

Review

***In Vivo* Applications of Single Chain Fv (Variable Domain) (scFv) Fragments**

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Abstract: Single chain variable domain (Fv) fragments (scFv) are powerful tools in research and clinical settings, owing to better pharmacokinetic properties compared to the parent monoclonal antibodies and the relative ease of producing them in large quantities, at low cost. Though they offer several advantages, they suffer from lower binding affinity and rapid clearance from circulation, which limits their therapeutic potential. However, these fragments can be genetically modified to enhance desirable properties, such as multivalency, high target retention and slower blood clearance, and as such, a variety of scFv formats have been generated. ScFvs can be administered by systemic injection for diagnostic and therapeutic purposes. They can be expressed *in vivo* through viral vectors in instances where large infection rates and sustenance of high levels of the antibody is required. ScFvs have found applications as tools for *in vivo* loss-of-function studies and inactivation of specific protein domains, diagnostic imaging, tumor therapy and treatment for neurodegenerative and infectious diseases. This review will focus on their *in vivo* applications.

Keywords: scFv; recombinant antibodies; *in vivo* expression; adeno-associated virus (AAV); immunotherapy

1. Introduction

Antibodies are the body's defense system to neutralize bacteria and viruses. They recognize and bind a target molecule through the antigen binding sites located on the two Fab segments, which are specific to a particular epitope on an antigen. The fragment crystallizable (Fc) domain binds to Fc receptors and recruits cytotoxic effector molecules and, by interacting with the neonatal Fc receptor, provides long serum half-lives [1,2]. Full length monoclonal antibodies (mAbs) have found various uses as tools in a number of therapeutic applications [3,4]. However, there are situations in which the Fc-mediated effect is undesirable. For instance, unwanted activation of Fc receptor-expressing cells may lead to toxicity through cytokine release [5]. The long serum half-life could also be undesirable in imaging applications, where faster clearance is required in order to limit exposure to radionuclide molecules [5]. Thus, mAbs have been manipulated to improve their therapeutic potential. Initially, the Fc domain has been removed through proteolysis with enzymes, such as papain and pepsin [6], to yield Fab fragments. Later, advances in antibody engineering have enabled the further reduction of mAbs to single chain Fv fragments (scFvs), monovalent Fabs, diabodies and minibodies [7–10]. The properties of these fragments have been tailored to optimize their binding affinity, *in vivo* pharmacokinetics, stability and expression levels [11]. ScFvs have provided an alternative to full-length mAbs in diagnostic and therapeutic applications and account for 35% of antibody fragments in clinical trials [5,12,13].

An scFv fragment consists of the smallest functional antigen-binding domain of an antibody (~30 kDa), in which the variable heavy (V_H) and variable light (V_L) chains are joined together by a flexible peptide linker [14] (Figure 1). The first scFv fragments were cloned by two labs in the same year, Bird *et al.* [15] and Huston *et al.* [16] in 1988. The length and amino acid composition of the linker are crucial in maintaining the correct folding of these proteins [17]. The linker is typically about 3.5 nm in length and must contain hydrophilic residues together with stretches of Gly and Ser sequences for flexibility, in addition to Glu and Lys for enhanced solubility [18,19]. ScFv fragments are most often produced in bacterial cells [20], although other expression systems, including mammalian cells [21], plants [22] and insect cells [23] have been employed.

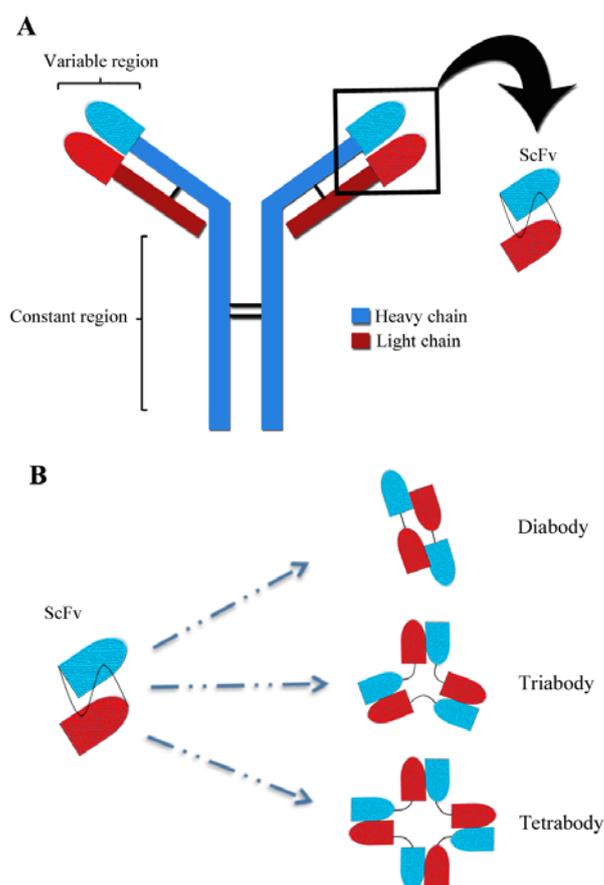
ScFv fragments retain the binding specificity of the parent antibody and offer several advantages compared to full-length mAbs. They display improved pharmacokinetic properties, such as better tissue penetration and rapid blood clearance, which may be beneficial in radiotherapy and diagnostic applications. For instance, these fragments can penetrate more rapidly into tumors compared to an intact antibody [24,25]. Also, when scFvs are joined to radionuclides in radiotherapy applications, their increased rate of clearance from blood minimizes exposure to healthy tissue [26,27]. Recombinant antibodies lack the Fc region, leading to low immunogenicity, making them better therapeutic agents compared to the full-length mAbs for many applications. In addition, scFvs can be cloned and expressed in bacterial and mammalian cells, making it possible to produce large quantities easily and cost-effectively.

One drawback of the *in vivo* use of scFvs is low retention on the target and rapid off rates, due to their monovalency. This can be especially pronounced in the non-equilibrium environment of tumor vasculatures [5]. Their small sizes, though a desirable property for tissue penetration, such as in cancer therapy, also leads to a short *in vivo* half-life, limiting the exposure of the target molecule to the scFv. These issues have been addressed by genetically manipulating and engineering scFvs to yield

multivalent and multifunctional multimers (diabodies, triabodies and tetrabodies) that have higher avidity and lower blood clearance, without compromising tissue penetration abilities [7]. For instance, it has been argued that the optimal tumor-targeting fragment would be a diabody (55 kDa) combining high tissue penetration, target retention and rapid blood clearance [28,29]. Moreover, scFv fragments can be further engineered so that they are linked to different moieties, such as drugs, toxins, radionuclides, quantum dots or liposomes [5,24,30–32]. The application of ScFv fragments is therefore quite broad, including use as molecular tools to perturb protein functions *in vivo* [33,34], delivery agents of radionuclides in diagnostic imaging [26,27] and as potential therapeutics for several diseases, including cancer, HIV and neurodegenerative diseases [13,25,35,36].

Traditionally scFvs were produced from hybridoma cells obtained from immunized animals by amplifying the V_H and V_L domains from the mRNA and joining them with a poly linker, after which they can be inserted in a vector of choice [33] (Figure 2). Recently, *in vitro* display technologies (phage, yeast) have taken over hybridoma technology, because of their adaptability to high throughput formats. They can also be manipulated to optimize scFv properties and generate a population of extremely diverse and highly functional antibodies [37,38]. These *in vitro* displays can be obtained either from natural or synthetic antibody libraries [37]. Synthetic antibody libraries specially eliminate the need to obtain antibodies from immunized host and also enable the introduction of extreme diversity in the scFvs, as they allow manipulating the CDR regions through synthetic DNA [37,38].

Figure 1. (a) Schematic representation of full-length monoclonal antibodies (mAb) and single chain variable domain (Fv) fragments (scFv) fragments. (b) scFv fragments can be engineered into multivalent species: diabody, triabody and tetrabody.



2. *In Vivo* Delivery

In vivo application of scFvs necessitates the choice of an appropriate scFv administration route, vehicle of delivery, maintenance of adequate concentration and toxicity effects. ScFvs can be produced and injected systemically, or they can be delivered using viral vectors and overexpressed *in vivo*. ScFvs can be produced using different cell systems, purified, tagged when necessary and systemically administered. This mode of delivery is useful when long-term expression is not required, for instance, in imaging applications. However, in cases where sustained level of antibody is required, viral mediated delivery provides an alternative. In the effort to develop scFv therapeutics for neurodegenerative diseases where the target is in the brain, a major challenge has been the difficulty of crossing the blood-brain barrier to deliver the therapy. Progress has been made in this area by tailoring scFvs to enhance their uptake by the brain. Viral mediated scFv delivery and enhancement of crossing of the blood brain barrier will be discussed briefly.

2.1. *Viral Mediated Delivery*

In order to sustain the expression of antibody fragments over long periods, scFvs can be cloned in a viral vector and delivered by injection of the virus (Figure 2). The adeno-associated virus (AAV) vector has been the most commonly used mode of scFv delivery compared to other viruses. The AAV is non-pathogenic, non-toxic and has low immunogenicity [39]. The use of other viral vectors is limited, due to some non-desirable properties. One advantage of the AAV is the ability to insert itself into the host genome at a specific locus resulting in a low frequency of random insertions. In contrast, for instance, retroviruses can insert at random sites, leading to mutagenesis and cancer. The AAV virus has a wide spread tropism and can infect both dividing and non-dividing cells, including neurons [39–41]. The most widely used serotype is AAV2, but AAV1, AAV5 and AAV8 have also been employed [42]. The challenge of delivering mAbs via AAV is the size restriction placed by what can be packaged by the virus. For AAV2 virus, the upper limit is 5 kb, and larger proteins are not efficiently expressed [39]. For expression of scFvs in avian cells, the replication-competent retroviral vector (RCASBP) has been used for long-term expression in the developing chick brain [33]. In this vector, genes longer than 2 kb are not efficiently expressed [43]. ScFvs do not suffer from this drawback compared to the larger size mAbs, and high-level long-term expression has been achieved in several animal models.

2.2. *ScFv Linked to a Blood-Brain Barrier (BBB) Receptor Antibody*

The therapeutic potential of antibodies to treat neurodegenerative diseases is limited, due to the difficulty of crossing the blood-brain barrier (BBB) and reaching target antigens in the brain. Very high doses of a therapeutic antibody have to be administered systemically in order for very small amounts (only 0.1–1% of plasma levels) to be detected in the brain, thus necessitating that they be administered intracranially [44,45]. To improve BBB permeability, receptor-mediated transcytosis has been employed, where an antibody against an endogenous BBB receptor-mediated transport protein is linked to scFvs to increase transport across the endothelial cell barrier (Figure 3). To this end chimeric antibodies have been engineered in which scFvs are linked to antibodies against transferrin receptor (TfR) or human insulin receptor (HIR), which are highly expressed by endothelial cells that make up

the BBB [46–49]. The TIR or HIR receptor antibody binds to the receptor on the BBB, leading to the release of scFvs in the brain. The efficiency of scFv release after crossing the BBB is another area to be researched, but regardless, this method is a promising avenue to deliver therapeutics to many neurodegenerative diseases where the target is in the brain. For instance, as a potential therapy in Alzheimer’s disease, peripherally administered scFv-TIR conjugates reduced the level of amyloid plaques in the brain of mouse models, and antibodies were detected in the brain [46–49].

Figure 2. Schematic representation of the process by which scFvs are generated from hybridoma cells and injected in the developing chick brain. Mice were immunized with [3] antigens from the N- and C-terminal parts of repulsive guidance molecule (RGMa) and antibodies generated. The V_H and V_L regions were amplified from the hybridoma mRNA, linked and cloned in the replication-competent retroviral vector (RCASBPB) vector. Virus was produced in DF1 cells and injected in the optic tectum of the chick embryo at E2 (Embryonic day 2). A Dil dye was implanted at E15 in the eye to trace the fibers targeting the tectum. Embryos were sacrificed two days later, and tracing revealed that retinal fibers displayed targeting errors in the tectum, due to neutralization of the RGMa domains [33].

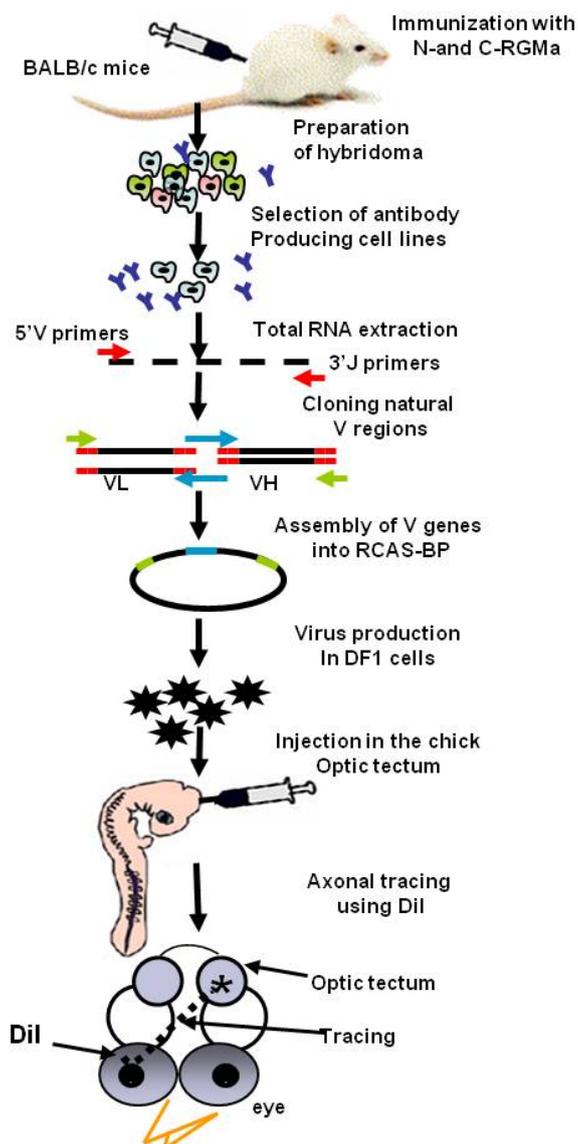
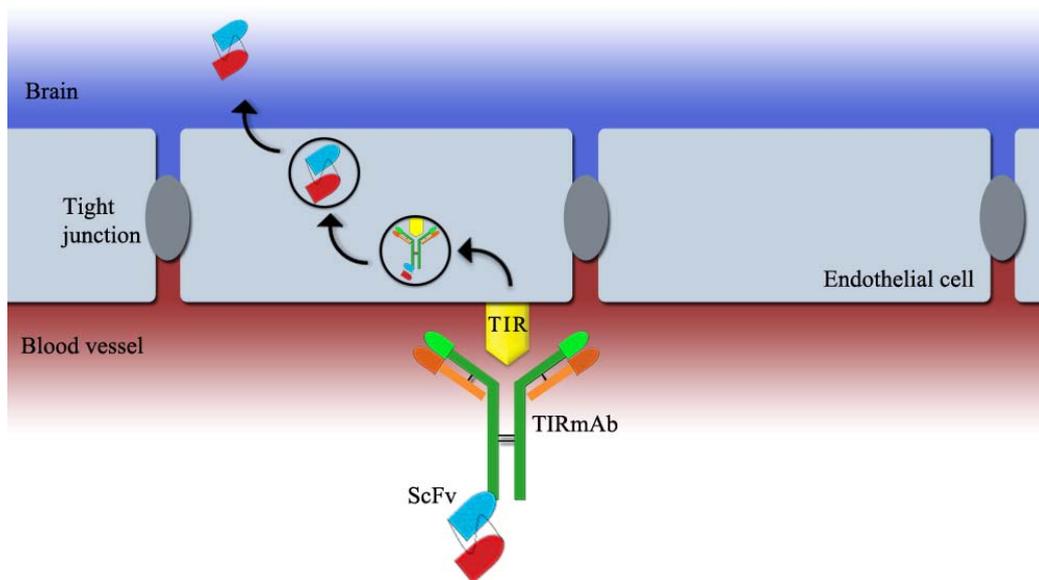


Figure 3. The scFv fragment is linked to the carboxy terminus of an antibody targeting the transferrin receptor (TfR), which is highly expressed by endothelial cells. The scFv crosses the blood-brain barrier through receptor-mediated transcytosis.



3. In Vivo Application of scFv Fragments

3.1. A Tool to Study Protein Functions

ScFvs provide a means to carry out loss-of-function studies *in vivo* animal models. They can be especially useful when only part of a protein needs to be studied. RNA interference (RNAi) technologies that are routinely used for the above function downregulate the entire protein, thus preventing the study of individual domains. In addition, RNAi technologies for *in vivo* use have faced challenges when it comes to delivery to the specific target tissue, as well as the existence of undesirable off-target toxic effects [39,50]. However, *in vivo* use of scFvs can overcome these limitations, since scFvs can be targeted to either a specific part or the entire protein. ScFv delivery via a viral vector eliminates the need for multiple administrations, and antibody concentration can be maintained to sustain silencing. These fragments have been shown to be useful for *in vivo* loss-of-function studies and have helped elucidate protein functions [33,34,40].

In the developing chick visual system, the long-term *in vivo* expression of scFvs was obtained through RCASBP virus-mediated delivery [33]. Hybridoma cells were obtained from mice immunized with antigens from the N- and C- domains of the repulsive guidance molecule (RGMa). ScFvs were generated and cloned in RCASBP and injected in the tectum of chick embryos (Figure 3). The scFvs were cloned with a 6His-tag, and the maintenance of antibody expression and spreading of the virus for the duration of the experiment (E2.5 to E17) was demonstrated by immunostaining for His-tag and the viral protein, gag. The recombinant antibody fragments were efficiently secreted and neutralized

RGMa domains, as axonal pathfinding phenotypes were observed in retinal axons failing to target their correct positions in the optic tectum [33]. This method allowed the dissection of RGMa domain functions in axon guidance, which would not have been possible with RNAi techniques. The use of full-length antibodies would also have limitations in this respect, as it would have to be administered several times for the duration of the experiment, since it cannot be packaged in a viral vector.

In another study, loss-of-function using scFvs were performed to dissect the activity of Pax6 on migrating oligodendrocyte precursor cells (OPC) in the developing chick CNS [34]. A plasmid encoding a neutralizing scFv against the extracellular domain of Pax6 was electroporated in the neural tube. The scFv was able to neutralize the extracellular domain of Pax6, resulting in decreased migration of OPCs, highlighting the involvement of the extracellular domain of Pax6.

Thus, scFv fragments offer a means of elucidating *in vivo* protein functions and may overcome the limitations associated with RNAi and mAb techniques for *in vivo* use.

3.2. Cancer Therapy

Monoclonal antibodies can recognize specific markers expressed on tumor cells and have been explored as treatments for cancer [25,51]. However, their use in solid tumors is limited, because of poor tissue penetration abilities [25]. ScFv fragments exhibit better tissue penetration, but are rapidly cleared from blood, compromising their ability to concentrate in the tumor, and have reduced affinity, due to their small size and monovalent binding, respectively [52]. Efforts to reduce blood clearance included conjugating scFvs to polyethylene glycol to increase their molecular size and hydrodynamic radius [53,54], dimerization of scFvs by incorporating C-terminal cysteines in order to form scFv multimers (diabodies; 60 kDa, triabodies; 90 kDa, tetrabodies; 120 kDa), which improved affinity and reduced renal clearance [52]. However, the increase in size, though, slows scFv clearance from blood; it may also compromise tissue penetration, and the two characteristics have to be balanced for effective tumor therapy. In this regard, diabodies outperform monomeric scFvs with a better tumor blood ratio [52]. Thus, recombinant antibodies of 60–100 kDa have been found to display efficient tumor penetration and fast circulation clearance compared to the intact antibody and are thus better suited for *in vivo* tumor targeting.

Tumor therapy using scFvs requires targeting specific markers on tumor cells and either neutralizing the specific protein or effectively delivering another therapeutic entity, such as a toxin, drug or siRNA. For example, the CC chemokine receptor 4 (CCR4) ligands are highly expressed in several cancers, such as breast cancer, ovarian cancer and cutaneous T-cell lymphoma [42,55,56], and can be targeted for tumor-therapy [42]. This was explored as a potential treatment for cutaneous T-cell lymphoma in which a minibody was encoded in AAV and delivered in a single intravenous injection [42]. A long-lasting and high level expression of the antibody was sustained and resulted in anti-tumor activity against CCR4+ bearing tumor cells [42].

ScFvs have also been recently developed as recombinant immunotoxins (RIT) to carry cytotoxic drugs to kill cancer cells [57,58]. This is achieved by replacing the cell-binding domain of *Pseudomonas exotoxin A* with an scFv that binds to an antigen on cancer cells [57]. Three such RITs, either alone or in combination with chemotherapy, are undergoing clinical trials for different types of cancer malignancies [58,59].

Another area that is being explored in scFv-mediated cancer therapy is using them as cancer vaccines in immunogenic tumors. For instance, epidermal growth factor receptor 2 (HER2) has been targeted in breast cancer therapy, as it is overexpressed in 20% of invasive breast cancer cases [35,60]. Thus, conferring anti-HER2 immunity has been pursued as a promising therapy. ScFvs have been used in this regard, and vaccinations with anti-HER2 scFvs have been shown to result in a robust humoral response and delay in the onset of breast tumors in mice [60].

3.3. Neurodegenerative Diseases

3.3.1. Alzheimer's Disease (AD)

Alzheimer's disease (AD) is characterized by progressive memory deficits and cognitive impairments. There is no cure for AD, and so far, treatments only slow the progression of the disease and ameliorate some of the symptoms. The 4 kDa A β peptide resulting from the cleavage of the amyloid precursor protein is considered a potential target for AD therapy [61]. This peptide is prone to self-aggregation, forming neurotoxic species, and therapies are aimed at reversing the formation of these aggregates [62]. Clinical trials involving active immunization therapies using A β peptide, though, cleared away toxic plaques and slowed cognitive decline among treated patients compared to control groups, had undesirable side effects induced by T-cell-mediated and/or Fc-mediated meningoencephalitis and were abandoned [63,64]. Passive immunization with A β antibodies showed clearance of A β deposits in mouse models and may be potentially developed for AD therapy [36]. However, this mode of administration would require repeated injections and may not be ideal for human therapy. Thus, alternative strategies that involve engineered antibodies that do not contain the Fc fragment and can be overexpressed long-term *in vivo*, eliminating the need for repeated injections, became attractive. Thus, ScFvs have evolved as potential therapeutic venues for the treatment of AD [65]. In addition to being efficiently expressed in several systems leading to high production and lower cost, scFvs may also cross the blood brain barrier, even in cases where they are peripherally administered [66,67].

The main mode of administering scFvs for AD therapy is intracranial injection using adeno-associated virus (AAV) vector. ScFvs with high immunoreactivity against the A β were identified through screening of human scFv libraries and were delivered in the brains of transgenic mouse models of AD [68,69]. Anti A β scFvs have been shown to reduce amyloid plaques, and treated mice had lower amyloid plaques and scored better on functional tests compared to control groups [68,69]. AAV mediated overexpression of scFvs enables long-term *in vivo* expression, and antibodies were detected in the brain a year after injection without causing neurotoxicity [68].

Though successful in mice, less invasive scFv delivery routes are required for human therapies, and other modes of administration have been explored. Intramuscularly-administered scFvs against A β have been shown to be successful in reducing amyloid plaques and in improving cognitive impairments in AD mouse models [66,67,70]. ScFvs were detected in the brain several months after viral-mediated administration and effectively reduced amyloid plaques. Intranasal delivery of scFvs has also been explored as a possible route of administration as a means of avoiding the blood-brain

barrier and clearance from circulation [71]. ScFvs directed against the C-terminus of A β were intranasally delivered and entered the brain, leading to reduction of A β accumulation.

3.3.2. Huntington's Disease (HD)

HD is a neurodegenerative disease that is characterized by abnormal folding and proteolytic cleavage of the mutant huntingtin protein (mHTT) to N-terminal fragments, leading to formation of aggregates and neuronal and neuropil inclusion bodies in the brain [72]. To reduce the levels of mHTT, RNAi interference and protein-based techniques have been employed. RNAi-based approaches may have limitations when it comes to off-target toxic effects and non-specificity for the mutant protein [39]. ScFvs show less off-target effects and can target the mHTT protein based on conformational differences with the wild-type protein [39]. Such scFvs have been generated that preferentially bind to different regions in the amino terminal of mHTT fragments compared to the full HTT and led to a reduction of aggregates [39,73–76].

The normal mode of scFv delivery for HD therapy has been intracranial injections using AAV virus in mouse models [74,75]. For these scFv-based HD therapies to be of use in clinical applications, systematic administration and getting the therapy in the brain are issues that need to be addressed.

The ideal antibody therapy to treat neurodegenerative diseases would be peripheral administration of antibodies that can cross the blood-brain barrier in adequate amounts and be efficiently released in brain areas to maintain therapeutic concentrations. Efforts are underway to genetically engineer scFvs to enhance their uptake by the brain. With new developments in delivering scFvs in the brain using receptor-mediated transcytosis, there is still more to be done to ensure that scFvs are efficiently released once they cross the blood-brain barrier [48].

3.4. In Vivo Imaging

The small size of scFvs enables genetic manipulations to conjugate them with radionuclei, quantum dots, nanoparticles, *etc.* [26,27]. Thus, they can provide a non-invasive tool to visualize the location and distribution of a specific target *in vivo*. To this aim, an optimal pharmacokinetics is critical and should balance deep tissue penetration and optimal clearance from blood without compromising binding affinity. The scFv format is the ideal imaging tool among the other antibody fragments, since it combines all of the above properties. Though the faster clearance of scFvs poses challenges for therapeutic applications, it is a much desired property when it comes to *in vivo* diagnostic applications. For instance, this has been shown in imaging of heart disease using site-specific ¹¹¹In labeled scFv fragments against tenascin-c in a myocardial infarction rat model [27]. Radiolabelled scFv specifically bound to tenascin-c, and higher uptake of radioactivity was seen in infarcted myocardium compared to the non-infarcted one. In addition, a much lower amount of radioactivity (about three-times less per gram of body weight) remained at 6 h after ¹¹¹In-scFv injection compared to similarly labeled Fab [27].

scFvs have been attached to fluorescent moieties and have been employed to image a target tissue [77,78]. For instance, scFv was conjugated to quantum dots and delivered in a xenograft mouse model [78]. The scFv was targeted against GRP78, a protein that is expressed by various cancers and has roles in cell proliferation and angiogenesis [79]. The quantum dot conjugated scFv enabled easy visualization of the *in vivo* target, as well as inhibiting breast tumor growth. Also in another study,

anti-tumor scFvs (425 scFv and 4D5 scFv), which bind to oncomarkers, HER1 EGFR and HER2/neu, respectively, were conjugated to quantum dots and enabled visualization of cancer cells [77]. This study was done *in vitro* using cells that overexpress the oncomarkers; however, it illustrates the point that scFvs allow for targeting and visualization of specific tissues *in vivo*.

Another emerging area of scFv use is in the magnetic resonance imaging (MRI) field [80,81]. The sensitivity of MRI imaging has been shown to improve by conjugating scFvs with supramagnetic iron oxide nanoparticles (SPIONs). For instance, in imaging of tumors, SPIONs provide contrast between healthy and cancerous cells, due to a differential uptake. Thus, by conjugating scFvs with SPIONs, specific cells can be targeted, improving the sensitivity of MRI. In a study, scFvs against carcinoembryonic antigen was attached to SPIONs, and it was demonstrated that a selective image contrast was obtained on MRI highlighting the potential of scFvs to improve specificity of targeting cancer cells.

3.5. Vehicles to Deliver Drugs/Nanoparticles

The application of scFvs as vehicles to deliver therapeutics is an emerging area for the development of immunotherapeutics for several diseases. The specific target recognition of scFvs combined with high levels of expression via viruses makes them useful tools to deliver drugs, siRNA and toxins to immune cells and tumors [82,83]. For instance, this has been shown in HIV-infected mice, in which anti-viral siRNA was coupled to a CD7 targeting scFv and was administered systematically [82]. This resulted in lower viral replication.

Another emerging application is the use of scFvs as immuno-nanoparticles for the delivery of drugs to a specific target. Chio *et al.* [84] have shown that latent HIV, which hides in CD4+ T-cells, can be targeted by delivering histone deacetylase inhibitor (HDACi) by covalently binding scFvCD7 with PLGA (a biodegradable polymer) that is loaded with HDACi. This method showed specificity, as the immuno-nanoparticles were only delivered to the T-cell targets and enabled the sustained release of HDACi. This work was done using human cells and needs to be shown *in vivo*. However, it highlights the potential of scFvs to be used as delivery vehicles.

4. Conclusions

Since their introduction in 1988, scFvs have become highly relevant in pre-clinical and clinical applications and research laboratories. Advances in antibody engineering have made possible the generation of highly tailored scFvs that have improved pharmacokinetic properties, making them more clinically relevant. A trend to couple these recombinant antibodies to different moieties has broadened their potential in diagnostic, therapeutic and *in vivo* imaging applications. Still, several challenges remain to be addressed in their *in vivo* use. Their small size, though advantageous for *in vivo* imaging, is undesirable for therapeutic applications, since it makes them unstable and prevents accumulation of scFvs at the target site. There are efforts to increase their *in vivo* half-life and stability through various modifications, such as PEGylation. However, certain technical challenges need to be overcome to produce them cost-effectively in this format. The lack of Fc leads to low immunogenicity; however, it also limits their therapeutic potential, since they can only exert their functions through binding to the target and cannot induce Fc-mediated cytotoxicity. Thus, they have to be conjugated to drugs and

toxins to be more effective in therapies. Even with the above challenges, these fragments are bound to become more relevant in the development of therapeutics for cancer, neurodegenerative and immunological diseases, due to their favorable properties.

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