# Part One
# General Information

# 1
# Half-Life Modulating Strategies – An Introduction

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## 1.1
## Therapeutic Proteins

With roughly 200 biologics approved for therapeutic applications and more than 600 under clinical development [1], biotechnology products cover an increased proportion of all therapeutic drugs. Besides monoclonal antibodies and vaccines, which account for more than two-thirds of these produces, hormones, growth factors, cytokines, fusion proteins, coagulation factors, enzymes and other proteins are listed. An overview of the different classes of currently approved protein therapeutics is shown in Table 1.1. Except for antibodies and Fc fusion proteins, many of these proteins possess a molecular mass below 50 kDa and a rather short terminal half-life in the range of minutes to hours. In order to maintain a therapeutically effective concentration over a prolonged period of time, infusions or frequent administrations are performed, or the drug is applied loco-regional or subcutaneously utilizing a slow adsorption into the blood stream. These limitations of small size protein drugs has led to the development and implementation of half-life extension strategies to prolong circulation of these recombinant antibodies in the blood and thus improve administration and pharmacokinetic as well as pharmacodynamic properties.

## 1.2
## Renal Clearance and FcRn-Mediated Recycling

The efficacy of protein therapeutics is strongly determined by their pharmacokinetic properties, including their plasma half-lives, which influence distribution and excretion. Although a small size facilitates tissue penetration, these molecules are often rapidly cleared from circulation. Thus, they have to be administered as infusion or repeated intravenous (i.v.) or subcutaneous (s.c.) bolus injections in order to maintain a therapeutically effective dose over a prolonged period of time,

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**Table 1.1** Proteins used as therapeutics.

| Protein class | Protein | Indication | Examples of approved drugs | $M_r$ (kDa) | Terminal half-life |
|---|---|---|---|---|---|
| Hormones | Insulin | Diabetes | Humalog, Novolog | 6 | 4–6 min |
| | hGH | Growth disturbance | Protropin, Humatrope, | 22 | 2 h |
| | FSH | Infertility | Follistim, Fertavid | 30 | 3–4 h |
| | Glucagon-like peptide 1 | Type 2 diabetes | Victoza | 4 | 2 min |
| | Parathyroid hormone | Osteoporosis | Preotach | 10 | 4 min |
| | Calcitonin | Osteoporosis | Fortical | 4 | 45–60 min |
| | Lutropin | Infertility | Luveris | 23 | 20 min |
| | Glucagon | Hypoglycemia | Glucagon | 4 | 3–6 min |
| Growth factors | Erythropoietin | Anemia | Epogen, Procrit | 34 | 2–13 h |
| | G-CSF/GM-CSF | Neutropenia | Filgrastim, | 20 | 4 h |
| | IGF-1 | Growth failure | Increlex | 8 | 10 min |
| Interferons | IFN-α | Hepatitis C (and B) | Roferon, Infergen | 20 | 2–3 h |
| | IFN-β | Multiple sclerosis | Betaferon, Avonex | 23 | 5–10 h |
| | IFN-γ | Chr. granulomatosis | Actimmune | 25 | 30 min |
| Interleukins | IL-2 | Renal cell carcinoma | Proleukin | 16 | 5–7 min |
| | IL-11 | Thrombocytopenia | Neumega | 23 | 2 d |
| | IL-1Ra | Rheumatoid arthritis | Kineret | 25 | 6 min |
| Coagulation factors | Factor VIII | Hemophilia A | Kogenate, ReFacto | | 12 h[a)] |
| | Factor IX | Hemophilia B | Benefix | 55 | 18–24 h |
| | Factor VIIa | Hemophilia | Novoseven | 50 | 2–3 h |
| | Thrombin | Bleeding during surgery | Recothrom | 36 | 2–3 d |

**Table 1.1** Continued

| Protein class | Protein | Indication | Examples of approved drugs | $M_r$ (kDa) | Terminal half-life |
|---|---|---|---|---|---|
| Thrombolytics and anti-coagulants | t-PA | Myocardial infarction | Tenecteplase | 65 | 2–12 min |
| | Hirudin | Thrombozytopenia | Refludan | 7 | 3 h |
| | Activated protein C | Severe sepsis | Xigris | 62 | 1–2 h |
| Enzymes | α-glucosidase | Pompe disease | Myozyme, Lumizyme | 109 | 2–3 h |
| | Glucocerebrosidase | Gaucher disease | Cerezyme | 60 | 18 min |
| | Iduronate-2-sulfatase | Mucopolysaccharidose II | Elaprase | 76 | 45 min |
| | Galactosidase | Fabry disease | Fabrazyme, Replagal | 100 | 1–2 h |
| | Urate oxidase | Hyperuricemia | Fasturtec | 140 | 17–19 h |
| | DNase | Cystic fibrosis | Pulmozyme | 37 | n.a. |
| Antibodies and antibody fragments | IgG | Cancer, inflammatory and infectious diseases, transplantation, etc. | Rituxan, Hereptin, Avastin, Remicade, Humira, Synagis, Zenapax, Xolair, etc. | 150 | days to weeks |
| | Fab | Prevention of blood clotting, AMD | ReoPro, Lucentis | 50 | 30 min |
| Fusion proteins | TNFR2-Fc | Rheumatoid arthritis | Enbrel | 150 | 3–6 d |
| | TMP-Fc | Thrombocytopenia | Nplate | 60 | 1–34 d |
| | CTLA-4-Fc | Rheumatoid arthritis | Orenica | 92 | 8–25 d |
| | IL-1R-Fc | CAPS | Arcalyst | 251 | 9 d |
| | LFA-3-Fc | Plaque psoriasis | Amevive | 92 | 11 d |
| | IL-2-DT | Cut. T-cell leukemia | Ontak | 58 | 70–80 min |

a) Bound to vWF; n.a., not available.

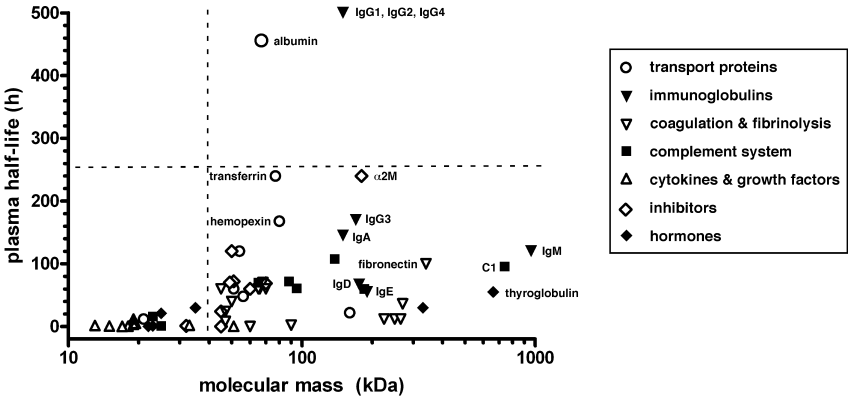


**Figure 1.1** The molecular mass and half-life of plasma proteins. Proteins are allocated according to their function.

or are restricted to loco-regional treatment. The rapid elimination of these small molecules mainly occurs by renal filtration and degradation [2] (see also Chapter 2). A comparison of the half-lives of plasma proteins reveals the threshold for rapid excretion to be in the range of approximately 40–50 kDa, demonstrating that the size of the molecules is one of the determining factors (Figure 1.1). The glomerular filtration barrier is formed by the fenestrated endothelium, the glomerular basement membrane (GBM) and the slit diaphragm located between the podocyte foot processes [3]. The fenestrae between the glomerular endothelial cells have diameters between 50–100 nm, thus, allowing free diffusion of molecules. It was suggested that the slit diaphragm represents the ultimate macromolecular barrier, forming an isoporous, zipper-like filter structure with numerous small, 4–5 nm diameter pores and a lower number of 8–10 nm diameter pores [4–6]. In addition to size, the charge of a protein contributes to renal filtration. It has been suggested the proteoglycans of the endothelial cells and the GBM contribute to an anionic barrier, which partially prevents the passage of plasma macromolecules [3]. Consequently, the size of a protein therapeutic, that is, its hydrodynamic radius, and also its physiochemical properties, that is, charge, represent starting points in order to improve half-life. Interestingly, two kinds of molecules, serum albumin and IgGs, exhibit an extraordinary long half-life in humans. Thus, human serum albumin (HSA) has a half-life of 19 days and immunoglobulins (IgG1, IgG2 and IgG4) have half-lives in the range of 3 to 4 weeks [7, 8]. These long half-lives, which clearly set albumin and IgG apart from the other plasma proteins (Figure 1.1), are caused by a recycling process mediated by the neonatal Fc receptor (FcRn) [9–11] (see also Chapters 8 and 11). FcRn, expressed for example by endothelial cells, is capable of binding albumin and IgGs in a pH-dependent manner. Thus, after cellular uptake of plasma proteins through macropinocytosis, albumin and IgG will bind to FcRn in the acidic environment of the endosomes. This binding diverges albumin and IgG from degradation in the lysosomal compartment and

redirects them to the plasma membrane, where they are released back into the blood plasma because of the neutral pH. This offers additional opportunities to extend or modulate the half-life of proteins, for example, through fusion to albumin or the Fc region of IgG [12].

## 1.3 Strategies to Modulate Plasma Half-Life

With an increasing number of protein therapeutics approved and developed (see Table 1.1) including various alternative antibody formats [13], many of them exhibiting a short plasma half-life, strategies to improve the pharmacokinetic properties are becoming increasingly important [14]. Based on the parameters described above which influence half-life, the strategies to extend half-lives of therapeutic proteins can be divided into those that (i) utilize an increased size and thus the hydrodynamic volume and (ii) in addition implement recycling by the neonatal Fc receptor (FcRn), the receptor that is responsible for the long half-lives of IgGs and albumin. These strategies comprise a variety of different approaches including chemical coupling of polymers and carbohydrates, post-translational modifications such as *N*-glycosylation, and fusion to recombinant polymer mimetics. Furthermore, conjugation, binding or fusion to an Fc region or serum albumin, respectively, results not only in an increased size but also incorporates FcRn-mediated recycling (Figure 1.2; Tables 1.2 and 1.3). More recently, nanoparticulate formulations have also been developed to improve the half-life and biodistribution of

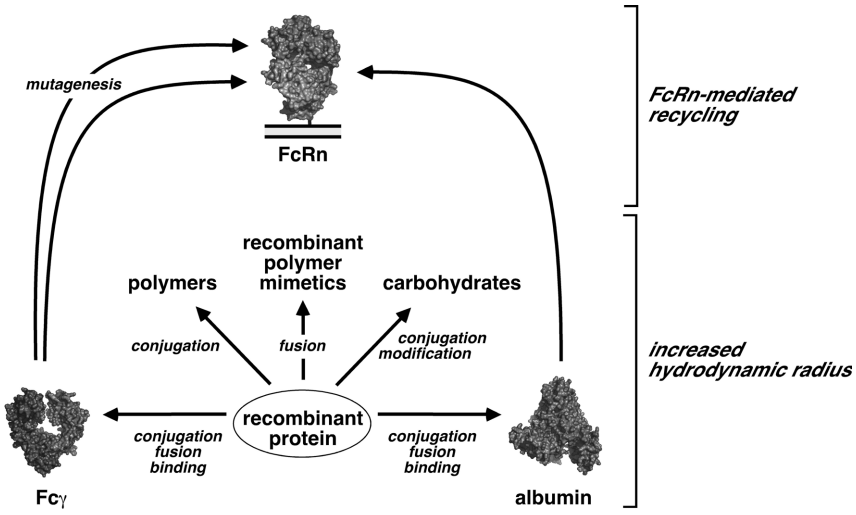


**Figure 1.2** Overview of the different strategies to extend the plasma half-life of recombinant proteins, aiming at increasing the hydrodynamic radius as well as implementing FcRn-mediated recycling.

**Table 1.2** Strategies to modulate half-life of therapeutic proteins.

| Strategy | Modification | Effect on hydrodynamic radius | Effect on FcRn-mediated recycling | Effect on half-life |
|---|---|---|---|---|
| PEGylation | Chemical conjugation of methoxy polyethylene glycol (mPEG) | Increased | None | Prolonged, depending on PEG size and structure |
| Polysialylation | Chemical conjugation or attached by post-translational modification of polysialic acid | Increased (plus change of pI) | None | Prolonged, depending on extent of polysialylation |
| HESylation | Chemical conjugation of hydroxyethyl starch (HES) | Increased | None | Prolonged, depending on HES size and structure |
| Recombinant PEG mimetics | Genetic fusion of flexible, hydrophilic amino acid chains | Increased | None | Prolonged, depending on size and composition |
| *N*-glycosylation | Post-translational attachment of *N*-glycans | Increased | None | Moderately prolonged |
| *O*-glycosylation | Post-translational attachment of *O*-glycans | Increased | None | Moderately prolonged |
| Fc fusion | Genetic fusion of IgG Fc region | Increased | Utilized | Prolonged |
| Engineered Fc | Mutations introduced into the Fc region of IgGs or Fc fusion proteins | Unaltered | Increased or decreased (depending on mutations) | Prolonged or reduced |
| IgG binding | Genetic fusion or conjugation to IgG-binding moieties | Increased upon binding to IgG | Utilized or diminished (depending on binding site) | Prolonged |
| Albumin fusion | Genetic fusion to serum albumin | Increased | Utilized | Prolonged |

**Table 1.2** Continued

| Strategy | Modification | Effect on hydrodynamic radius | Effect on FcRn-mediated recycling | Effect on half-life |
|---|---|---|---|---|
| Albumin binding | Genetic fusion or conjugation to albumin-binding moieties (peptides, protein domains, antibody fragments, antibody mimetic scaffolds, small chemicals, fatty acids, etc.) | Increased after binding to albumin | Utilized | Prolonged |
| Albumin coupling | Chemical conjugation to cysteine 34 | Increased | Utilized | Prolonged |
| Nanoparticles | Encapsulation into or conjugation to nanoparticles (e.g., liposomes, polymeric capsules) | Strongly increased by the size of the nanoparticles (50–200 nm) | None | Prolonged (slow release) |

therapeutic proteins, for example, through encapsulation into liposomes or polymeric capsules [15].

### 1.3.1 Strategies to Increase the Hydrodynamic Radius

The foremost approach in improving the half-life of a protein therapeutic is to reduce the renal clearance rate, for example, by increasing the size above the renal cut-off of 40–50 kDa. This can be achieved by several ways including chemical and post-translational modification as well as genetic engineering (Table 1.2).

PEGylation, that is, the chemical conjugation of polyethylene glycol (PEG), mainly of its methoxy derivative methoxy polyethylene glycol (mPEG), was established more then two decades ago [16] (see also Chapter 3). In 1990, the first PEGylated protein drug (pegadamase for the treatment of severe combined immunodeficiency [SCID]) was approved by the FDA [17, 18] and since then eight more PEGylated protein drugs have been approved, including enzymes, interferon-α2b, G-CSF, hGH, erythropoetin, and a Fab fragment [19, 20] (see Table 1.3). PEG is composed of ethylene oxide units connected in a linear or branched configuration

**Table 1.3** Half-life extended protein therapeutics (approved or in clinical trials).

| Modification/ Protein | Drug | Indication | Status |
|---|---|---|---|
| PEGylation | | | |
| Adenosine deaminase | Pegademase bovine (Adagen) | Severe combined immunodeficiency disease (SCID) | Approved 1990 |
| L-asparaginase | Pegaspargase (Oncaspar) | Acute lymphoblastic leukemia | Approved 1994 |
| Interferon α-2b | Peginterferon alfa-2b (Peg-Intron) | Hepatitis C | Approved 2000 |
| Interferon α-2b | Peginterferon alfa-2b (Pegasys) | Hepatitis C | Approved 2002 |
| G-CSF | Pegfilgrastim (Neulasta) | Chemotherapy-induced neutropenia | Approved 2002 |
| Human growth hormone (hGH) | Pegvisomant (Somavert) | Acromegaly | Approved 2002 |
| Erythropoietin | mPEG-epoetin beta (Mircera) | Anemia | Approved 2007 |
| Anti-TNF Fab' | Certolizumab pegol (Cimzia) | Crohn's disease | Approved 2008 |
| Uricase | Pegloticase (Krystexxa) | Chronic gout | Approved 2010 |
| EPO mimetic peptide | Peginesatide (Hematide) | Anemia | Phase II |
| Phenylalanine ammonia lyase | rAvPAL-PEG | Phenylketonuria | Phase II |
| IL-29 | PEG-rIL-29 | Hepatitis C | Phase I |
| Coagulation factor IX | PEG-rFIX | Hemophilia B | Phase I |
| Arginase | PEG-rArgI | Liver cancer | Phase I |
| Hyaluronidase | PEGPH20 | Advanced solid tumors | Phase I |
| *N*-glycosylation and polysialylation | | | |
| Erythropoietin | Darbepoetin alfa (Aranesp) | Anemia | Approved 2008 |
| Erythropoietin | PSA-EPO (ErepoXen) | Anemia | Phase II |
| Insulin | PSA-insulin (SuliXen) | Diabetes mellitus | Phase I |

**Table 1.3** Continued

| Modification/ Protein | Drug | Indication | Status |
|---|---|---|---|
| Albumin fusion | | | |
| Interferon α-2b | Albinterferon alfa-2b (Joulferon, Zalbin) | Hepatitis C | Phase III |
| Coagulation factor IX | rIX-FP | Hemophilia B | Phase I |
| HER2 + HER3 specific single-chain Fv | MM-111 (scFv-HSA-scFv) | Cancer | Phase I/II |
| Albumin binding through a conjugated fatty acid chain | | | |
| Insulin | Insulin detemir (Levemir) | Diabetes mellitus | Approved 2003 |
| Glucagon-like peptide-1 | Liraglutide (Victoza) | Diabetes mellitus type 2 | Approved 2009 |
| Fc fusion proteins | | | |
| TNF receptor 2 | Etanercept (Enbrel) | Rheumatoid arthritis, ankylosing spondylitis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, plaque psoriasis | Approved 1998 |
| LFA-3 | Alefacept (Amevive) | Severe chronic plaque psoriasis | Approved 2003 |
| CTLA-4 | Abatacept (Orenica) | Rheumatoid arthritis, juvenile idiopathic arthritis | Approved 2005 |
| IL-1R | Rilonacept (Arcalyst) | Cryopyrin-associated periodic syndromes | Approved 2008 |
| TPO-mimetic peptide | Romiplostim (Nplate) | Chronic idiopathic thrombocytopenic purpura | Approved 2008 |
| VEGF receptor | Aflibercept (VEGF trap) | Macular degeneration | Phase III |
| BR3 | Briobacept (BR3-Fc) | Rheumatoid arthritis | Phase II |
| Coagulation factor IX | rFIXFc | Hemophilia B | Phase II/III |
| Coagulation factor VIII | rFVIII-Fc | Hemophilia A | Phase I |

and of varying length. In the approved drugs, one or several PEG chains of 5 to 40 kDa are conjugated. Because PEG is highly hydrophilic, the molecular mass of PEGylated proteins is drastically increased through the binding of water molecules, reflected by a strong increase of the hydrodynamic radius. Different coupling methods have been established including random and site-directed approaches. The PEGylation strategy, that is, the site of PEGylation as well as the number and size of attached PEG chains, has to be carefully chosen in order to avoid a reduction or abrogation of the activity of the therapeutic protein [18]. Ideally, a single PEG chain is conjugated in a site-directed manner, for example, through the use of existing or genetically introduced cysteine residues. This is exemplified by Cimzia (certolizumab pegol), a bacterially produced anti-TNF Fab' fragment, where a 40 kDa PEG chain is attached to the free cysteine at the C-terminus of the heavy chain Fd chain, that is, opposite the antigen-binding site [21]. In general, PEGylation of proteins is considered to be safe and well tolerated [20], although in animals the occurrence of renal tubular vacuolization has been observed due to accumulation of the nondegradable PEG chains in the kidney.

Recently, alternative strategies to PEGylation have been established. For example, it was noted that the polypeptide backbone resembles, at least in part, the structure of PEG. This has led to the generation of long polypeptide chains of a hydrophilic and flexible structure, which can be genetically fused to recombinant proteins [22, 23] (see also Chapter 4). Thus, chemical conjugation and additional purification steps are avoided. Furthermore, the fused polypeptide chains are biodegradable and have also been shown to be immunologically inert. *In vivo* studies established that these recombinant PEG mimetics behave in a similar manner to PEG, that is, they result in a drastic increase of the hydrodynamic radius and extension of the half-life.

As an alternative, carbohydrate chains can be attached to therapeutic proteins. Because this process takes place naturally, for example, by post-translational modifications in mammalian cells, therapeutic proteins have been genetically modified to contain additional *N*- or *O*-glycosylation sites [24–26] (see Chapter 5). For example, *N*-glycosylation sites (Asn-X-Thr) can be introduced into the protein sequence of interest resulting in hyperglycosylated proteins (Table 1.3). A prominent example is darbepoetin alfa (Aranesp), a hyperglycosylated derivative of human erythropoietin containing two additional *N*-glycosylation sites [27]. Compared with recombinant human erythropoietin, darbepoetin alfa has a threefold longer terminal half-life and a strongly increased capacity to elicit an erythropoietic response [24]. Similarly, sequences obtained from *O*-glycosylated proteins, for example, from chorionic gonadotropin, have been fused to therapeutic proteins resulting in *O*-glycosylated proteins with prolonged half-life and improved bioactivity [28] (see Chapter 5).

Carbohydrates have also been chemically conjugated to therapeutic proteins in a similar way to the PEGylation approach (Table 1.3). For example, polysialic acid (PSA) has been investigated as an alternative to PEG [29, 30]. PSA is found on the surface of a variety of cells including mammalian cells, thus is a biocompatible and biodegradable natural polymer. Colominic acid, a linear polymer of α-(2,8)-

linked *N*-acetylneuraminic acid, was used for polysialylation of various proteins including asparaginase, insulin and antibody fragments and was shown to be capable of prolonging their half-lives [31] (see also Chapter 6).

Another carbohydrate structure, which has been established for half-life extension of therapeutic proteins, is hydroxyethyl starch (HES). HES is a modified, branched amylopectin, for example, isolated from waxy maize starch, composed of glucose units linked by α-1,4- and α-1,6-glycosidic bonds. HES is an approved plasma volume expander with a proven safety record. Because of its close similarity to glycogen, HES is not immunogenic [32, 33]. The size and structure of HES and thereby its stability can be adjusted by acidic hydrolysis and by chemical hydroxyethylation at positions 2, 3, and 6 of the glucose unit. The HESylation technology was pioneered by the company Fresenius Kabi to improve the pharmacokinetic and pharmacodynamic properties of therapeutic proteins. For example, it was successfully applied to produce improved derivatives of erythropoietin by chemical coupling of a 60 kDa HES (see also Chapter 7).

Another obvious approach to increase the size of a therapeutic protein is the fusion to another protein moiety, for example, a plasma protein. Mainly, fusion to immunoglobulin Fc regions or serum albumin has been utilized to extend the half-life of peptides and proteins [34, 35] (Table 1.3). Because albumin and Fc-containing proteins also utilize recycling by the FcRn, these strategies are summarized below (Section 1.3.2).

Pharmaceutical formulations of drugs are widely used to influence their pharmacokinetic properties, that is, administration, distribution, metabolism and excretion (ADME) [36, 37]. For example, small molecular weight drugs such as doxorobucin and amphothericin B have been encapsulated or incorporated, respectively, into liposomal carrier systems. In addition to liposomes, various other nanoparticulate carrier systems have been utilized to improve the pharmacokinetic and pharmacodynamic properties of proteins (see Chapters 16 and 17). The carrier systems combine various advantages. Because of their large size in the range of 50 to 200 nm, the half-life is strongly increased. Furthermore, nanoparticulate formulations influence biodistribution, for example, accumulation in tumors through an enhanced permeability and retention (EPR) in the tumor tissue [38]. In addition, they are also capable of protecting the protein drug from degradation, for example, through plasma proteases, and can act as slow release formulation, that is, as a drug depot in the body.

### 1.3.2 Strategies Implementing FcRn-Mediated Recycling

Fusion of a therapeutic protein (or peptide) to another protein results in a fusion protein with increased molecular mass. Mainly human plasma proteins, or fragments thereof, exhibiting per se a long half-life, are used for this purpose. This includes the Fc portion of IgG, especially of the γ1 subclass, and serum albumin. As described before, IgG and serum albumin possess a size above the renal filtration threshold and utilize recycling by the neonatal Fc receptor (FcRn).

Importantly, it has been shown that the binding sites for IgG and albumin are located at different regions of the FcRn, that is, the two molecules do not compete for the same binding site (see Chapter 8). In addition to direct fusion leading to a covalent linkage, binding to IgG or albumin has been employed for half-life extension strategies (Figure 1.2, Table 1.2). This noncovalent interaction allows for a reversible interaction, that is, dissociation from the plasma protein, which can be beneficial in respect to tissue penetration and bioactivity [39].

Therapeutic proteins and peptides have been mainly fused to the N-terminal of the Fc fragment, which often includes the hinge region in order to establish a covalent linkage between the heavy chain fragments [40] (see Chapters 9 and 10) (Table 1.3). Fusion to an Fc region increases the molecular mass by approximately 50 kDa but also results in a homodimeric molecule possessing two moieties of the therapeutic protein. This might further increase the therapeutic activity, for example, through stronger binding due to avidity effects and/or more efficient activation/neutralization of the targeted molecule [34]. This strategy has been realized for the generation of various soluble receptors (see Table 1.1), which are potent inhibitors of natural ligands such as TNF, a key mediator of inflammatory diseases, but also for ligands, for example, thrombopoietin mimetic peptides, leading to fusion proteins with strong receptor-activating properties. The binding site for the FcRn resides in the Fc region and is located between the CH2 and CH3 domains. Key residues involved in the interaction have been identified and mutants of the Fc region have been generated exhibiting either an increased or decreased affinity for FcRn at acidic pH. Incorporation of such mutations into whole IgG molecules results in antibodies with prolonged or reduced half-life [41–43] (see Chapter 11). Principally, these modifications can also be introduced in the Fc region of Fc fusion proteins in order to further improve the half-life of the therapeutic protein.

Using albumin, therapeutic proteins can be fused to either the N- or C-terminus or to both ends located at opposite sites of albumin (see Chapter 12). This strategy has been extensively applied to extend the half-life of a variety of different therapeutic proteins, including interferons, growth factors, hormones, cytokines, coagulation factors, and antibody fragments [39, 44]. Albinterferon alpha-2b (Zalbin, Joulferon) for the treatment of chronic hepatitis C is the most advanced albumin fusion proteins, which is currently in phase 3 clinical trials [45]. As an alternative to genetic fusion, therapeutic proteins can also be chemically conjugated to albumin. Because albumin exhibits a free and accessible cysteine residue at position 34, proteins, peptides but also other drugs can be chemically conjugated in a defined and site-directed manner, for example, with bifunctional crosslinkers. This strategy has, for example, been applied to extend the half-life of insulin and peptides with anti-HIV activity [46, 47].

A variety of albumin-binding strategies has been developed as an alternative for direct fusion [12]. Because serum albumin is a transport protein for different molecules, including fatty acids, such natural ligands with albumin-binding activity were used initially for half-life extension of small protein therapeutics. A prominent example is insulin determir (Levemir), where a myristyl chain is con-

jugated to a genetically modified insulin resulting in a long-acting insulin analog [48] (Table 1.3). Alternatively, albumin-binding synthetic peptides, for example, isolated from phage display libraries [49], or albumin-binding domains (ABD) from bacterial proteins, for example, streptococcal protein G [50], were employed for the generation of fusion proteins with albumin-binding activity (see Chapter 14). More recently, small chemicals with albumin-binding activity have been generated, which can be conjugated to therapeutic proteins and other molecules [51] (see Chapter 15). In addition, various recombinant antibody fragments (e.g., scFv, Fab, single domain antibodies, and nanobodies) or alternative scaffolds (e.g., Darpins) have been established as albumin-binding moieties [52–54] (see Chapter 13). All these strategies rely on complex formation with albumin after administration of the fusion protein into the blood stream. Several studies have shown that affinities in the micro- to nanomolar range are sufficient for half-life extension. For these strategies it is essential that binding of the fusion protein to albumin does not interfere with FcRn recycling and that binding is stable under acidic pH in order to avoid dissociation from albumin in the endosomal compartment.

## 1.4 Half-Life Extension Strategies Applied to a Bispecific Single-Chain Diabody–A Case Study

Bispecific antibodies were developed in the early 1980s, initially for the retargeting of immune effector cells to tumor cells for cellular cancer therapy [55]. For example, bispecific antibodies directed against a tumor-associated antigen (TAA) and CD3, which is part of the T-cell receptor complex, allow the retargeting and triggering of cytotoxic T lymphocytes leading to antibody-mediating killing of tumor cells. At that time, preparation methods included chemical crosslinking of two monoclonal antibodies or their fragments, or the use of somatic hybridization of two antibody-producing cell lines [56]. The high expectations were, however, not fulfilled in clinical trials, mainly because of low efficacy, the occurrence of severe side effects, such as cytokine storm syndrome, and the generation of a neutralizing human anti-mouse antibody response (HAMA) against the nonhuman bispecific antibodies [57]. A better understanding of effector cell biology and the implementation of antibody engineering resulted in the development of various recombinant bispecific antibody formats circumventing many of the problems associated with the original molecules. Among others, small bispecific antibodies such as tandem scFv, diabodies, and its single-chain version (single-chain diabodies) were developed and several of these molecules have meanwhile entered clinical trials, for example, for the treatment of hematologic malignancies [58]. These bispecific antibody molecules are composed only of the variable domains of two antibodies genetically connected by flexible linkers by a defined arrangement. Thus, in tandem scFvs two scFv moieties, which represent separate folding units, are connected by a middle linker of varying length in the order ($V_H$A-$V_L$A)-($V_H$B-$V_L$B). In contrast, in single-chain diabodies, which have a similar size as tandem scFv, the

variable domains have the order $V_HA$-$V_LB$-$V_HB$-$V_LA$ [59]. The molecular mass of these antibody molecules is in the range of 50 to 60 kDa. Several studies have shown that these molecules are rapidly cleared from circulation with terminal half-lives of only a few hours. For example, a bispecific single-chain diabody (scDb) directed against carcinoembryonic antigen and CD3 (CEACD3) exhibits in mice a half-life of 1 to 2 hours [60]. Using this scDb, we have recently compared different half-life extension strategies, including PEGylation, *N*-glycosylation, fusion to HSA, and fusion to an albumin-binding domain from streptococcal protein G (Figure 1.3, Table 1.4).

PEGylation of the scDb was achieved by introducing an additional cysteine residue either in one of the flanking linkers or at the C-terminus. This allowed for a site-directed and defined conjugation of a branched 40 kDa PEG chain resulting in a PEGylated scDb with a calculated molecular mass of 100 kDa. Interestingly however, in size exclusion chromatography this molecule migrated with an apparent mass of around 800 kDa, that is, the hydrodynamic radius increased from 2.7 nm of the unmodified scDb to 7.9 nm of the PEGylated scDb (see also Table 1.2). This resulted in an increase of the terminal half-life to approximately 13 hours in CD1 mice receiving a single dose of 25 µg protein [61].

*N*-glycosylated scDb derivatives were generated by introducting *N*-glycosylation sites (Asn-X-Thr sequon) in the two flanking linkers as well as at a C-terminal extension of varying length [61]. Thus, three derivatives containing three, six or nine potential *N*-glycosylation sites (scDb-$ABC_1$, scDb-$ABC_4$, and scDb-$ABC_7$) were obtained and produced in 293 cells. *N*-glycosylation through post-translational modification resulted in molecules with an increased size, as revealed by SDS-PAGE and SEC analysis. However, this analysis in combination with MS

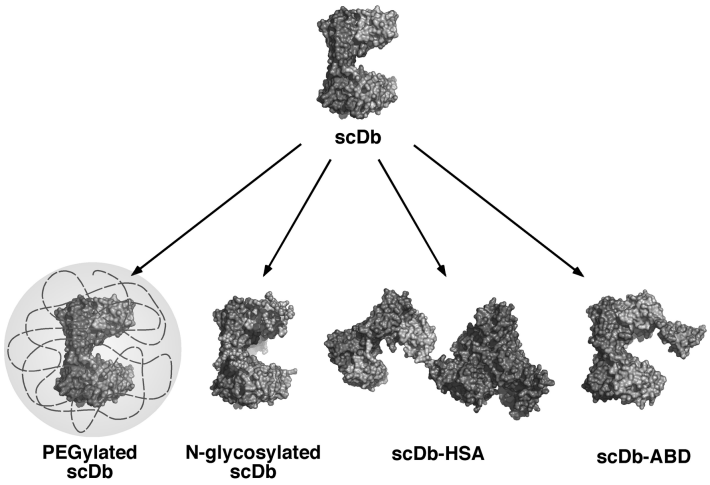


**Figure 1.3** Half-life extension strategies applied to a bispecific single-chain diabody (scDb) including PEGylation, introduction of *N*-glycosylation sites, fusion to human serum albumin (HSA) or a 6 kDa albumin-binding domain (ABD) from streptococcal protein G.

**Table 1.4** Biochemical and pharmacokinetic properties of an scDb and its half-life extended derivatives.

| Construct | Length (aa) | calc. $M_r$ [a] (kDa) | Apparent $M_r$ (kDa) | | Sr (nm) | $t_{1/2}\beta$ (h) | Tumor accumulation |
|---|---|---|---|---|---|---|---|
| | | | SDS-PAGE | SEC | | | AUC (%*h) |
| scDb | 505 | 54.5 | 62 | 36 | 2.7 | 1.3 | 168 ± 20 |
| scDb-$ABC_1$ | 497 | 53.6 | 62–68 | 8 | 2.9 | 8.9 | n.d. |
| scDb-$ABC_4$ | 519 | 55.6 | 64–80 | 65 | 3.5 | 7.2 | n.d. |
| scDb-$ABC_7$ | 544 | 57.8 | 68–87 | 78 | 3.8 | 6.2 | 150 ± 6 |
| scDb-A'-$PEG_{40k}$ | 505 | 54.5 | 230 | 650 | 7.9 | 13.1 | 450 ± 14 |
| chimeric IgG | 1382 | 145.1 | ~280 | 280 | 5.7 | 163 | n.d. |
| HSA | 585 | 66.5 | 67 | 66 | 3.5 | n.d. | |
| scDb-HSA | 1080 | 119.6 | 121 | 90 | 3.9 | 25.0 | n.d. |
| scDb-ABD | 550 | 59.3 | 64 | 32 | 2.5 | – | |
| scDb-ABD + HSA | – | 125.8 | – | 150 | 4.8 | 27.6 | 753 ± 47 |

a) Calculated from the amino acid sequence without post-translational modifications. n.d., not determined.
Half-lives were determined in CD1 mice receiving a single dose of 25 µg. Half-lives were calculated for the first 3 days, except for IgG, which was calculated over a period of 7 days.

analysis also revealed substantial heterogeneity in respect to the number of glycosylated sites and carbohydrate composition. The moderate increase in size translated into a moderate extension of half-life, which compared well with the effects seen for example for the hyperglycosylated form of erythropoietin, darbepoetin alfa.

An scDb-albumin fusion protein was generated by fusing the scDb to the N-terminus of HSA. The purified scDb-HSA fusion protein had a $M_r$ of 120 kDa and showed a strongly extended half-life (25 h) [60]. As an alternative, the scDb was fused to an albumin-binding domain (ABD) from streptococcal protein G. This 46 aa domain binds with a nanomolar affinity to human and mouse albumin. *In vitro* studies confirmed that the scDb-ABD fusion protein forms 1:1 complexes with HSA or MSA. *In vivo*, this resulted in a similar half-life extension as seen for scDb-HSA indicating that scDb-ABD binds also *in vivo* to the abundantly present

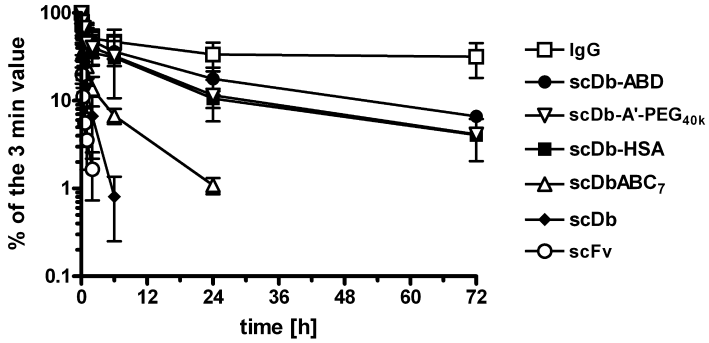


**Figure 1.4** Clearance of scDb and its half-life extended derivatives after a single dose i.v. injection into mice. An scFv and a chimeric IgG were included for comparison (scDb-ABD, fusion of the scDb to an albumin-binding domain from protein G; scDb-A'-PEG40k, scDb chemically conjugated to a branched 40 kDa PEG; scDb-HSA, fusion of scDb with human serum albumin; scDb-ABC7, scDb with in total 9 *N*-glycosylation site in the linkers A and B as well as at a C-terminal extension).

serum albumin (Figure 1.4) [50]. In an attempt to further increase the half-life we compared an scDb-ABD derivative containing an ABD with increased affinity for albumin. However, *in vitro* studies showed that the increased affinity led only to a moderate increase of the terminal half-life [62]. In addition, we generated an scDb ABD fusion protein possessing two ABDs, one at the N-terminus and one at the C-terminus of the scDb (scDb-$ABD_2$). SEC studies showed that this derivative is indeed capable of binding two albumin molecules at the same time leading to a further increase of the apparent size. Interestingly, this did not translate in any improved half-life as compared with scDb-ABD [62].

All scDb derivatives were able to bind with unaltered strength to effector and target cells indicating that the modifications did not influence binding. However, *in vitro* recruitment assays, determining T-cell activation by measuring release of IL-2 and cytotoxicity towards CEA-positive tumor cells, revealed a reduce capacity of T-cell stimulation and activity. This finding demonstrates a negative effect of the various modifications in this specific setting, where two cells have to come into close contact. Importantly, however, further biodistribution studies clearly showed that a prolonged circulation time result in an increased accumulation of the antibody molecule in antigen-positive tumors, which was most pronounced for the scDb-ABD fusion protein [63]. Thus, these studies provide a rationale for modulating the half-life of recombinant proteins to various extent.

## 1.5 Conclusion

Proteins are established therapeutics for the treatment of a variety of diseases and the number of proteins under development, most of them produced in recom-

binant form, is steadily growing. Many of these therapeutic proteins exhibit a rather short half-life, which necessitates frequent injections or infusions. Improvement of the pharmacokinetic properties through half-life extension strategies can reduce costs and ease administration, and can also result in improved pharmacodynamic properties of the drug. Half-life extension strategies have therefore attracted increasing attention by the pharmaceutical industry. This has already led to the establishment of a variety of different strategies to modulate the half-life of therapeutic proteins, including chemical but also genetic modifications. Some of these modifications such as PEGylation and hyperglycosylation are clinically approved strategies and many more are in clinical testing (see Table 1.3). It is expected that half-life extension strategies will be an integral part of many future protein drugs. The following 16 chapters of this book provide in-depth information of the most important half-life modulation strategies, also including background information on general pharmacokinetic aspects and considerations.

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