibited retained binding ability, the observed reduction compared to the unmodified protein indicated a potential trade-off between high thermal stability and therapeutic viability when translated to therapeutic proteins of increasing complexity. Regardless, these promising results show that solvent-free biofluids could become platform biotechnology for the realization of cold chain-free storage for temperature-sensitive therapeutic proteins. As such, we are now pursuing a research program to expand on this study to demonstrate its efficacy on antibodies and nucleic acids and to optimize the biomaterial formulation towards therapeutic applications.

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AUTHOR CONTRIBUTIONS

Liem Bui-Le and Alex P. S. Brogan designed the experiments. Liem Bui-Le performed and analyzed the experiments. Liem Bui-Le and Alex P. S. Brogan wrote the manuscript with Jason P. Hallett providing critical feedback and support throughout. All authors proofread, discussed, and have given approval to the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All data are archived at the Imperial College London online library. Please consult the authors for details.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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