

Antibody fragments: Prolonging circulation half-life special issue-antibody research

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ABSTRACT

Antibodies are currently the fastest growing class of therapeutic proteins. When antibody fragments are included, there are over thirty-five antibody-based medicines approved for human therapy. Many more antibody and antibody-like fragments are being evaluated clinically. Production of antibody fragments can be efficient and their compact size can allow for better tissue extravasation into solid tumors than full antibodies. Unfortunately, a key limitation of antibody fragments for systemic use is their short half-life in circulation. Prolonging their circulation half-life can be accomplished clinically by the covalent conjugation of the antibody fragment to the water-soluble polymer, poly(ethylene glycol) (PEG). Many polymers and strategies are also being pursued to increase antibody fragment half-life.

Keywords: Antibody Fragment; Half-Life Extension; PEGylation; Fc Fusion

1. INTRODUCTION

Kohler and Milstein in 1975 described how antibodies could be made by fusing murine B lymphocytes with myeloma cells to generate hybrid cells (hybridomas). These hybridomas propagate indefinitely in culture and produce homogenous antibodies with respect to antibody isotype, primary amino acid sequence, affinity and specificity. This discovery created the ability to produce antibodies for research and clinical development. There are now more than 35 antibodies and antibody fragments that have been approved for clinical use (Table 1).

Antibody production has evolved from murine to chimerisation, humanization, phage display and now transgenic mice. Even with these innovations, supplying large quantities required for therapeutic use can be difficult.

Often the doses of the antibody used in therapy can be relatively high compared to other protein-therapeutics. When considering production as a limiting factor, solutions have included the production of antibody fragments such as Fab, Fv and scFv using microbial expression systems. These systems can provide greater amounts of the desired protein compared to earlier eukaryotic systems [1-3]. Using prokaryotic expression systems can be very efficient to produce antibody fragments due to their small size and lack of glycosylation [3].

Antibody fragments can display improved tissue penetration due to their smaller size compared to full antibodies. The lack of an Fc domain means antibody fragments have reduced half-lives and a lower immunogenicity risk. The Fc domain has two functions in a full IgG, these are: i) neonatal Fc receptor (FcRn) mediated recycling to prolong circulation half-life and ii) recruitment of cytotoxic effector functions through the complement and/or through interactions with γ Fc receptors [4,5].

There have been many clinically successful monoclonal antibodies including MabThera[®], Herceptin[®], Erbitux[®] (Table 1). Antibody fragments have also made their way into the clinic, including Lucentis[®], Cimzia[®], ReoPro[®] and Removab[®] (Table 1). Antibody engineering is expanding to include non-endogenous molecular structures [6]. Many antibody derived scaffolds are in clinical trials, such as over six scFv's [7]. Additionally the functionality of monoclonal antibodies is being expanded, for example there are over 14 antibody drug conjugates (ADCs) that have been evaluated in the clinic [8]. Antibodies tend to be developed for cancer, immunological and cardiovascular targets [9].

Antibody fragments have some advantages over full IgG molecules. One advantage is that antibody fragments due to their smaller size can penetrate tissue more rapidly than full IgG molecules. This property has been exploited to develop diagnostics, with three *in vivo* imaging

agents currently on the market (CEA-Scan, Myoscint, Verluma) [10,11].

In this review we focus on the recombinant and chemical modifications applied to improve the shortcomings of different antibody fragments. We discuss the dif-

ferent modified antibody variants, with particular interest on the techniques used by FDA approved antibody fragments (**Table 1**). Additionally, we look at alternative methods, which are in their infancy and hold real promise for future therapeutic antibody fragments.

Table 1. Monoclonal antibodies and other antibody derivatives on the market [12].

Trade Name	Target	Indication	Company	Yr	Ab type
Monoclonal Antibodies					
Orthoclone OKT3 [®]	CD3	Acute kidney transplant rejection	Ortho Biotech	1986	Murine
MabThera [®]	CD20	Non-Hodgkins Lymphoma	Genetech/Biogen-Idec	1997	Chimeric
Panorex ^{®*}	17A-1	Colorectal cancer	GlaxoSmithKline	1995	Murine
Zenapax ^{®*}	IL2Ra (CD25)	Acute kidney transplant rejection	Hoffman-LaRoche	1997	Humanized
Simulect [®]	IL2R	Prophylaxis acute organ rejection	Novartis	1998	Chimeric
Synagis [®]	RSV	Respiratory Syncytial Virus	Medimmune	1998	Humanized
Remicade [®]	TNF α	Rheumatoid arthritis	Centocor	1998	Chimeric
Herceptin [®]	HER2	Metastatic breast cancer	Genetech	1998	Humanized
Mylotarg ^{®*}	CD33	Acute myelogenous lymphoma	Wyeth-Averst	2000	Humanized
Campath ^{®*}	CD52	B cell lymphocytic leukaemia	Takeda	2001	Humanized
Humira [®]	TNF α	Rheumatoid arthritis	Abbott	2002	Human
Xolair [®]	IgE	Allergy	Genetech/Novartis	2003	Humanized
Erbitux [®]	EGFR/HER1	Colorectal cancer	Bristol-Myers	2004	Humanized
Avastin [®]	VEGF	Colorectal cancer	Genetech	2004	Humanized
Raptiva ^{®*}	CD11a	Psoriasis	Genetech/Xoma	2004	Humanized
Vectibix [®]	EGFR	Colorectal cancer	Amgen	2006	Human
Tysabri [®]	α 4-integrin	Multiple Sclerosis	Biogen Idec/Élan	2006	Humanized
Solaris [®]	C5 complement	PNH-paroxysmal nocturnal	Alexion	2007	Humanized
Stelara [®]	IL-12 and IL-23	Psoriasis	Centocor	2008	Human
Simponi [®]	TNF α	Rheumatoid arthritis	Centocor	2008	Human
Illaris [®]	IL-1 β	Cryopyrin-associated periodic syndrome	Novartis	2009	Human
Arzerra [®]	CD20	Chronic lymphocytic leukaemia	Genmab	2009	Human
Prolia [™] , Xgeva [™]	RANK inhibitor	Osteoporosis, bone metastases	Amgen	2010	Human
Actemra [®] , RoActemra [®]	IL-6R	Rheumatoid arthritis	Chugai/Roche/PDL	2010	Humanized
Yervoy [®]	CTLA-4	Melanoma	Bristol-Myers Squibb	2011	Human
Benlysta [®]	B cell act. factor	Systemic lupus erythematosus	GlaxoSmithKline	2011	Human
Radioimmunotherapy Monoclonal Antibodies					
Zevalin [®]	CD20	Non-Hodgkins Lymphoma	Biogen-Idec	2002	Murine
Bexxar [®]	CD20	Non-Hodgkins Lymphoma	Corixa/GSK	2003	Murine
Antibody Drug Conjugates					
Adcetris [®]	CD30	Large cell and Hodgkins lymphoma	Seattle Genetics	2011	Chimeric
Bispecific Antibodies					
Removab [®]	CD3/EpCAM	Malignant ascites	Fresenius /Trion	2009	Murine
Monoclonal Antibody Fragments					
ReoPro [®]	GP IIb/IIIa	Prevention of blood clot	Eli Lilly	1994	Chimeric Fab
Lucentis [®]	VEGF-A	Age related macular degeneration	Genetech	2006	Hum Fab'
Cimzia [®]	TNF α	Rheumatoid arthritis	UCB	2008	PEG-Fab
Fc Fusion Proteins			Fused molecule		
Enbrel [®]	TNF α	RA, Psoriasis, ankylosing sponditis	Amgen	1998	TNF α receptor
Amevive [®]	CD2	Psoriasis	Biogen-Idec	2003	LFA3
Orencia [®]	CD80/86	Rheumatoid arthritis	Bristol-Myers Squibb	2005	CTLA4
Arcalyst ^{®*}	IL-1	CAPS-Cryopyrin associated	Regeneration	2007	IL-1 receptor
Nplate [®]	TPO	Chronic idiopathic thrombocytopenic purpura	Amgen	2008	TPO receptor (c-Mpl)
Nulojix [®]	T-Cell	Transplant Rejection	Bristol-Myers Squibb	2011	mutated CTLA-4
Eylea [®]	VEGF-A and PIGF	Age-Related Macular Degeneration	Regeneron Pharmaceuticals, Inc.	2011	hVEGF receptors 1 and 2

*withdrawn from market.

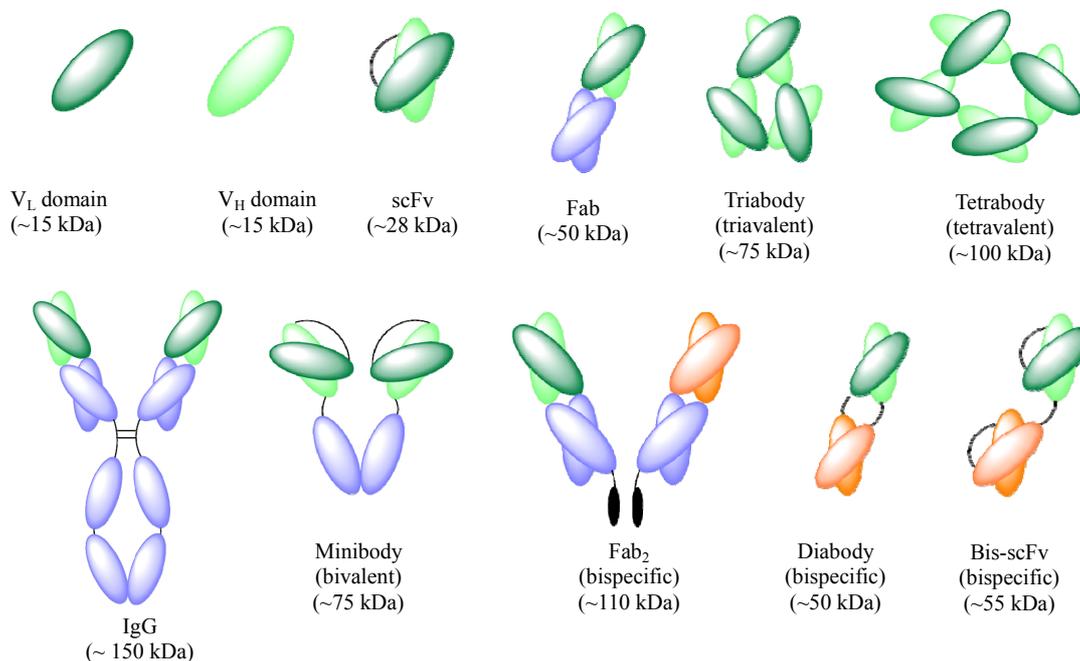


Figure 1. Schematic representation of different antibody formats [1].

2. ANTIBODY FRAGMENT DESIGN, THEIR APPLICATIONS AND LIMITATIONS

2.1. Antibody Fab Fragments

Fab fragments (~50 kDa) (**Figure 1**) can be produced by two methods: i) proteolysis of intact antibodies yielding monovalent Fab fragments and ii) direct expression from bacterial (*E. coli*, *Bacillus megaterium*), mammalian cells (CHO cells), or yeast (*Pichia pastoris*) [13,14]. Kunert *et al.*, compared the expression of Fab fragments in CHO and *Pichia pastoris*, where they found that CHO cells were better for expressing glycosylated Fab fragments but that the *Pichia* system was more efficient during development to make clinical grade products [15]. Purification by Protein A, Protein G and Protein L of Fab fragments of different isotypes are standard methods used to isolate Fabs. Ion exchange and size exclusion chromatography are also used [16,17].

To improve antigen-binding affinity of antibody fragments, two approaches can be taken: i) the creation of large libraries of randomly mutated complementarity-determining regions (CDRs) and ii) the creation of small libraries focused on mutagenesis that increase the chance to identify higher affinity variants [6].

Fab fragments are often produced to overcome the unwanted Fc effector functions for cytokine inactivation, receptor blockade or viral neutralisation [2]. The smaller size of Fab fragments compared to IgGs is often used to explain the improved tissue penetration of Fabs, which can be observed at tumour sites [2,5,18]. Additionally the

smaller size of Fab fragments may allow interactions with cryptic epitopes such as enzyme active sites which mAbs may be unable to access [19]. Fab fragments have full binding specificity due to the antigen-binding site being unaltered; however due to Fab monovalency, it has been observed that Fab fragments have fast off-rates and poor retention times on the target [20].

2.2. Multivalent Antibody Fab Fragments

Multivalent Fab species can be built from univalent Fab fragments using genetic or chemical cross-linking Weir *et al.* formed a trivalent tri-Fab'-maleimide (TFM) and compared its binding, internalisation and pharmacokinetics (PK) properties to that of native IgG. They found that TFM showed a 3-fold improvement in the binding off-rate and there were increased rates of internalisation compared to IgG. These are promising characteristics when considering a therapeutic for drug delivery to tumour cells. The PK profiles of the monomeric Fab and TFM were found to be comparable [18].

2.3. Single-Chain Variable Region (scFv)

Single-chain, scFv (~28 kDa) (**Figure 1**) are comprised of both V_H and V_L domains joined by a flexible polypeptide linker that prevents dissociation. Many scFv formats are being evaluated preclinically. This is attributable to scFv's having more favourable PKs for tissue penetration due to their even smaller size compared to Fab, while also retaining specific, monovalent, antigen-binding affinity [1,2]. scFv's can bind a variety of antigens such as

haptens, proteins, whole pathogens and can be fused to proteins which can be used in an effort to control infectious disease. Additionally, scFv's can be used alone or as fusions in enzyme-linked immunosorbent assays (ELISA) [21]. However, scFv's often exhibit fast off-rates and poor retention times on the target; therefore multivalent scFv's have been engineered [20].

To produce scFv antibodies, mRNA can be obtained from hybridomas, spleen cells, immunised mice and B lymphocytes and then by reverse transcription spliced into cDNA to serve as a template for antibody gene amplification (PCR) [20]. From here, several types of library can be made: phage (immune, naïve and synthetic) or ribosomal display libraries to create a diverse range of antibody V_H and V_L genes. To date, scFv antibodies have been successfully expressed in various expression systems such as mammalian cell, yeast, plant and insect cells [22-24].

The most promising system is *E.coli* as this system has well-studied physiology and genetics, plus the capability to produce scFv rapidly, at high yields, low cost and with robust methodology [21]. As already discussed, CDR optimisation is a standard process in antibody engineering. This process can be used with scFv's to improve their specific activity, which can allow for lower therapeutic doses to be used to reduce cost and possible toxicities [6].

2.4. Multimeric scFv's

Multivalent scFv's have been obtained by both chemical and genetic cross-linking. The most successful strategy is to genetically reduce the scFv linker length to between zero to five residues to give bivalent dimers (diabodies, ~50 kDa), trivalent trimers (triabodies, 75 kDa) and tetravalent tetrabodies (110 kDa) (**Figure 1**). These multivalent scFv's show potential in cancer imaging and radiotherapy applications. They have improved tissue penetration and faster blood clearance compared to the larger native IgG (150 kDa) [1,2]. IgG molecules have been shown to penetrate tumours more slowly than scFv fragments; however due to their monovalent binding they are cleared readily. The ideal tumour targeting reagent would have rapid tumour penetration, high target retention and rapid blood clearance, this has been observed with bivalent diabodies [1]. Another tumour therapy option is minibodies (~75 kDa) (**Figure 1**) which have shown promise due to their higher tumour intake compared to IgG [1,2].

3. APPROACHES TO EXTEND THE HALF-LIFE OF ANTIBODY FRAGMENTS

Different strategies have been developed to modify antibody fragments (e.g. Fab, scFv and Fv) [25]. Both re-

combinant and chemical approaches allow many modifications, especially to prolong circulation time [26]. Recombinant approaches include modification of antibody fragments that could lead to increased binding specificity and affinity (e.g. bispecific antibody **Figure 1**), to prolong the serum half-life (e.g. fusion with Fc or albumin) of therapeutics by using the endocytic recycling mechanism [27- 29], or by increasing the size of the protein molecule in circulation (e.g. glycoengineering methods).

Another recombinant approach to increase the apparent molecular size is multimerisation of recombinant antibody fragments. This method increases the molecular mass leading to enhancement in the overall size and improvement of binding affinity and valency [30]. However, multimerisation can result in heterogeneous complex formation and crosslinking of the target receptor that can lead to agonist functions that may not be desirable.

The chemical conjugation of poly(ethylene glycol) (PEG) to Fabs is a clinically proven method to extend half-life. Protein PEGylation has been shown to be clinically viable for several classes of protein-based medicine including cytokines, hemapoietics and enzymes. A hydrophilic, flexible polymer such as PEG has a large hydrodynamic volume in solution so that when conjugated to an antibody fragment increases its overall size [31]. The benefits are longer blood half-life, improved bioavailability, protease protection, stability, solubility, reduced immunogenicity and toxicity [32-36]. Several other polymers have also been conjugated to antibody fragments including polysialic acid (PSA), N-(2-hydroxypropyl) methacrylamide (HPMA) and dextran [35,37].

3.1. Conjugation of PEG and Other Polymers PEGylation

Protein PEGylation is recognised as the leading method to prolong blood residence times of proteins with the potential to also reduce immunogenicity [33,34,37,38]. Although many more non-antibody examples exist (e.g., PEGylated IFN-2a/b, PEGylated erythropoietin), antibody fragments such as scFv, Fab or other domains are also a significant group of proteins that have been PEGylated [32,39].

PEGylation involves the covalent conjugation of a linear or branched PEG molecule to the antibody fragment by either a random or site-specific conjugation reaction [34,40]. The most common random PEG conjugation is via an amine-reactive linker such as *N*-hydroxy-succinimide (NHS) ester which can conjugate with lysine residues. Such PEG reagents have been used for several antibody fragments including (Fab')₂, Fab and scFv. As expected the PEGylated antibody fragments have shown a prolonged *in vivo* half-life [3,41-43]. Unfortunately NHS PEG reagents undergo reactions with water as well as different lysine and other nucleophilic groups on the

protein. This results in heterogeneous products with PEG often conjugated near or at the binding site [3,32,41,44]. While PEG shielding will decrease “on” rates, direct conjugation of PEG at the binding site may decrease activity too much so that efficacy will be compromised even with the extended half-life. The problem of structural heterogeneity however can lead to a mixture of protein conjugates with varied activity.

An alternative approach is site-specific PEG conjugation, preferably in a position distal from the antigen binding site on an antibody fragment. One example is PEG chain conjugation through a maleimide linker to a free cysteine residue in the hinge region of a Fab' fragment, or to a genetically engineered antibody fragment with a free cysteine [3,36,45-53]. Maleimide linkers can undergo hydrolysis and also PEG exchange reactions with other proteins, while incorporating a free cysteine into a protein can lead to disulfide scrambling and protein aggregation [54].

Nonetheless, since thiol conjugation is much more efficient compared to amine conjugation, it has been used as a basis to develop a PEG-Fab product. The first market-approved PEGylated Fab fragment is the anti-TNF- α PEGylated Fa-Certolizumab Pegol (Cimzia[®]) (**Table 1**). It was approved in April 2008 to treat rheumatoid arthritis [55-57].

Other examples under development are an anti-VEGFR2 PEGylated di-Fab for the solid tumors (Phase II) [58], an anti-CD40L monovalent PEGylated Fab (CDP-7657) to treat patients with systemic lupus erythematosus (Phase I) [59], an anticainin-based binding protein against VEGF, chemically PEGylated with a 40 kDa polymer for solid tumor (Angiocal-PRS-050; Phase I) [60] and a 40 kDa PEGylated adnectin (fibronectin domain-based binding protein being developed by Adnexus/Bristol Myers Squibb, Phase I, CT322) [61].

Another cysteine-directed site-specific coupling approach is PEG conjugation via two sulfur atoms in the disulfide bond of Fab fragments. This has been demonstrated using a thiol-specific PEG mono-sulfone for anti-CD4 Fab, ranibizumab (Lucentis[®]) and Fabs and Fab' obtained by the proteolytic digestion of IgGs such as bevacizumab (Avastin[®]) and trastuzumab (Herceptin[®]). Using this method, the PEGylated Fab showed improved size and retained antigen-binding capacity [62,63].

Another site-specific strategy to PEGylate an antibody fragment involves PEGylating a polyhistidine tag. This is commonly used in recombinant protein expression and purification. It was shown that this approach was able to increase the half-life of an anti-TNF domain antibody 200-fold, using two 20 kDa PEG chains [64].

A protein of interest can be expressed as an N-terminal intein fusion protein. The intein domain induces an N to S acyl shift at this protein-intein junction to form the

thioester intermediate. Then C-terminal hydrazide or aminoxy derivatives are then generated through chemical cleavage under aqueous buffered conditions of this thioester intermediate using hydrazine or dioxyamine. The C-terminal hydrazide (or aminoxy) protein is then site-specifically PEGylated with benzaldehyde-PEG derivatives to form homogenous product [65]. This technology has been applied to extend half-life of single domains [66].

3.1.1. Polysialylation

Polysialic acid (PSA) is a polymer derived from N-acetylneuraminic (sialic acid) and has been used for protein conjugation [67,68]. The hydrophilic nature of PSA is thought to result in similar hydration properties to PEG, giving it a high apparent molecular weight in the blood and therefore increased circulation time. The first PSA antibody fragment conjugate was prepared using amine-based reductive amination by conjugation of PEG-aldehyde to H17E2Fab [69]. Reductive amination is not very efficient as a conjugation procedure and since PSA is charged, in contrast to PEG, the use of ion exchange chromatography can be somewhat tedious during purifications. Another example of PSA conjugation is polysialylation of an antitumor scFv fragment using maleimide chemistry [68]. Currently, no PSA-fragment conjugates are in the clinic.

3.1.2. HPMA

HPMA copolymer is another hydrophilic polymer that has been used extensively [70]. Seymour *et al.* reported conjugation of HPMA to murine IgG B72.3 and its Fab' and F(ab')₂ fragments against carcinoembryonic antigen [71]. The half-life of the Fab' fragment in blood circulation was improved 10-fold (from 35 min to 6 h). Despite the enhanced PK, the random amine-coupled HPMA conjugates showed no tumour-targeting activities. This may be due to steric hindrance of the polymer or disruption of the antigen binding domain by polymer chains. Lu *et al.* showed that site-specific HPMA conjugation through the maleimide group to the thiol residues created after IgG pepsin digestion results in retained antigen targeting affinity [72]. HPMA conjugation often occurs on pendent chains along the polymer main chain as is the case for PEG where conjugation is at the PEG terminus.

3.1.3. Dextran Modification

Dextran modification has also been applied to improve the PK properties of antibody fragments. Dextran is natural polysaccharide derived from monomers of glucose [73]. In comparison to PEG and PSA, dextran has a more compact structure and possesses multiple primary and secondary hydroxyl groups [35]. Due to its poly-functional character protein conjugation usually yields a heteroge-

neous mixture of products [73]. One example is dextran conjugation to an anti-CEA Fab fragment. They found that the PK of the conjugate was improved and the immune-reactivity of the Fab molecule was reduced [74].

3.1.4. Albumin Conjugation

Conjugation to albumin can also improve the PK of antibody fragments. Albumin has a naturally extended serum half-life, with a half-life of 19 days in humans [75-77]. This is due to albumin recycling that is analogous to IgG recycling. A Fab fragment has been site-specifically conjugated to serum albumin using maleimide [78].

3.2. Recombinant Approaches

3.2.1. Recombinantly Modified Glycosylation

Recombinant glycosylation is the incorporation of N-glycosylation sites into the protein sequence, to improve the PK and pharmacodynamics of therapeutic proteins such as EPO, Interferon and blood factors [79,80]. Arensep[®], an FDA approved hyper-glycosylated EPO has shown a three-fold increase in circulation time, but a four-fold decrease in receptor binding [79]. Stork *et al* produced a N-glycosylated bispecific single-chain di-body molecules (scDb) and compared their *in vivo* half-lives to other modification such as PEGylation and fusion to albumin-binding domains (ABD) [81]. They found that the longest circulation time was produced by the fusion of scDb to an ABD (14-fold increase of AUC), followed by a scDb-HSA fusion protein and 40 kDa branched PEGylated scDb (10-fold increase of AUC), while N-glycosylation in HEK293 cells resulted only in a moderate increase in circulation time (2 - 3-fold increase of AUC) [81]. This demonstrates that N-glycosylation can be used as a general method to modulate the PK of antibody fragments.

3.2.2. Recombinant Homo-Amino-Polymers

Inert, hydrophilic polymers represent one of the strategies for improving the PK of proteins of interest; by genetically fusing amino acid polymers to therapeutic proteins to increase the overall hydrodynamic radius of the protein. Schlapsky *et al.* described the genetic fusion of a glycine rich homo-amino-acid polymer (HAP) to an anti-HER2 Fab fragment 4D5. Their *in vivo* findings found a moderate increase in half-life from 6 h to 2 h with the 200 residue HAPylated Fab fragment compared to the Fab fragment alone [82].

HAPylation has been shown to have easier production with less downstream processing with the polymer being biodegradable, biocompatible and potentially non-immunogenic. Conversely, with HAPylation there are concerns that longer polymers are required for a PK effect. However, shorter polymer chains may have an application in medical imaging [35,82].

3.2.3. Recombinant Polysialylation

Recombinant polysialylation is a novel recombinant glycosylation technique with the aim of improving the half-life of proteins; however this method is still in its infancy. This technique attaches PSA-carrier domains from neural cell adhesion molecules (NCAMs) to antibodies or any other protein of interest. Offering an alternative to chemical conjugation of PSA to proteins, recombinant polysialylation offers easier production without downstream processing. Conjugates are biodegradable, bio-compatible and potentially nonimmunogenic. However, there are concerns over yield and homogeneity [35,83].

3.3. Other Approaches That Can Extend Half-Life and Therapeutic Effect

3.3.1. Bispecific Antibodies (bsMAB)

Currently there are two production approaches: i) the fusion of two hybridoma cell lines into a single "quadroma" cell line, however this is a difficult and time-consuming process; and ii) coupling two Fab fragments together via chemical or genetic conjugation or fusion. Chemical conjugation is still being developed, and there are no products yet in the clinic. However, there has been work described using bifunctional reagents such as 5,5'-dithiobis (2-nitrobenzoic acid) or o-phenylenedimaleimide to generate the bsMAB [84-87].

The key approach for bispecifics to increase half-life is for one binding moiety to bind to circulating albumin as was done with Dabs and diabodies [81]. However bispecific antibodies can either bind to two adjacent targets or can cross-link two different antigens. Thus, they are powerful therapeutic agents, showing great promise in cancer treatment either i) in the recruitment of cytotoxic T cells or ii) to effectively target tumour-associated antigens and deliver a cytotoxic payload to tumour cells [2].

A number of bispecific antibodies in clinical trials have one arm targeting the FcγR to enhance tumour killing. Currently the major challenge with bispecifics is production of material in sufficient quantity and purity to meet clinical needs [87]. Nevertheless Removab[®], a mouse CD3-human EpCAM bispecific was approved for the treatment of malignant ascites due to epithelial carcinomas in 2009. Removab[®] kills EpCAM positive tumour cells in the peritoneal cavity which are the known cause of malignant ascites, leaving healthy cells alive since the peritoneum lack EpCAM [88,89]. Other bispecific antibodies in advanced clinical trials (Phase II) include Merimack's MM-111, targeting HER2 and HER3 receptors on breast cancer cells, Trion's Lymphomun/ FBTA0, which targets CD20 on lymphoma cells and CD3 on T cells to treat B cell lymphoma, and Micromet's blinatumomab which binds on T cells and CD19 which targets lymphoma cells to treat acute lymphoblastic leukaemia

(ALL) [90].

3.3.2. Antibody Fusion

Antibody-cytokine fusion proteins have been linked to full length antibodies and antibody fragments such as scFv through the N- or C-termini of the antibody. The hope is to stimulate immune cell types such as monocytes, macrophages, natural killer cells, dendritic cells T and B cells. The Fc fragment of an antibody can be used to extend the half life of other molecules

Enbrel[®] is dimeric fusion protein consisting extracellular domain of p75 TNF receptor linked to the Fc domain of IgG1, with the aim of binding and inactivating cell bound and soluble TNF and lymphotoxin α . Due to the Fc domain of IgG1, the half-life of Enbrel[®] is 4.8 days and has a high binding affinity making Enbrel[®] a first line therapy for rheumatoid arthritis [91]. Rheumatoid arthritis patients whose treatment with TNF α is unsuccessful, Orencia[®] is given. Orencia[®] is a fusion protein using the Fc domain of IgG1 to extend the half-life of the extracellular domain of CTLA-4 [92].

An alternative strategy is the fusion to albumin or albumin binding domains for example scFV fusion to albumin binding domains [93]. Alternative applications for fusion technology are in tumour therapy, where antitumor efficacy has been shown with Interleukin-12 fused to the N-termini of fibronectin-specific scFv [94]. However antibody-cytokine fusions must be concentrated at the intended site to overcome serious toxicity issues which may arise from cytokines modulating an immune response elsewhere [87].

4. CONCLUSION

This review summarises the strategies used to increase the half-life of therapeutic antibody fragments and the use of antibody fragments to extend the half-life of other molecules. Antibody fragments are an important class of therapeutic medicines. There are Fab fragments in development derived from native IgG as well as a range of single chain fragments and other protein scaffolds. As discussed, if these molecules are to be used as therapeutics their half-lives may need to be modulated; this can be accomplished clinically by the covalent conjugation of poly(ethylene glycol) (PEG) *i.e.* Cimzia[®]. Other clinically proven strategies include Fc fusion to increase the half-life of other molecules.

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