Journal of Immunological Methods 464 (2019) 57–63

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Unravelling enhancement of antibody fragment stability – Role of format structure and cysteine modification

Hui Ma[a], Ciarán Ó'Fágáin[a,*], Richard O'Kennedy[a,b]

[a] School of Biotechnology, Dublin City University, Dublin 9 D09 Y5N0, Ireland,
[b] Qatar Foundation, Hamad Bin Khalifa University, Education City, Doha, Qatar.

ARTICLE INFO

Keywords:
Single-chain antibody fragment
Antibody engineering
Protein stabilisation
Cysteine mutations
Complementarity determining region H3

ABSTRACT

Antibody-based diagnostics and therapeutics have huge commercial value. However, applications of antibodies are often limited by instability, particularly for recombinant antibody formats. This paper describes the conversion of a single-chain variable fragment (scFv) antibody to a single-chain antibody fragment (scAb) with notably improved stability characteristics. This scAb retains antigen-binding activity (i) at high temperature (up to 60 °C), (ii) in guanidine hydrochloride (GdnHCl, up to 1 M), and (iii) when stored at 37 °C for 6 months. However, limited improvement was observed when the original scFv was converted to a larger fragment antigen-binding (Fab) format. Certain Cys-to-Ala mutations in the third complementarity determining region of the antibody heavy chain (CDR−H3) also led to stability improvements. Our findings indicate that the stability of an antibody derivative depends on its format and on the positions of cysteines in the CDRs.

1. Introduction

Antibody stability is crucial for optimal performance in diagnostic and therapeutic applications. For therapeutics, instability can lead to significantly reduced yields during production and to loss of efficacy and/or patient-associated complications (Ruegg et al., 1990; Gébleux et al., 2015). High stability of antibodies is also required when generating antibody-based sensors and particularly during use in harsh conditions, such as areas contaminated with chemicals (e.g. toluene, benzene, or xylene) or heavy metals, in high temperatures, in high/low acidity, with high salt, or in extreme environmental conditions (e.g. deserts or hot springs) (Khaw et al., 1999; Matulis et al., 2005; Hahm and Bhunia, 2006; Randis et al., 2009). Recombinant antibodies are increasingly used in diagnosis and therapy, and can be engineered for high sensitivity, specificity, affinity and stability (Hussack et al., 2011; Ma, 2013; Sharma et al., 2018). Moreover, the orientation and labelling of recombinant antibodies for more effective detection can be readily accomplished by genetic and/or chemical insertion of tags (e.g. biotin, polyhistidine and hemagglutinin) (Zeng et al., 2012; Ma and O'Kennedy, 2016).

There are three main approaches to enhance or maintain antibody stability. Firstly, addition of reagents to prevent or retard degeneration (e.g. trehalose, sucrose or nanoparticles; Dimitrov, 2010; Hrkach et al., 2012); secondly, chemical modification or conjugation of the antibody (e.g. pegylation; Jevševar et al., 2012) and finally, restriction of antibody-related work to room, or other 'mild', temperatures (Reverberi and Reverberi, 2007; Randis et al., 2009). However, all of these approaches have disadvantages: (i) addition of reagents (e.g. glycerol, sodium azide, protease inhibitors and BSA) to prevent antibody degradation cannot protect antibodies in vivo, nor are they always applicable to certain assays (e.g. sodium azide should not be used in many ELISA or Biacore-based assays); (ii) chemical modification may adversely affect antibody binding activity and (iii) performance of antibody-related work at 'mild' temperatures is not always feasible.

A recombinant antibody is assembled by genetically combining antibody heavy chain and/or light chain gene sequences. Among the various existing formats for recombinant antibodies, the scFv, Fab and scAb are the most popular due to their short generation time, convenient expression in *Escherichia coli* (*E. coli*) and ease of protein engineering for better sensitivity, specificity, affinity and stability (Townsend et al., 2006). Improved stabilities have been reported after conversion of scFv to Fab (Quintero-Hernández et al., 2007) or to scAb

Abbreviations: CDR, complementarity determining region; cTnI, human cardiac troponin I; Fab, fragment antigen-binding; GdnHCl, guanidinium hydrochloride; HA, human influenza hemagglutinin; IPTG, isopropyl β-D-1-thiogalactopyranoside; NTA, nitrile triacetic acid; DPBS, Dulbecco's phosphate-buffered saline; DPBSM, 5% (w/v) low-fat milk powder in DPBS; DPBST, DPBS containing 0.1% (v/v) Tween-20; scAb, single-chain antibody fragment; scFv, single-chain variable fragment; WT, wild type

* Corresponding author.
E-mail address: ciaran.fagan@dcu.ie (C. Ó'Fágáin).

https://doi.org/10.1016/j.jim.2018.10.012
Received 27 June 2018; Received in revised form 11 September 2018; Accepted 22 October 2018
Available online 22 November 2018

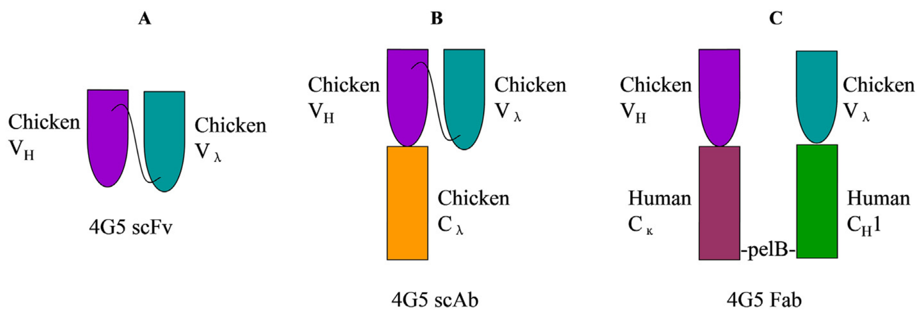

Fig. 1. Schematics of the structures of 4G5 scFv (A), scAb (B) and Fab fragment (C). V_H: variable heavy chain; V_λ: variable light lambda chain; C_λ: constant lambda light chain; C_H1: constant heavy chain one; C_κ: constant kappa light chain; pelB: pectate lyase B leader sequence. 4G5 represents the designation given during the experimental processes.

(Jeong and Rani, 2011; Sharma et al., 2016). Moreover, dramatically increased expression levels in *E. coli* were also observed for a scAb format (Hayes et al., 2012), enabling greater yields and productivity. These clear advantages of the scAb format led us to explore the relative performances of the scFv, Fab and scAb variants of an anti-cTnI antibody.

In this research, the binding stabilities and affinities of chicken antibody derivatives (i) single chain variable fragment (scFv), (ii) fragment antigen-binding (Fab) region and (iii) single chain antibody fragment (scAb), against human cardiac troponin I (cTnI), are compared in a variety of conditions designed to affect their stability. Two Cys in the third complementarity determining region of the antibody heavy chain (CDR−H3) (S S Y Q **C** S G D Y **C**) were mutated and their possible role in functional stability of the scFv, Fab and scAb was investigated.

2. Materials and methods

2.1. SDS-PAGE analysis of scFv, Fab and scAb expression and purification

The avian anti-cTnI scFv, 4G5 (Fig. 1A) and 4G5 scAb (Fig. 1B) in Top 10F' were generated and selected as described previously (Conroy, 2011). Briefly, for avian anti-cTnI scFv, the chicken was immunised using intact cTnI. Once sufficient antibody titre was achieved, the chicken was sacrificed, and RNA was extracted from spleen and bone marrow, followed by cDNA synthesis and scFv library generation (in pComb3XSS vector). ScFv antibodies with high specificity and affinity were then selected using phage display. The scAb was prepared from the scFv by adding a chicken constant domain (C_λ) to the C-terminus of the V_H. The 4G5 Fab (Fig. 1C), generated by conversion from the scFv, was produced using existing protocols (V_H, V_λ, C_H1 and C_κ amplification. This was followed by V_H-C_H1 and V_λ-C_κ amplification and, finally, V_H-C_H1-V_λ-C_κ overlap extension to give a product which was then *sfi* I digested and ligated with *sfi* I digested pComb3XSS vector) and chimeric chicken/human Fab primers (Barbas et al., 2001), followed by transforming into Top 10F' competent cells. 4G5 scFv, scAb and Fab each contained a HA-tag at their C-terminus. The scFv culture was expressed at 37 °C overnight (1 L, A_{600} 0.6–0.7) then induced at 25 °C using 0.1 mM IPTG for 3 h, followed by purification on a QIAgen Ni^{2+}-NTA agarose resin (Ayyar et al., 2010). The scAb culture was expressed at 37 °C overnight (2 L, A_{600} 0.6–0.7) then induced at 30 °C using 0.2 mM IPTG for 5 h, followed by purification on Ni^{+}-NTA agarose resin (Ayyar et al., 2010). The Fab culture was expressed at 37 °C overnight (2 L, A_{600} 0.6–0.7) then induced at 30 °C using 1 mM IPTG overnight, followed by purification using protein G agarose then by Ni^{+}-NTA agarose affinity chromatography (Ma and O'Kennedy, 2015). The quality of purification of the scFv, Fab and scAb achieved was evaluated by SDS-PAGE.

2.2. Site-directed mutagenesis of anti-cTnI antibody fragments

AccuPrime™ Pfx DNA polymerase (Bio-Sciences Limited, Ireland) and primers designed 'in-house' (Supplementary Table S.1; synthesized by Eurofins Genomics) were used to make Cys-to-Ala mutations in the CDR-H3 region (S S Y Q **C** S G D Y **C**) of the scFv, scAb and Fab. M1 represents those antibodies with the first Cys mutated to Ala (S S Y Q **A** S G D Y **C**), M2, those with the second Cys substituted by Ala (S S Y Q **C** S G D Y **A**); while in M3, both Cys were changed to Ala (S S Y Q **A** S G D Y **A**).

2.3. Binding stability assay of scFv, Fab and scAb in GdnHCl

A Nunc 96-well ELISA plate (Fisher, Ireland, #DIS-971-030 J) was coated with 100 μL/well of human cardiac troponin I (cTnI; Lifediagnostics Inc., USA; 2 μg/mL in DPBS (Biosciences, Ireland; pH 7.2) and incubated at 4 °C overnight. The plate was blocked using 5% (w/v) low-fat milk powder in DPBS (DPBSM; 200 μL/well) for 1 h at 37 °C and then washed three times with DPBS containing 0.1% (v/v) Tween-20 (DPBST) followed by three washes of DPBS. Fixed concentrations of the scFv, Fab or scAb (the mid-point of the antibody titre curve in each case) were added in the presence of different concentrations (0–1.0 M) of GdnHCl (Sigma, Ireland) in DPBS (100 μL/well) and incubated for 1 h at 37 °C (Quintero-Hernández et al., 2007). The plate was washed three times with DPBST followed by three washes of DPBS. For detection of scFv and Fab through the N-terminal HA-tag, HRP-labelled rat anti-HA antibody (1:1000 dilution, 100 μl/well obtained from Roche, Ireland) was added as the secondary antibody and incubated for 1 h at 37 °C. For detection of the scAb, HRP-labelled donkey anti-chicken IgY Fab (1:1000 dilution, 100 μL/well obtained from Gallus Immunotech Inc., U.S.A.) was added as the secondary antibody and incubated 1 h at 37 °C (Conroy, 2011). The plate was washed three times with DPBST followed by three washes of DPBS. TMB (100 μL) was then added to each well and incubated in the dark for 5 min at room temperature. The reaction was stopped by adding 50 μL/well of 1 M HCl. The plate was read at 450 nm using a Tecan Safire 2 plate reader.

2.4. Thermal stability assays of scFv, Fab and scAb

An ELISA plate was coated with 100 μL/well of cTnI (2 μg/mL in DPBS, pH 7.2) and incubated at 4 °C overnight. The plate was blocked using 5% (w/v) DPBSM (200 μL/well) for 1 h at 37 °C and then washed three times with DPBST followed by three washes of DPBS. Fixed concentrations of scFv, scAb and Fab (the mid-point of the antibody titre curve in each case) were pre-incubated at 0, 30, 40, 50 or 60 °C for 30 min. As described by Ó'Fágáin (2017), samples were immediately placed onto ice for rapid cooling. All samples were re-warmed to room temperature before addition into wells and incubation for 1 h at 37 °C. The subsequent plate washings, addition of secondary antibody and TMB substrate were performed exactly as detailed in the GdnHCl procedure described previously.

2.5. Stability of scFv, Fab and scAb in various storage buffers

Fixed concentrations of scFv, Fab and scAb (twice the concentration of the mid-point of the antibody titre curve in each case) were diluted in

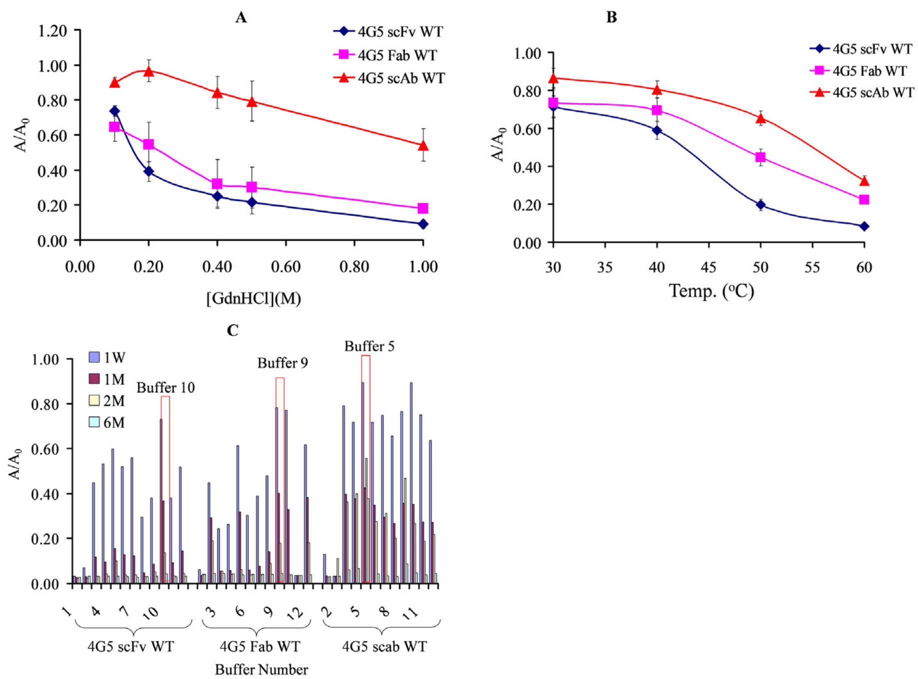

Fig. 2. Antigen-binding stability of soluble 4G5 scFv WT, Fab WT and scab WT antibodies. (A) Stability in moderate concentrations of GdnHCl. Binding to cTnI by 4G5 Fab WT and scAb WT compared with 4G5 scFv WT was detected using direct ELISA. Results are the mean ± S.D. where n = 3. A and A_0 on the ordinates represent the absorbance values of antibodies in the presence and absence of GdnHCl, respectively. (B) Thermal stability. Binding of cTnI by 4G5 Fab WT and scAb WT compared with 4G5 scFv WT, after incubating at various temperatures, was detected by direct ELISA. Results are the mean ± S.D., where n = 3. In each case, A is the absorbance due to antibody fragments incubated at various temperatures for 30 min, while A_0 is the absorbance due to antibody fragments incubated on ice prior to the assay procedure. (C) Storage stability in various buffers at 37 °C. Binding of cTnI by 4G5 Fab WT and scAb WT compared with 4G5 scFv WT in 12 different storage buffers at week 1 and months 1, 2 and 6 was detected using direct ELISA. 1 W represents the values determined after 1 week whereas 1 M, 2 M, 6 M represent observations over 1, 2 and 6 months. A and A_0 are the absorbance of antibodies after and before 37 °C storage, respectively.

an equal volume of 12 different storage buffers followed by incubation at 37 °C in sealed vials. Buffers 1–10 were commercial storage buffers (Gwent Electronic Materials Ltd., UK; product codes Q2030529P1 (one buffer) and STKAB (nine buffers)), while buffer 11 was sterile DPBS (pH 7.2) only and buffer 12 was DPBS containing 0.05% (w/v) sodium azide and 2% (w/v) BSA. To assay the binding ability remaining after storage, an ELISA plate was coated with 100 μL/well of cTnI at a concentration of 2 μg/mL in DPBS and incubated at 4 °C overnight. Plates were blocked using 5% (w/v) DPBSM (200 μL/well) for 1 h at 37 °C, then washed three times with DPBST followed by three washes with DPBS. Samples of the scFv, Fab and scAb, withdrawn for each time point from a single incubation in the 12 storage buffers, were incubated at 37 °C for 1 h. The subsequent plate washings, addition of secondary antibody and TMB substrate were performed exactly as detailed in the GdnHCl procedure described previously.

2.6. *Evaluation of binding affinity*

2.6.1. *Anti-HA IgG surface preparation*

Using a pre-written method, the surface of a sensor chip (CM5, GE Healthcare) was activated by mixing equal volumes (70 μL) of 400 mM ethyl(dimethylaminopropyl)-carbodiimide and 100 mM N-hydroxysulfosuccinimide and passing 80 μL of the mixture over the sensor surface at a flow rate of 10 μL/min. Next, anti-HA IgG antibody (25 μg/mL in 10 mM NaOAc, pH 4.5) was immobilised onto the sensor chip at a flow rate of 10 μL/min for 20 min. The surface was then capped with 1 M ethanolamine HCl, pH 8.50, for 7 min. The surface was further cleaned with four 30 s injections of 10 mM NaOH (flow rate 10 μL/min) to remove any unbound or extraneous material. FC1 (flow cell 1), used as a reference (negative control), was activated and capped using the same method applied with FC2. The response of FC2 was subtracted from that of FC1, which then indicated the actual experimental response obtained.

2.6.2. *Kinetic analysis using the Biacore 3000 system*

In order to determine the capture concentration of each antibody, dilutions of the antibody sample ranging across 1/10, 1/100, 1/1000 and 1/2000 were prepared and the concentrations which achieved the desired capture levels were found out (≤100 response units) (Leonard et al., 2017). The anti-cTnI 4G5 scFv (0.8 μg/mL, pH 7.4), Fab (0.8 μg/mL, pH 7.4) or scAb (0.01 μg/mL, pH 7.4) were then captured on the surface of the pre-immobilised anti-HA antibodies (5 μL/min flow rate). The cTnI (20.8, 10.4, 5.2 and 2.6 nM for scFv; 4.2, 2.8. 1.8 and 1.2 nM for Fab; 4.2, 2.8, 1.8 and 1.2 nM for scAb) was passed over the anti-cTnI scFv (or Fab, or scAb) at a flow rate of 30 μL/min and binding levels determined. The surface was regenerated using 10 μL 20 mM NaOH. One concentration of cTnI (5.2 nM for scFv; 1.8 nM for Fab; 1.8 nM for scAb) was run in duplicate and a zero concentration was included, enabling double referencing. Association and dissociation phases were monitored for 3 and 10 min, respectively. Sensograms for each of the concentrations were fitted to a 1:1 interaction model using BIAevaluation 4.1 software (Mimoto et al., 2013). All procedures were performed at room temperature.

3. Results

3.1. *Expression and purification of the scFv, Fab and scAb*

ScFv, Fab and scAb were expressed successfully and purified, as depicted in Fig. S1 (A, B and C for scFv, Fab and scAb respectively).

3.2. *Binding activity of scFv WT, Fab WT and scAb WT in GdnHCl*

Concentrations of GdnHCl from 0.1-1 M were used. A small stability gain (up to 2-fold in 1 M GdnHCl) was shown for the Fab WT versus the scFv WT, while a larger improvement (up to 6-fold in 1 M GdnHCl) was observed for the scAb WT (Fig. 2A). Therefore, the functional stabilities of the Fab, and especially of the scAb WT, in 0.1–1 M GdnHCl were improved compared to the scFv WT.

3.3. *Stability of the scFv WT, Fab WT and scAb WT at various temperatures*

Following 30 min incubation at 60 °C, retained activities of the Fab and scAb WT were greater by up to 3- and 4-fold, respectively, compared to the scFv WT (Fig. 2B). In addition, the scAb WT format showed the greatest stability in the range 30–60 °C.

3.4. Stability of 4G5 scFv WT, Fab WT and scAb WT in various storage buffers at 37 °C

The activity of the scFv WT in storage buffer 10 was higher than that in all the other storage buffers. About 14% of the initial scFv WT activity remained after two months at 37 °C in buffer 10 (Gwent Electronic Materials Ltd., product code Q2030529P1). For the Fab, buffer 9 (Gwent Electronic Materials Ltd., product code Q2030317P4) performed best, retaining 18% activity after 2 months at 37 °C. Both the scFv WT and Fab WT retained little activity after 3 months at 37 °C in any buffer. Notably, 38% activity remained for the scAb WT in buffer 5 (Gwent Electronic Materials Ltd., product code Q2030317P4) after 6 months at 37 °C (Fig. 2C).

The two "home-made" buffers 11 and 12 comprised sterile DPBS (pH 7.2), and DPBS containing 0.05% (w/v) sodium azide/2% (w/v) BSA, respectively. Interestingly, scAb WT showed the highest retention of activity compared with scFv WT or Fab WT when stored in either buffer 11 or buffer 12. Following 2 months of storage in buffer 11, scAb WT retained approximately 19% activity, while little activity remained for scFv or Fab WTs. After 2 months in buffer 12, scAb WT and Fab WT exhibited approx. 22% and 18% activity, respectively, while little scFv WT activity remained. Although buffer 12 increased the activity of Fab WT (from little activity after 1 week in buffer 11 to 18% activity after 2 months in buffer 12), it showed limited improvement for the scFv WT and scAb WT when comparing to that of buffer 11. This indicates that sodium azide and BSA, which are often used to assist in stabilisation, are not always effective in retaining antibody binding stability.

In summary, the scAb WT displayed the highest overall stability compared to either the scFv WT or Fab WT in any storage buffer.

3.5. Stability of mutated and wild type 4G5 scFv, Fab and scAb in GdnHCl

Similar stability changes were observed following mutation of either Cys in the CDR-H3 region of 4G5 scFv WT (Fig. 3A) and Fab WT (Fig. 3B). In the case of the first Cys (in the CDR-H3 region of 4G5 scFv WT), up to 5-fold greater stability was noted in 0.5 M GdnHCl but this was not sustained at 1.0 M GdnHCl. In contrast, mutation of both Cys yielded no improvement.

Similarly, mutation of both corresponding Cys in 4G5 scAb WT led to little stability improvement, while mutation of either Cys yielded more modest stability gains (up to 43%) in 0.5-1 M GdnHCl (Fig. 3C).

3.6. Thermal stability of mutated and wild type 4G5 scFv, Fab and scAb

For the 4G5 scFv (Fig. 4A), mutation of either or both CDR-H3 Cys yielded significant thermal stability improvement (nearly 6-fold in the range 30–60 °C).

In contrast, for 4G5 Fab (Fig. 4B) and scAb (Fig. 4C), there was a only a modest gain in stability (up to 61%) following mutation of the Cys residues. Regarding the scAb, substitution of the second, or of both, Cys was beneficial, but mutation of the first Cys had little effect (Fig. 4C).

The most significant improvement in thermal stability, upon Cys-to-Ala mutation, occurred for scFv in comparison to the Fab and scAb.

3.7. Storage stability of mutant and wild type 4G5 scFv, Fab and scAb in optimised storage buffers at 37 °C

Wild-type and Cys-to-Ala mutants (M1, M2, M3) of each antibody fragment were incubated at 37 °C in their respective optimal buffer (Fig. 2C; i.e. buffer 10, 9 and 5 for 4G5 scFv, Fab and scAb, respectively). Overall stability of 4G5 scFv, Fab and scAb decreased after mutation of either or both Cys in CDR-H3 region, except for the Fab M3 double mutant. Mutation of both Cys in CDR-H3 region of 4G5 Fab led to a notable increase in retained activity, especially after two months (over 4-fold; see Supplementary Fig. S.2). Mutation of Cys in 4G5 scAb (where the wild-type had retained 38% activity in buffer 5 after 6 months at 37 °C) led to much poorer storage outcomes versus wild-type (Fig. S.2). This result indicated that these two Cys in the CDR-H3 region play an important role in scFv, Fab and scAb stability. Mutation of either leads to decreased storage stability, while mutation of both leads to decreased stability for scFv and scAb, but to increased stability for Fab. This may be due to the extra constant domain of Fab (Röthlisberger et al., 2005).

3.8. Kinetic analysis using the Biacore 3000 system

Data for kinetic analysis of 4G5 scFv (Supplementary Fig. S.3A),

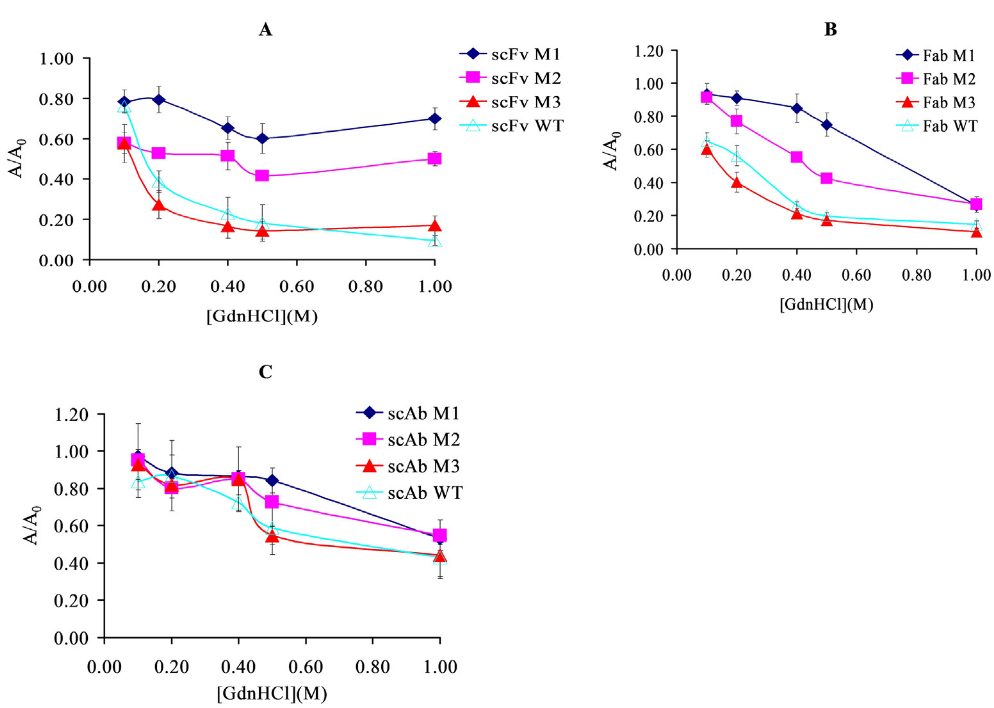

Fig. 3. Stability of mutated and WT anti-cTnI 4G5 scFv (A), Fab (B) and scAb (C) antibodies in moderate GdnHCl concentrations. cTnI binding by the mutated and WT 4G5 scFv (A), Fab (B) and scAb (C) was detected using direct ELISA. Results are the mean ± S.D. where n = 3. M1, M2 and M3, represent mutation of the first, second and both Cys in the CDR–H3, respectively; WT denotes the wild type (original) form; A and A_0 are the absorbance of antibodies with and without GdnHCl, respectively.

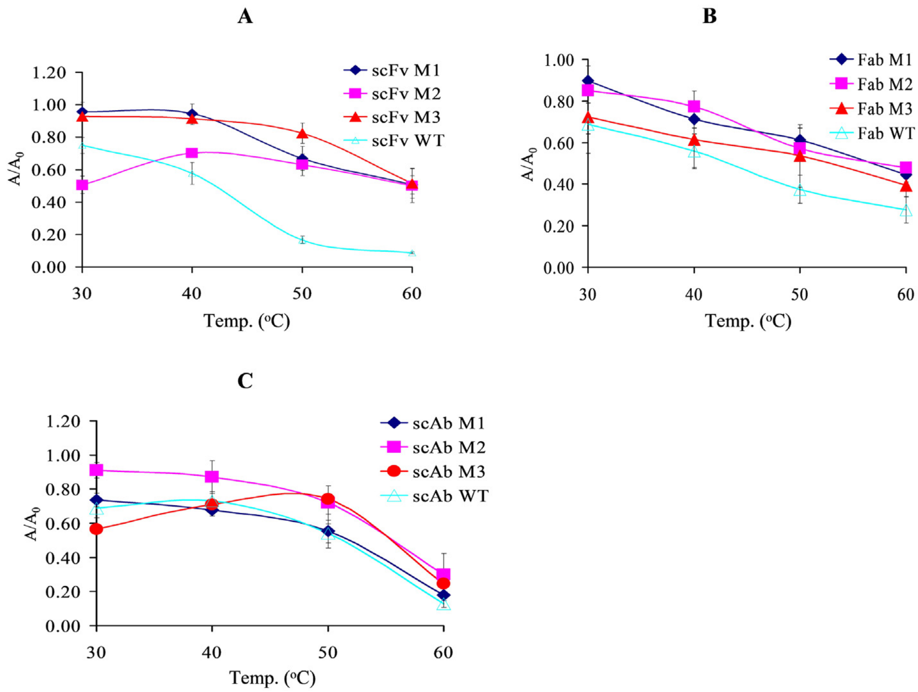

Fig. 4. Thermal stability of mutated and WT anti-cTnI 4G5 scFv (A), Fab (B) and scAb (C) antibodies. Binding to cTnI by mutated and wild type 4G5 scFv (A), Fab (B) and scAb (C) following incubation for 30 min at various temperatures was measured by direct ELISA. Results are the mean ± S.D., where n = 3. M1, M2 and M3 denote, respectively, mutation of the first, second and both Cys in CDR−H3; WT, wild type; A, the absorbance of antibodies incubated at the indicated temperatures; A_0, the absorbance of antibodies kept on ice prior to assay.

Table 1
Kinetic constants and binding affinity calculated for mutant and WT 4G5 scFv, Fab, scAb with cTnI.

4G5 Antibody fragment	Kinetic parameter K_D (M)	Chi^2	Fold difference .v. respective wild type	Fold difference .v. 4G5 scFv
ScFv WT	$1.16 \pm 0.69 \times 10^{-9}$	0.71 ± 0.18	–	–
scFv M3	$2.06 \pm 1.47 \times 10^{-9}$	2.79 ± 0.83	1.78	1.78
Fab WT	$1.55 \pm 0.82 \times 10^{-9}$	1.12 ± 0.21	–	1.34
Fab M3	$2.58 \pm 0.18 \times 10^{-9}$	5.56 ± 0.83	1.66	2.22
scab WT	$2.95 \pm 1.25 \times 10^{-9}$	0.90 ± 0.40	–	2.54
scAb M3	$1.97 \pm 0.99 \times 10^{-9}$	0.89 ± 0.59	0.67	1.70

Results are the mean ± S.D., where n = 3.
M3 = mutation of the both Cys in the third complementarity determining region of the antibody heavy chain; K_D = the equilibrium dissociation constant.

4G5 Fab (Fig. S.3B) and 4G5 scAb (Fig. S.3C) satisfactorily fitted a Langmuir model, and this 1:1 interaction model was used to extrapolate the relevant kinetic constants and binding affinity between cTnI and 4G5 Fab/scFv/scAb. The low Chi^2 goodness-of-fit values (Table 1) indicate the validity of the kinetic analysis. The results show that the binding affinity to cTnI changed very little upon conversion of 4G5 scFv WT to Fab WT or scAb WT format (maximum 2.54-fold, for scAb WT versus scFv WT). Similarly, little change occurred following mutation of any 4G5 WT fragment to its double Cys-to-Ala M3 counterpart (maximum 2.54-fold, for scFv; Table 1).

4. Discussion

We chose the retention of antigen-binding ability as our index of stability, since this is one of the key functional properties of any antibody or fragment. Classic denaturation studies follow the heat- or denaturant-induced unfolding of a polypeptide in solution (e.g. Tanford, 1970). The loss of biological properties, however, may not coincide with unfolding of the protein, and protein aggregation can complicate such studies. In addition, some proteins do not follow a two-state, reversible, unfolding transition (preventing the calculation of unfolding Gibbs (ΔG) energies).

Upon conversion of 4G5 scFv WT antibody, against cTnI epitope 3, to scAb WT format, we observed greatly improved stability to both heat and GdnHCl with little change in binding activity and affinity. The GdnHCl concentrations used were quite low (0–1.0 M) and are unlikely to have led to extensive unfolding of the antibody fragments/derivatives, especially in the absence of disulphide reducing agents. (Denaturation studies typically use GdnHCl concentrations up to 6 M to achieve complete unfolding of the polypeptide.)

Compared with the scFv WT and Fab WT formats, the scAb WT showed much better stability in each of the storage buffers, especially upon 6 months' storage at 37 °C in commercial buffer 5 (38% activity retained). This is an important finding for long-term work in the field or outdoors, where antibodies need to be stable for long periods. Note that Gwent Electronic Materials does not reveal the compositions of the buffers, so we are unable to discuss their comparative properties and performances. It is likely, however, that they include various formulations of polyelectrolyte and polyalcohol compounds, similar to those described in some of the publications and patents listed at http://www.gwent.org/aet_papers.html and http://www.gwent.org/aet_patents.html, respectively.

The pair of cysteines that is commonly found in the in CDR-H3 region of chickens, camelids, sharks, cows, pigs and platypus (Wu et al., 2012; Conroy et al., 2014) has been reported to be crucial for antibody stability and binding (Govaert et al., 2012). In contrast, such Cys pairs are much rarer in murine (0.18%) and human (5.98%) antibodies (Zemlin et al., 2003). The present 4G5 scAb is a chimeric chicken scAb, comprising a chicken scFv (wild type sequence) combined with a chicken lambda light chain (C_λ). Addition of C_λ to the 4G5 scFv when converting it to a scAb may provide the molecule with a stabilizing scaffold, leading to improved folding and/or decreased tendency to denature.

The generally improved stability of 4G5 Fab WT versus scFv WT or scAb WT is reportedly due to the C_H-C_L interaction in Fab, which does not exist in the scFv or scAb (Rothlisberger et al., 2005; Shimba et al., 1995). In the present study, however, the scAb WT showed greater stability than the Fab WT in certain conditions, indicating that the C_H-C_L interaction is only one factor contributing to higher stability. Unsurprisingly, antibody fragments containing disulphide bonds show improved stability (Almagro et al., 2012; Dooley et al., 1998), and the Cys in CDR-H3 region may play an important role in antibody stability (Kipriyanov et al., 1997; Zemlin et al., 2003). Therefore, our attention focused on Cys in the CDR-H3 region.

With respect to GdnHCl, the stabilizing effect of mutation is most pronounced for scFv, and substitution of the first Cys (only) is the most effective mutation. The scFv is the smallest of the three antibody derivatives, and lacks any constant-chain (C-chain) moiety, either chicken or human. The absence of a C-chain scaffold, which would likely contribute to protein stability, may account for the more obvious effects of the Cys substitutions in the case of the scFv.

The patterns of activity loss in GdnHCl differ from those at elevated temperatures, for the wild-type antibody forms (Fig. 2, A and B) and for the various mutants (Figs. 3 and 4). All of the proteins studied (scFv, Fab and scAb) are multi-domain proteins. Denaturation processes in multi-domain proteins are often multi-step and can be quite complex. The key adverse molecular event(s) that lead to inactivation (i.e. loss of binding ability) may be different across our three antibody types and/or whether the particular protein is in GdnHCl or subjected to heat.

This study indicates that the Cys in antibody 4G5 WT CDR-H3 region (S S Y Q **C** S G D Y **C**) can influence the antibody's stability with minimal effects on its affinity (antigen binding). Mutation of either of the Cys in the CDR-H3 region, particularly the first, improved the stability of 4G5 scFv WT, Fab WT and scAb WT in 0.5-1 M GdnHCl. Similarly, mutation of either, or both, CDR-H3 Cys enhanced thermal stability of 4G5 scFv WT, Fab WT and scAb WT in the range 30–60 °C (except scAb M1). In marked contrast, however, during long-term storage at 37 °C, Cys-to-Ala mutations decreased the overall stability of 4G5 scFv WT, Fab WT and scAb WT (except for Fab M3; Fig. S2). While 4G5 scAb WT retained 38% activity after 6 months' storage at 37 °C, its Cys-to-Ala mutants showed little activity after 2 months. In short, Cys-to-Ala mutations led to different characteristics with respect to GdnHCl, thermal and extended-storage stabilities. The R-group of Cys comprises $-CH_2$-SH, while that of Ala is simply $-CH_3$. Removal of the thiol (-SH) group, and presence of the smaller Ala side chain, could lead to improved folding or packing of the polypeptide, or may relieve some degree of crowding or steric overlap. Wu et al. (2012) found that the inter- or intra-CDR disulfides may be crucial for antibody stability. Moreover, Zemlin et al. (2003) reported that the disulfide bond within the CDR-H3 region contributes to structural stability. Therefore, in the present case, the overall decreased storage stability of scFv WT, Fab WT and scAb WT following mutation of Cys in CDR-H3 region could be due to the removal of inter- or intra-CDR disulfide bond(s). Additional research into the mutation-induced structural changes is required before any further conclusions can be drawn. It would be intriguing to compare the three-dimensional structures of these scFv and scAb formats, as these could reveal distinct structural features that promote high stability. Information from such an approach would be especially applicable to recombinant antibody formats (e.g. scAb, Fab and $(Fab)_2$), and to directed mutagenesis of monoclonal antibodies from hybridoma cells. Unfortunately, molecular structures are not available at this time.

The 4G5 scAb WT represents an antibody derivative with increased stability and with greater potential for commercial application. Firstly, it may provide a promising antibody scaffold for use in harsh conditions, e.g. in extreme environments. Secondly, it may enable wider applications in antibody-based diagnostics and therapeutics. Finally, investigation of the three-dimensional structural features of this antibody format, which improve its functional and thermal stability (in particular the location of cysteines), may enable the generation of more stable antibody derivatives for a wide range of applications.

Acknowledgements

This work was supported by Science Foundation Ireland [CSET Grant Nos. 05/CE3/B754, 10/CE/B1821] and by Enterprise Ireland [RECANT Grant No. CF/2015/0105]. Gwent Electronic Materials Ltd., U.K. is thanked for its generous gift of the 10 storage buffers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.10.012.

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