Influence of protein properties and protein modification on biodistribution and tumor uptake of anticancer antibodies, antibody derivatives, and non-Ig scaffolds

Frank-Jan Warnders | Marjolijn N. Lub-de Hooge | Elisabeth G. E. de Vries | Jos G. W. Kosterink

1 Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2 Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
3 Department of Medical Oncology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
4 PharmacoTherapy, Epidemiology & Economy, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands

Correspondence
Jos G. W. Kosterink, Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands.
Email: j.g.w.kosterink@umcg.nl

Abstract
Newly developed protein drugs that target tumor-associated antigens are often modified in order to increase their therapeutic effect, tumor exposure, and safety profile. During the development of protein drugs, molecular imaging is increasingly used to provide additional information on their in vivo behavior. As a result, there are increasing numbers of studies that demonstrate the effect of protein modification on whole body distribution and tumor uptake of protein drugs. However, much still remains unclear about how to interpret obtained biodistribution data correctly. Consequently, there is a need for more insight in the correct way of interpreting preclinical and clinical imaging data. Summarizing the knowledge gained to

ABBREVIATIONS: ABD, antigen-binding domain; ADA, antidrug antibody; ADC, antibody–drug conjugate; BAT, 6-(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; 11C, carbon-11; CD, cluster of differentiation; CDR, complementarity-determining residue; CEA, carcinoembryonic antigen; c/p, chelate/protein; 64Cu, copper-64; DAR, drug to antibody ratio; d/p, dye/protein; DTPA, diethylenetriaminepentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; E, glutamic acid; EMA, European Medicines Agency; EPR, enhanced permeability and retention; 18F, fluorine-18; Fab, antigen-binding fragment; Fab2, two antigen-binding fragments (linked together with disulphide bounds); Fc, fragment crystallizable; FDA, Food and Drug Administration; Fr-Rn, neonatal Fc receptor; G, glycine; 68Ga, gallium-68; H, histidine; H6, six consecutively placed positively charged histidines; HER2, human epidermal growth factor receptor 2; HSA, human serum albumin; % ID/g, percentage injected dose per gram; Ig, immunoglobulin; IgG, immunoglobulin G; IgM, immunoglobulin M; I, iodine; 123I, iodine-123; 124I, iodine-124; 125I, iodine-125; 131I, iodine-131; 111In, indium-111; K, lysine; 177Lu, lutetium-177; Ma, mercaptoacetyl; mAb, monoclonal antibody; MaSSS, mercaptoacetyl-serine-serine-serine; NIR, near-infrared; NOTA, 1,4,7-triazacyclononane-1,4,7-tri-acetic acid; NOGADA, 1-(1,3-carboxypropyl)-4,7-carboxymethyl-1,4,7-triazacyclonane; PEG, polyethylene glycol; PET, positron emission tomography; S, serine; scDb, single chain diabodies; scFv, single chain variable fragment; SCID, severe combined immunodeficiency; SPECT, single photon emission computed tomography; 99mTc, technetium-99m; T-DM1, trastuzumab emtansine; VHH, variable domain of a heavy chain of heavy-chain antibodies; ZHER2, HER2 targeting affibody; 89Zr, zirconium-89
This review therefore provides an overview of specific protein properties and modifications that can affect biodistribution and tumor uptake of anticancer antibodies, antibody fragments, and nonimmunoglobulin scaffolds. Protein properties that are discussed in this review are molecular size, target interaction, FcRn binding, and charge. Protein modifications that are discussed are radiolabeling, fluorescent labeling drug conjugation, glycosylation, humanization, albumin binding, and polyethylene glycolation.

**KEYWORDS**

biodistribution, drug, modification, molecular imaging, protein

## 1 | INTRODUCTION

Protein drugs are increasingly used in oncology. Much clinical experience has been obtained with monoclonal antibodies (mAbs). Due to their clinical successes, researchers additionally focus on the development of antibody derivatives and non-Ig (where Ig is immunoglobulin) protein scaffolds. These protein drugs, when targeting tumor-associated antigens, are often modified to optimize therapeutic effects, tumor exposure, and safety profile. Examples of such protein modifications include humanization, glycosylation, polyethylene glycol (PEG)ylation, and both covalent and noncovalent albumin binding (Figure 1). Currently, ~66% of Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved mAbs have been humanized to some extent, conjugated to cytotoxic drugs, or conjugated to therapeutic radionuclides. Apart from mAbs also antibody fragments and non-Ig scaffolds are being modified.

Clinical development of protein drugs is challenging. Up to 2015 more than 200 different anticancer mAbs have been tested in clinical trials. Currently, only 27 mAbs are FDA or EMA approved for the treatment of cancer. One of the biggest challenges is the identification of the best drug candidates in early phase clinical studies. Early insight in whole body and organ pharmacokinetics, as well as tumor uptake of protein drugs, might facilitate this identification. Both characteristics can noninvasively be visualized and quantified with molecular imaging. In this way, real-time dynamics can be obtained on whole body biodistribution of protein drugs or protein drug candidates in the same animal or patient. Furthermore, a correlation between tumor uptake of radiolabeled antibodies, as determined by positron emission tomography (PET) imaging, and intratumoral target levels, as determined by enzyme-linked immunosorbent assay or immunohistochemistry, has been observed in cancer patients.

Protein modification can both intentionally and unintentionally alter in vivo behavior of protein drugs. Therefore these modifications may hamper a sound interpretation of the obtained imaging data in both the preclinical and clinical setting. This review aims to give insight into protein characteristics that affect biodistribution and tumor uptake of tumor-targeting protein drugs. In this review we included antibodies, antibody derivatives, and non-Ig scaffolds that were retrieved with the search strategy we used. Furthermore, we summarize the influence of common protein modifications on biodistribution and tumor uptake of these proteins.

### 1.1 | Search strategy

Articles for this review were found by searches of PubMed, using the terms as mentioned in Table 1. Moreover, we included useful studies that were mentioned in the references of obtained articles. We did not focus on studies that only included small peptides such as somatostatin analogs.
FIGURE 1   Overview of common modifications to proteins. ABD = albumin-binding domain

TABLE 1   Used search terms

<table>
<thead>
<tr>
<th>Primary search terms</th>
<th>Combined with</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Protein AND affinity AND cancer”</td>
<td></td>
</tr>
<tr>
<td>“FcRn”</td>
<td></td>
</tr>
<tr>
<td>“Protein AND charge”</td>
<td></td>
</tr>
<tr>
<td>“Labeling AND proteins AND chelator”</td>
<td></td>
</tr>
<tr>
<td>“Site AND specific AND labeling”</td>
<td></td>
</tr>
<tr>
<td>“Biodistribution”</td>
<td>“(Chelator AND conjugation) OR chelated”</td>
</tr>
<tr>
<td>OR pharmacokinetics*</td>
<td>“(Antibody AND drug AND conjugate) OR ADC”</td>
</tr>
<tr>
<td></td>
<td>“(Glycosylation OR glycosylated) AND protein AND cancer”</td>
</tr>
<tr>
<td></td>
<td>“Humanized AND cancer”</td>
</tr>
<tr>
<td></td>
<td>“Albumin AND cancer”</td>
</tr>
<tr>
<td></td>
<td>“Protein AND cancer AND (PEG OR PEGylation)”</td>
</tr>
<tr>
<td></td>
<td>“Fluorescent AND protein AND imaging AND cancer”</td>
</tr>
<tr>
<td>“EPR effect”</td>
<td></td>
</tr>
</tbody>
</table>

2 | PROPERTIES OF PROTEIN DRUGS DETERMINING BIODISTRIBUTION AND TUMOR UPTAKE

Pharmacokinetics and tumor uptake can be influenced by a multitude of protein properties. In this review we focus on the influence of size, target interaction, the neonatal fragment crystallizable (Fc) receptor (FcRn) binding capacity, and charge (Table 2).
<table>
<thead>
<tr>
<th>Protein properties</th>
<th>Effect on protein pharmacokinetics / biodistribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High weight (&gt;60–70 kDa)</td>
<td>Serum half-life&lt;sup&gt;a&lt;/sup&gt;↑</td>
<td>9,10</td>
</tr>
<tr>
<td></td>
<td>Renal excretion / uptake ↓</td>
<td>7,8</td>
</tr>
<tr>
<td></td>
<td>Organ uptake (except kidneys) ↑</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Tumor uptake, including nonspecific tumor uptake ↑</td>
<td>14,15</td>
</tr>
<tr>
<td>Target Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low protein dose / high antigen levels</td>
<td>Serum half-life&lt;sup&gt;b&lt;/sup&gt;↓</td>
<td>26–28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increasing target affinity</td>
<td>Tumor penetration (anti-HER2 antibodies at an affinity of &lt;7.3 × 10&lt;sup&gt;−9&lt;/sup&gt; M) ↓</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Tumor uptake ↑</td>
<td>29,30</td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;125&lt;/sup&gt;İ-scFv at an affinity of &lt;1.0 × 10&lt;sup&gt;−9&lt;/sup&gt; M) ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;111&lt;/sup&gt;In-antibodies at an affinity of &lt;2.3 × 10&lt;sup&gt;−8&lt;/sup&gt; M)&lt;sup&gt;c&lt;/sup&gt; ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor uptake ↓</td>
<td>29,30</td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;125&lt;/sup&gt;İ-scFv at an affinity of &lt;1.2 × 10&lt;sup&gt;−10&lt;/sup&gt; M)&lt;sup&gt;d&lt;/sup&gt; ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;125&lt;/sup&gt;I-antibodies at an affinity of &lt;7.3 × 10&lt;sup&gt;−9&lt;/sup&gt; M)&lt;sup&gt;e&lt;/sup&gt; ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor uptake ←</td>
<td>33,34</td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;99m&lt;/sup&gt;Tc-designed ankyrin repeat proteins at affinities of 1 × 10&lt;sup&gt;−8&lt;/sup&gt;−1 × 10&lt;sup&gt;−11&lt;/sup&gt; M) ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(anti-HER2&lt;sup&gt;111&lt;/sup&gt;In-antibodies at affinities of 3.8 × 10&lt;sup&gt;−9&lt;/sup&gt;−1.6 × 10&lt;sup&gt;−10&lt;/sup&gt; M) ↓</td>
<td></td>
</tr>
<tr>
<td>FcRn binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of FcRn binding</td>
<td>Serum half-life ↓</td>
<td>39,40</td>
</tr>
<tr>
<td></td>
<td>Organ uptake (in most organs) ↓</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Uptake in liver, spleen, and intestines ↑</td>
<td>40,41</td>
</tr>
<tr>
<td>Increasing FcRn affinity</td>
<td>Serum half-life ↑</td>
<td>42</td>
</tr>
<tr>
<td>Charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly charged&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Renal accumulation of renally excreted proteins/ peptides ↑</td>
<td>55–62</td>
</tr>
<tr>
<td>Anionization</td>
<td>Serum half-life of antibodies ↓</td>
<td>49–51</td>
</tr>
<tr>
<td></td>
<td>Serum half-life of fab fragment ↑</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Serum half-life of disulfide-bonded variable region ~</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Organ uptake of antibodies (in most organs) ↓</td>
<td>49–51</td>
</tr>
<tr>
<td>Cationization</td>
<td>Serum half-life of antibodies ↑</td>
<td>44–48</td>
</tr>
<tr>
<td></td>
<td>Organ uptake of antibodies ↓</td>
<td>44–48</td>
</tr>
<tr>
<td>Lipophilic patches</td>
<td>Liver uptake ↓</td>
<td>34,63</td>
</tr>
<tr>
<td></td>
<td>Serum clearance ↑</td>
<td>65</td>
</tr>
</tbody>
</table>

<sup>a</sup>Exceptions have been described.42
<sup>b</sup>Trastuzumab 100 mg at 10 week interval and shed HER2 blood levels of <500 μg/L versus <500 μg/L.
<sup>c</sup>Not observed with anti-HER2<sup>125</sup>I-antibodies.
<sup>d</sup>Observed at a dose of 1 μg but not at a dose of 20 μg.
<sup>e</sup>Not observed with anti-HER2<sup>111</sup>In-antibodies.
<sup>f</sup>Renal accumulation likely increases with the increase of overall charge of proteins that are prone to renal excretion.
TABLE 3  Targeted proteins and their pharmacokinetics in mice, as adapted from Freise and Wu\textsuperscript{7}

<table>
<thead>
<tr>
<th>Format</th>
<th>Approx. MW (kDa)</th>
<th>Typical serum t\textsubscript{1/2}</th>
<th>Clearance route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact IgG</td>
<td>150–160</td>
<td>1–3 weeks</td>
<td>Hepatic</td>
</tr>
<tr>
<td>F(ab′)\textsubscript{2}</td>
<td>110</td>
<td>8–10 hr</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Minibody</td>
<td>75</td>
<td>5–10 hr</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Fab</td>
<td>50–55</td>
<td>12–20 hr</td>
<td>Renal</td>
</tr>
<tr>
<td>Diabody</td>
<td>50</td>
<td>3–5 hr</td>
<td>Renal</td>
</tr>
<tr>
<td>scFv</td>
<td>28</td>
<td>2–4 hr</td>
<td>Renal</td>
</tr>
<tr>
<td>VHH</td>
<td>12–15</td>
<td>30–60 min</td>
<td>Renal</td>
</tr>
<tr>
<td>Affibody</td>
<td>7</td>
<td>30–60 min</td>
<td>Renal</td>
</tr>
</tbody>
</table>

F(ab′)\textsubscript{2}, antibody of which the Fc domain is removed by pepsin digestion; Fab, antigen-binding fragment; scFv, single chain variable fragment; VHH, variable domain of a heavy chain of heavy-chain antibodies.

2.1 Molecular size

Both the molecular weight and size of proteins may affect biodistribution. Due to the fact that biodistribution studies often mention protein weights rather than the hydrodynamic radius, in this review we focus on the impact of molecular weight of proteins on their biodistribution. The different proteins that will be discussed in this review differ in molecular weight, which for antibodies generally is ∼150–160 kDa, antibody fragments ∼12–110 kDa, and non-Ig scaffold ∼<20 kDa (Table 3).\textsuperscript{7,8} It is thought that the pores in the glomerular filtration membrane are approximately 75 Å and that proteins are generally renally filtered until a threshold of around 60–70 kDa proteins.\textsuperscript{9–12} As a result, proteins smaller than ∼60–70 kDa are more prone for fast renal clearance and are therefore subjected to reabsorption in the proximal tubule as compared to larger proteins. Likely due to the fact that proteins are flexible constructs, proteins larger than the above-mentioned threshold, such as a 80 kDa carcinoembryonic antigen (CEA) targeting minibody, can still be prone to renal clearance.\textsuperscript{13} Fast renal filtration of small proteins generally results in low accumulation and exposure to normal and tumor tissue. Increasing the weight of proteins to >60–70 kDa can prolong serum half-life and increase tissue levels, tumor levels, and target exposure.\textsuperscript{14} However, high tumor levels of large proteins are not only the result of antigen binding in the tumor. Due to leakiness of blood vessels and a poorly developed lymphatic system in tumors, macromolecular particles tend to penetrate easily into tumors and poorly drain back onto the blood or lymphatic system, a process known since the 80s.\textsuperscript{15} This so-called enhanced permeability and retention (EPR) effect is size depending and thought to nonspecifically increase tumor uptake of nanosized anticancer drugs, including particles, vesicles, micelles, antibodies, or macromolecules.\textsuperscript{16} It is thought that this effect may increase the intratumoral concentration of nanosized anticancer drugs by 20–30%.\textsuperscript{16} This EPR effect is more pronounced in murine models with fast-growing tumors and immature blood vessel formation as compared to slow-growing tumors.\textsuperscript{17} In mice with slowly growing human tumors, which are models closer to the clinical setting, this EPR effect is less pronounced.\textsuperscript{17} As a result, the impact of the EPR effect on nonspecific tumor accumulation is likely more pronounced in mice than in patients. In addition, the impact of the EPR effect on nonspecific uptake of nanosized anticancer drugs likely differs between individual tumors. For example, tumor uptake of \textsuperscript{64}Cu-labeled liposomes in 11 canine cancer patients with spontaneous growing tumors seemed to be EPR mediated in six out of seven carcinomas and one out of four sarcomas.\textsuperscript{18}

Due to the fact that large proteins (typically with a molecular weight of >60–70 kDa) are excreted relatively slow, these proteins show relatively long and high tumor exposure after a single admission.\textsuperscript{14} Therefore increasing molecular weight and size may result in a reduction of the required administration frequency. Consequently, there is a growing interest in increasing the molecular weight and size of small protein drug candidates, in order to reduce their excretion.
rate. Modifications that have been used include the fusion to an elastin-like peptide, gelatin-like protein, homo-αmino acid polymer, proline–alanine–serine polymer, PEG, and to antibody fragments.\textsuperscript{19–23}

In conclusion, modifications that increase size can drastically increase blood, tissue, and tumor levels. Such an increase should be taken into account when interpreting molecular imaging data obtained with radiolabeled modified proteins. As modifications that increase protein size can increase nonspecific tumor uptake, it may complicate the interpretation of antigen-dependent tumor uptake, particularly in tumors with low antigen levels.

### 2.2 Target interaction

As proposed by Gerard Levy in 1994 and described in a mathematical model by Mager and Jusko in 2001, target binding can influence antibody pharmacokinetics.\textsuperscript{24,25} Relative high antigen levels, as compared to antibody dose, is thought to shorten the half-life of antibodies due to target binding. Nonlinear kinetics have been observed for antibodies targeting membrane-associated antigens, such as trastuzumab (targeting human epidermal growth factor receptor 2 (HER2)) and panitumumab (targeting epidermal growth factor receptor).\textsuperscript{26,27} Furthermore, shedding of membrane-associated antigens may also affect the half-life of antibodies.\textsuperscript{28} In a phase II study the half-life of trastuzumab in patients with low circulating levels of the extracellular domain of HER2 (≤500 μg/L) was 9.1 days (n = 40), while in patients with high circulating levels (>500 μg/L) the half-life of trastuzumab decreased to 1.8 days (n = 5).\textsuperscript{28} Both groups received the same dose of trastuzumab (loading dose of 250 mg, followed by a 10 weekly dose of 100 mg each).

In addition, the extent of target binding also depends the degree of tumor accumulation of protein drugs targeting tumor-associated antigens. Their target affinity can majorly affect tumor uptake. Both decreasing and increasing target affinity can reduce tumor uptake of these protein drugs.\textsuperscript{29,30} Increasing target affinity of antibodies and single chain variable fragments (scFvs) to extremely high levels, prevents them to detach from their targets in the tumor rim, making them prone for antigen internalization and catabolization, precluding them to bind targets located deeper in tumors.\textsuperscript{29,30}

The impact of antigen affinity on the penetration depth of proteins may partly depend on the internalization rate of the targeted antigens when these antigens are expressed on the cell membrane of tumor cells. At dissociation rates faster than the rate of antigen internalization, increasing antigen affinity of antibodies may promote antibody catabolism, which prevents them to penetrate deeply into tumors. This has been demonstrated with anti-HER2 antibodies.\textsuperscript{29} As dissociation rates of the studied antibodies were not measured, the authors used the known dissociation rates of the scFvs of these antibodies. In contrast, at dissociation rates slower than the rate of antigen internalization, differences in dissociation rates may have less to no effect on antibody catabolism and tumor penetration.\textsuperscript{29}

The fact that a high affinity can prevent deep tumor penetration is known as the "binding site barrier effect."\textsuperscript{31} This barrier effect has been visualized in mice with four antibodies targeting the same HER2 epitope with different affinities (G98A, C6.5, ML39, and H3B1).\textsuperscript{29} These anti-HER2 antibodies have target affinities of, respectively, \(2.7 \times 10^{-7}\) M, \(2.3 \times 10^{-8}\) M, 7.3 \(\times 10^{-9}\) M, and 5.6 \(\times 10^{-10}\) M, as determined by surface plasmon resonance.\textsuperscript{32} In severe combined immunodeficient (SCID) mice xenografted with HER2 expressing human ovarian carcinoma cells (SKOV-3), tumor penetration of the four antibodies negatively correlated with affinity.\textsuperscript{29} This was based on immunohistochemistry at 120 hr after injection. Interestingly, tumor uptake of the high-affinity iodine-125 (\(^{125}\text{I}\))-labeled H3B1 was lower than that of \(^{125}\text{I}\)-C6.5, with intermediate affinity. In contrast, no difference was observed between the tumor uptake of the two antibodies when radiolabeled with indium-111 (\(^{111}\text{In}\)). However, a low affinity of \(2.7 \times 10^{-7}\) M did prevent high tumor accumulation, as observed with \(^{111}\text{In}\)-G98A (the lowest affinity antibody). Tumor uptake of \(^{111}\text{In}\)-G98A was lower than that of the other \(^{111}\text{In}\)-labeled higher affinity antibodies.

The same group also studied the effect of HER2 affinity of anti-HER2 scFvs on tumor uptake in SKOV-3 xenografted C.B17/Icr-scid mice.\textsuperscript{30} ScFvs were derived from a single clone with increasing affinities for the same HER2 epitope (\(3.2 \times 10^{-7}\) M, \(1.6 \times 10^{-8}\) M, \(1.0 \times 10^{-9}\) M, \(1.2 \times 10^{-10}\) M, and \(1.5 \times 10^{-11}\) M). This study also showed that a high affinity could prevent deep tumor penetration of proteins. As demonstrated by fluorescence microscopy, tumor penetration of the scFv with an affinity of \(1.5 \times 10^{-11}\) M was much less than of the scFv with an affinity of \(3.2 \times 10^{-7}\) M. Tumor uptake of the \(^{125}\text{I}\)-labeled scFvs depended on both target affinity and the administered dose. Increasing HER2 affinity up to \(1.0 \times 10^{-9}\) M resulted in increased tumor uptake of the studied anti-HER2 scFv, which remained similar at an
affinity of $1.2 \times 10^{-10}$ M. However, increasing the affinity to $1.5 \times 10^{-11}$ M resulted in a reduction of tumor accumulation. Noteworthy, this decrease in tumor uptake was observed at a 1 $\mu$g dose level but not at a higher dose level of 20 $\mu$g, suggesting that this effect is dose dependent. As both doses are within the range of often used tracer doses in preclinical imaging studies, this effect might very well be relevant for other HER2-binding antibody fragments or non-Ig scaffolds. Apart from tumor uptake, this study additionally demonstrated that a high affinity could prevent deep tumor penetration, as was observed in ex vivo immunohistochemistry and immunofluorescence experiments. Nephrectomized SCID mice bearing SKOV-3 tumors were injected with anti-HER2 scFvs with an affinity of $3.2 \times 10^{-7}$ M or $1.5 \times 10^{-11}$ M, at a 100 $\mu$g dose. The lowest affinity molecule exhibited diffuse tumor staining, whereas the highest affinity molecule was primarily retained in the perivascular regions of the tumor.

Although not observed with the above-mentioned HER2 antibodies and scFvs, receptor binding of proteins could mediate transcytosis instead of catabolism, which may result in deeper tumor penetration. Under such circumstances, high target affinity may increase tumor penetrations.

In contrast to the above $^{125}$I-labeled anti-HER2 scFvs and antibodies, HER2 affinity of technetium-99 m ($^{99m}$Tc) labeled HER2-targeting designed ankyrin repeat proteins and $^{111}$In-labeled affibodies in SKOV-3 tumors bearing mice did not affect tumor uptake. The affinities of these proteins range between, respectively, $1 \times 10^{-8}$ M to $1 \times 10^{-11}$ M and $3.8 \times 10^{-9}$ M to $1.6 \times 10^{-10}$ M.

In conclusion, antigen binding can affect the pharmacokinetics of proteins, especially when affinities become extremely high or low. Either extremely high or extremely low affinities may prevent high tumor uptake, which can be dose dependent. The effect of extremely high target affinity can be less pronounced or absent at high doses. Protein modifications that decrease antigen affinity can, but not necessarily do, decrease target-specific tumor uptake. Therefore, the effect of protein modifications on antigen affinity should be taken into account when interpreting molecular imaging data.

### 2.3 FcRn binding

Both albumin and the Fc domain of IgG antibodies are able to bind to the FcRn receptor. It is generally accepted that the FcRn receptor protects serum albumin and endogenous IgG antibodies from catabolism, as they strongly bind to FcRn at low endosomal pH ($<6.5$) and weakly at extracellular physiological pH (7.4). Upon endosomal FcRn binding, endogenous and exogenous IgG antibodies are transported back to the cellular surface and are then released at physiological pH. This process has been visualized with fluorescent microscopy in FcRn-expressing human endothelial cells. Upon endocytosis, fluorescently labeled wild-type IgG1 was transported to the cellular surface, while fluorescently labeled IgG1 mutant without detectable FcRn binding was transported to the lysosomes for degradation.

The impact of FcRn-dependent protection from catabolism has been demonstrated in mice. In FcRn-deficient mice, the serum half-life of IgG1 was considerably shorter (1.4 days) than in sex-matched FcRn wild-type animals with functional FcRn expression (9 days). The fact that FcRn protects IgG1 from catabolism has also been shown with molecular imaging. Yip et al. radiolabeled both wild-type IgG1 and non-FcRn-binding IgG1 with $^{111}$In. Indeed, $^{111}$In-labeled non-FcRn-binding antibody cleared much faster from plasma. In addition, a lack of FcRn binding promoted accumulation in the liver, spleen, and intestines. These results suggest that FcRn binding protects antibodies from catabolism in these organs.

Similar findings were observed after the reduction of FcRn affinity of an anti-Lewis-Y antibody (hu3S193), radiolabeled with lutetium-177 ($^{177}$Lu). Complete abrogation of mouse FcRn affinity decreased blood levels from $15.67 \pm 2.47%$ of injected dose per gram (% ID/g) to $1.05 \pm 0.27%$ ID/g, as observed 48 hr after tracer injection in mice. This resulted in a reduced uptake in most organs. However, uptake of the non-FcRn-binding $^{177}$Lu-hu3S193 I253A/H310A increased in liver (from $5.99 \pm 1.43%$ ID/g to $14.16 \pm 1.28%$ ID/g) and spleen (from $9.32 \pm 1.33%$ ID/g to $13.77 \pm 2.57%$ ID/g) as compared with wild-type $^{177}$Lu-hu3S193.

In addition to the above preclinical studies, changes in FcRn affinity can also affect in vivo behavior in humans. Substitutions of specific amino acids located in the Fc domain of motavizumab (M252Y/S254T/T256E) resulted in a tenfold increase of in vitro human FcRn binding at pH 6.0. It prevented high lysosomal degradation of motavizumab and increased its serum half-life in healthy adults from 19–34 days to 70–100 days.
In conclusion, modifications that reduce or prevent the binding of antibodies to FcRn can increase serum clearance and increase organ uptake, which is mostly observed in the liver and spleen. Therefore, if molecular imaging with modified antibodies shows unexpected high uptake in these organs, the modification could have reduced FcRn binding.

2.4 | Charge

Protein modifications that change protein charge may affect charge-dependent interactions in vivo and potentially alter both tissue accumulation and pharmacokinetics. The effect of charge on the biodistribution of antibodies, antibody derivatives, and non-Ig scaffolds is mainly studied by using cationizing or anionizing protein modifications.

Cationization of already positively charged particles induced nonspecific uptake in normal tissue.\textsuperscript{43} This was likely due to increased interaction of the positively charged particle with negative cell surface charges. Similarly, cationization of antibodies by attaching hexamethylenediamine tends to increase the deposition of antibodies in both target (e.g., tumor) and normal tissues in rats and mice.\textsuperscript{44–48} As studied at 1–2 hr after injection, cationization mainly increased organ uptake in liver, lung, and kidneys. Increased organ uptake additionally resulted in increased plasma clearance. Interestingly, anionization of antibodies, by diethyleneetriaminepentaacetic acid (DTPA) conjugation and/or succinylation also increased plasma clearance in mice and rats.\textsuperscript{49–51} While cationization increased nonspecific tissue uptake and subsequently plasma clearance, anionization of antibodies mainly increased whole body clearance. Anionization of \textsuperscript{111}In-labeled antibodies generally decreased their organ uptake (except for liver), as studied 1–24 hr after injection.\textsuperscript{49–51} The fact that hepatic uptake did not decrease might be due to the fact that anionization may have increased the interaction of the modified antibodies with intrahepatic scavenger receptors.\textsuperscript{51} At high antibody doses liver uptake of \textsuperscript{111}In-labeled highly succinylated bovine IgG could be saturated, suggesting that the binding to these scavenger receptors can be saturated.\textsuperscript{51}

In contrast to antibodies, charge changing protein modifications can have a different effect on the biodistribution of smaller proteins. As small proteins (<60 kDa) are prone to renal excretion, they are exposed to charge-dependent renal tubular reabsorption.\textsuperscript{52} In cancer patients, high renal accumulation has been observed with small size radiolabeled proteins such as a trastuzumab antigen-binding fragment (Fab) and an anti-HER2 affibody.\textsuperscript{53,54} This high renal uptake potentially hampers the visualization of tumor uptake in tumors that are located in close proximity of the kidneys. Modifications that affect the charge of small protein drugs can thus alter renal accumulation, potentially hampering the visualization of their tumor uptake. Attaching negatively charged glycolate molecules to both an interleukin-2 receptor targeting Fab and disulfide-bonded variable region fragment, generally reduced renal accumulation in mice.\textsuperscript{55,56} As glycolation of the disulfide-bonded variable region fragment hardly increased its size (a maximum of 2.8%), the observed effect was most likely due to the anionizing of the protein.\textsuperscript{56} Renal accumulation of the nonglycolated interleukin-2 receptor targeting Fab was indeed triggered by positive charge interaction in the kidneys, as coinjection with positively charged lysine drastically reduced renal accumulation (from 196.2 ± 18.8% ID/g to 24.9 ± 2.0% ID/g).\textsuperscript{55} In line with this finding, coinjection of cationic amino acids also reduced renal accumulation of other proteins including Fab, F(ab′)\textsubscript{2}, scFv, and a variable domain of a heavy chain of heavy-chain only antibodies (VHH).\textsuperscript{57–61} Similar to cationic proteins, anionic peptides are also prone for charge-dependent renal accumulation. Renal uptake of, for example, negatively charged \textsuperscript{111}In-minigastrin, \textsuperscript{111}In-bombesin, and \textsuperscript{111}In-exendin could be reduced by coinjection of negatively charged polyglutamic acid.\textsuperscript{62} In contrast to that observed with antibodies, the blood clearance of Fab fragments and disulfide-bonded variable region fragments targeting interleukin-2 receptor alpha did not increase upon anionization.\textsuperscript{55,56} Anionization of the disulfide-bonded variable region fragment had negligible impact on blood levels as measured up to 3 hr after injection in mice.\textsuperscript{55} In case of the Fab fragment, anionization decreased the clearance rate in mice.\textsuperscript{56}

As discussed above, the overall charge of proteins can change due to anionizing and cationizing protein modifications. Likewise, protein modifications can also reduce local and overall charge, potentially introducing "lipophilic patches" and increasing overall lipophilicity of proteins. The presence of a lipophilic patch likely promoted hepatic uptake of radiolabeled peptides in monkeys.\textsuperscript{63} In addition, increasing the lipophilicity of anti-HER2 affibodies increased the uptake of these affibodies in the liver and spleen of mice at 4 hr and 24 hr after injection.\textsuperscript{34} Moreover, protein...
modifications promoting hydrophobic interactions, such as increasing the amount of lipophilic drugs to create antibody–drug conjugates (ADCs), can make proteins prone to aggregation. Aggregation may subsequently alter the biodistribution of proteins. When using lipophilic drugs and drug linkers, increasing the drug to antibody ratio (DAR) can also promote the uptake of ADCs in sinusoidal endothelium and Kupffer cells in the liver of rats, increasing their clearance rate. The use of less lipophilic drug linkers can prevent a large increase in clearance rate.65

In conclusion, both anionizing and cationizing protein modifications can decrease plasma half-lives of antibodies. However, the underlying mechanisms differ. Anionization of antibodies usually increases whole body clearance, while cationization of antibodies promotes nonspecific accumulation in normal tissue. In contrast however, anionization of smaller proteins may decrease clearance rate. For small-sized protein drugs that are prone to renal excretion, both anionization and cationization can trigger accumulation in the kidneys. This accumulation is primarily due to an increase in charge-dependent renal tubular reabsorption rather than an increase in renal excretion. The discussed findings may be translational to other types of protein drugs and may explain an unexpected biodistribution seen on imaging scans.

3 PROTEIN MODIFICATIONS THAT AFFECT PHARMACOKINETICS AND TUMOR UPTAKE OF PROTEIN DRUGS

3.1 Radiolabeling

Radiolabeling of protein drugs changes their chemical structure, potentially altering their in vivo behavior. Below we discuss how differences in radionuclide, radiolabeling method, site of labeling, chelator/protein (c/p) ratio, and chelator type can alter the in vivo behavior of protein drugs.

3.1.1 Radionuclides/radiolabeling method

Protein drugs can be radioactively labeled with different PET and single photon emission computed tomography (SPECT) radionuclides. Predominant radionuclides that are currently used in SPECT imaging include $^{99m}$Tc, iodine-123 ($^{123}$I), and $^{111}$In. Often used PET radionuclides are carbon-11 ($^{11}$C), fluorine-18 ($^{18}$F), copper-64 ($^{64}$Cu), gallium-68 ($^{68}$Ga), zirconium-89 ($^{89}$Zr), and iodine-124 ($^{124}$I).

The choice for a specific radionuclide determines which information can be retrieved from the labeled proteins, as radionuclides can be trapped in cells after internalization of radiolabeled proteins in these cells. Internalization of protein drugs that are directly labeled with neutrally charged radiohalogens (e.g., radioactive iodine) are generally catabolized into neutrally charged catabolites that can pass the cell membrane.66 These radiocatabolites do not accumulate in cells upon internalization of radiolabeled proteins. However, indirectly radiolabeled protein drugs, with, for example, $^{89}$Zr or $^{111}$In, are often catabolized into charged radiocatabolites that remain trapped intracellularly.66 Consequently, indirect labeling results in higher tumor signals and is therefore a more sensitive approach to visualize tumor uptake.67–72 Its use additionally results in higher signals in normal tissue, which is for antibodies and their fragments most pronounced in liver, spleen, and kidneys.67–72 Extremely high organ uptake may hamper the visualization of tumor uptake in tumors that are located in or in close proximity of these organs. As charged radiocatabolites remain trapped intracellularly after internalization and catabolism, their use could provide additional insight in catabolic sites of therapeutic proteins and delivery of toxic drugs by, for example, ADCs.6

One should always take into account that with molecular imaging the biodistribution of (radio)labels rather than the biodistribution of proteins is studied. When the radiolabel is attached to the protein, both are the same. However, if these labels detach or if the protein degrades, respectively, the label or labeled protein fragment are traced in vivo. In addition, the attachment of radionuclide labels involves the conjugation of (mainly negatively) charged chelating agents. The introduction of such charged chelating agents will alter protein charge and may hamper target and FcRn binding. As discussed earlier in this review this can potentially alter the biodistribution of protein drugs. Chelating agents
TABLE 4  Frequently used SPECT and PET isotopes

<table>
<thead>
<tr>
<th>SPECT</th>
<th>Radionuclide</th>
<th>Half-life</th>
<th>PET</th>
<th>Radionuclide</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>99mTc</td>
<td>6.0 hr</td>
<td></td>
<td>11C</td>
<td>20.4 min</td>
<td></td>
</tr>
<tr>
<td>123I</td>
<td>13.2 hr</td>
<td></td>
<td>18F</td>
<td>109.8 min</td>
<td></td>
</tr>
<tr>
<td>111In</td>
<td>2.8 days</td>
<td></td>
<td>64Cu</td>
<td>12.7 hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>68Ga</td>
<td>67.7 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>89Zr</td>
<td>78.5 hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>124I</td>
<td>4.2 days</td>
<td></td>
</tr>
</tbody>
</table>

require high selectivity and binding capacity for the radiometal as the human body possess an excess of biometals and biochelators, which concentrations in the human body are typically much higher than that of injected radiolabeled proteins. Copper, for example, has many chelating biomolecules that may cause transchelation of radioactive copper to these biomolecules.73,74 Such transchelation occurs when chelators are not kinetically inert and may cause increased liver levels.75–77 As a result much effort has put into the development of kinetically inert chelators.78 Like copper, radioactive gallium may transchelate from chelating moieties as it is well established that gallium(III) has affinity for transferrin.79 It has been shown that 68Ga-apo-transferrin may accumulate in liver, spine, and muscle of mice.80 Similarly, 111In may transchelate to transferrin and accumulate in liver, spleen, and bone tissue.81,82 Of the remaining often used radionuclides, free 89Zr likely accumulates in bone tissue,83–85 while nonlabeled radioactive iodine and 99mTc may accumulate in thyroid, salivary glands, and stomach.86,87

In addition to the above-mentioned differences between radionuclides, the choice for a specific radionuclide is often based on their availability. The radionuclides 123I, 111In, 11C, 18F, 64Cu, 89Zr, and 124I can only be produced in centers that have a cyclotron, hampering their worldwide use. In contrast, 99mTc and 68Ga can be retrieved from specific generators producing these radionuclides on site. Furthermore, the choice for a specific radionuclide can be based on its physical half-life (Table 4) that most often matches the serum half-life of the protein of interest. However, one may prefer a longer-lived radionuclide in order to study long-term in vivo biodistribution and in particular tumor uptake.88 A radionuclide with a relative long half-life can additionally be used to study long-term stability in serum and tumor tissue.89

3.1.2 Type of chelator or chelating amino acid sequences

Indirect labeling can be performed using a wide range of charged chelators, chelating amino acid sequences, and charged radioisotopes. Therefore indirect radiolabeling might alter biodistribution and tumor uptake, depending on the type of chelator used.

Several studies have demonstrated that the use of specific chelating agents can alter the biodistribution of HER2 targeting affibodies (ZHER2). Switching between the macrocyclic chelators 4,7-triazacyclononane-\(N,N',N''\)-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), and 1-(1,3-carboxypropyl)-4,7-carboxymethyl-1,4,7-triazacyclononane (NOGADA) did alter the biodistribution of ZHER251, as determined 4 hr after injection in mice.90,91 Largest differences in 111In-ZHER251 levels were observed in blood and liver. Blood levels were lowest for NOGADA-conjugated ZHER251 in NMRI mice, but not in BALB/C nu/nu mice.90,91 Hepatic accumulation of 111In-NOTA-ZHER251 (6.6–6.8% ID/g) was higher than of 111In-DOTA-ZHER251 and 111In-NOGADA-ZHER251 (<3.0% ID/g) in both mouse strains.90,91 It was suggested that this difference was due to the fact that 111In-NOTA is positively charged (+1), while both 111In-DOTA and 111In-NOGADA complexes are neutrally charged. Interestingly, labeling with another trivalent positively charged radiometal (gallium-68) resulted in less pronounced differences in hepatic accumulation of ZHER251.91

In addition to macrocyclic chelators, radiometals can be labeled to chelating amino acid sequences present in protein drugs. The impact of using different chelating amino acid sequences on the biodistribution of protein drugs has
extensively been studied using $^{99m}$Tc-labeled ZHER2:342 in mice. At 4 hr after injection, differences in $^{99m}$Tc-ZHER2:342 levels were mainly observed in liver, kidney, spleen, and blood (Table 5). The radioactivity of the intestines with content has been determined to estimate hepato-biliary excretion of radioactivity. Radiolabeled affibodies were administered subcutaneously or intravenously, which is expected to result in a similar biodistribution profile at 4 hr after injection.92 Introducing six consecutively placed positively charged histidines (H$_6$) at the N-terminus of ZHER2:342 increased hepatic uptake nearly a fivefold in NMRI mice.93 In addition, it resulted in an unusually high hepatic accumulation (19% ID/g) in SCID mice.93 Similarly, the use of positively charged lysines in the chelating sequence at the N-terminus of ZHER2:342 also resulted in high liver (7–23% ID/g), high kidney (36–127% ID/g), and relatively high spleen (~2.5% ID/g) uptake of mice.94,95 Replacing three of the six histidines in the N-terminal H$_6$ tag by three hydrophobic isoleucines resulted in increased levels in both the liver (32 ± 4% ID/g) and spleen (38 ± 9% ID/g) and decreased renal levels (9 ± 2% ID/g).95 Replacing the same histidines (at the N-terminus) with negatively charged glutamic acids reduced uptake in liver (<1.5% ID/g) and spleen (~0.2% ID/g).95,96 The use of N-terminal mercaptoacetyl (ma)-glycine (G)-glycine-glycine (maGGG), a chelating sequence containing side chain deficient amino acids, decreased renal and hepatic accumulation to, respectively, ≤8.2% ID/g and ≤0.93% ID/g.97,98 Replacing the glycines with negatively charged glutamic acids (E), increased renal uptake (≥8.2% ID/g for maGGG, 8.9 ± 0.8% ID/g for maGE, and 95 ± 23% ID/g for maEEE), while it slightly decreased hepatic uptake (0.93–0.66% ID/g for maGGG, 0.3 ± 0.1% ID/g for maGE, and 0.21 ± 0.02% ID/g for maEEE) and decreased the amount of the intestines with content (29.84–32% ID/g for maGGG, 8.7 ± 0.8% ID/g for maGE, and 3 ± 2% ID/g for maEEE).92,98 Reducing overall charge of the chelating sequence by using the neutrally charged mercaptoacetyl-serine (S)-serine-serine (maSSS) chelator, resulted in low uptake in the liver (0.47 ± 0.05% ID/g) and spleen (<0.5% ID/g), relatively low uptake in the kidneys (33 ± 2% ID/g), and high radioactive content in the intestines (11 ± 1% ID/g).99

The above-discussed effects of amino acid based chelators on the biodistribution of affibodies strongly depend on their location in affibodies (Table 5). This may be due to a difference in charge distribution. As N-terminal amino acids form a more stable complex with $^{99m}$Tc as compared to the same sequences located at the C-terminus, this may partly explain the observed differences in biodistribution.95 Weakly bound $^{99m}$Tc transchelates to blood proteins, increasing circulation time and blood levels of $^{99m}$Tc as seen at 4 hr after injection (Table 5).95,96

Switching between chelators has a less pronounced effect on the biodistribution of antibodies in mice. Both DOTA and DTPA conjugation of an anti-EGFR variant III antibody resulted in a similar biodistribution pattern, as studied with $^{177}$Lu labeling of anti-EGFR variant III mAb.100 In addition, no differences in biodistribution and tumor uptake have been observed after conjugation of cetuximab and anti-cluster of differentiation (CD) 44v6 antibody with similarly charged tetrafluorophenol-N-succinyl-desferal or p-isothiocyanatobenzyl-desferrioxamine B and subsequent $^{89}$Zr labeling.101

### 3.1.3 Site-specific labeling

Indirect radiolabeling by attaching chelating agents to amino acid residues located in or in close proximity of antigen-binding domains may interfere with target binding. This may affect antigen affinity and subsequently affect tumor accumulation. In a study with an anti-HER2 VHH (11A4), random labeling with a fluorescent dye (IRDye CW800) reduced target affinity of 11A4 a 1000-fold.102 Despite the fact the impact of fluorescent labeling was studied instead of radiolabeling, it does demonstrate the potential effect of labeling, including indirect radioactive labeling. The observed reduction in affinity prevented tumor accumulation of randomly labeled 800CW-11A4 in HER2 overexpressing xenografts. In contrast, using site-specific labeling of IRDye 800CW to a C-terminal cysteine of 11A4, high HER2 affinity could be retained and HER2 positive tumors could be visualized at 4 hr after tracer injection. However, if conjugation sites are not in or in close proximity of antigen-binding domains, no difference in biodistribution and tumor uptake between site-specific and random labeling is expected. For that reason, site-specific (cysteine) and random (lysine) labeling of an anti-HER2 VHH targeting with $^{111}$In resulted in comparable biodistribution and tumor uptake.103 Nevertheless, indirect labeling is increasingly performed site specifically to amino acids not located in or in close proximity of antigen-binding domains, for example, cysteines or oligosaccharides. Given the low quantity and
| Charge of amino acid side chain used in chelator | Amino acids | Site of chelation | Chelator | Blood (% ID/g) | Kidney (% ID/g) | Spleen (% ID/g) | Liver (% ID/g) | Intestines with content<sup>c</sup> (% ID/g) | Tumor (% ID/g) | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Neutral hydrophilic | Serine | N-terminus<sup>a</sup>,<sup>d</sup> | maSSS | 0.15 ± 0.01 | 33 ± 2 | N.V.(<0.5) | 0.47 ± 0.05 | 11 ± 1 | 11.5 ± 0.5 (SKOV3)<sup>b</sup> | 99<sup>h</sup> |
| Positive/negative hydrophobic | Histidine/Isoleucine | N-terminus<sup>b</sup> | HIHIHI | 1.09 ± 0.02 | 9 ± 2 | 38 ± 9 | 32 ± 4 | 4.3 ± 0.4 | N.D. | N.D. | 95<sup>h</sup> |
| | | C-terminus<sup>b</sup> | HIHIHI | 1.2 ± 0.1 | 106 ± 8 | 0.9 ± 0.2 | 64 ± 0.8 | 6 ± 2 | N.D. | N.D. | 95<sup>h</sup> |
| Positive | Histidine | N-terminus<sup>b</sup> | H<sub>b</sub> | 0.54 ± 0.10 | 82 ± 9 | 1.3 ± 0.5 | 8.1 ± 0.8 | 4.1 ± 0.7 | 2.2 ± 0.3 (LS174T) | 96<sup>h</sup> |
| | | C-terminus<sup>b</sup> | H<sub>b</sub> | 1.1 ± 0.2 | 67 ± 13 | 0.8 ± 0.3 | 4.3 ± 0.8 | 5.1 ± 0.6 | 3.2 ± 0.9 (LS174T) | 96<sup>h</sup> |
| | Lysine | N-terminus<sup>b</sup> | maKKK | 0.23 ± 0.01 | 127 ± 9 | N.V. | 7 ± 2 | 4.0 ± 0.3 | N.D. | 94<sup>h</sup> |
| | | Histidine/Lysine | HKHKHK | 0.5 ± 0.1 | 36 ± 3 | 2.5 ± 0.5 | 23 ± 4 | 7.4 ± 0.6 | N.D. | 95<sup>h</sup> |
| | C-terminus<sup>b</sup> | HKHKHK | 1.7 ± 0.2 | 84 ± 14 | 1.5 ± 0.1 | 13 ± 2 | 3.6 ± 0.3 | N.D. | 95<sup>h</sup> |
| Positive/negative | Histidine/| N-terminus<sup>b</sup> | HEHEHE | 0.33 ± 0.02 | 70 ± 10 | 0.20 ± 0.02 | 0.88 ± 0.05 | 6 ± 1 | 2.6 ± 0.4 (LS174T) | 96<sup>h</sup> |
| | Glutamic acid | N-terminus<sup>b</sup> | HEHEHE | 0.83 ± 0.06 | 129 ± 15 | 0.2 ± 0.2 | 15 ± 0.1 | 5 ± 1 | N.D. | 95<sup>h</sup> |
| | | C-terminus<sup>b</sup> | HEHEHE | 2.4 ± 0.2 | 117 ± 10 | 1.1 ± 0.1 | 39 ± 0.4 | 3.6 ± 0.3 | N.D. | 95<sup>h</sup> |
| Negative | Glutamic acid | N-terminus<sup>b</sup> | maEEE | 0.09 ± 0.02 | 95 ± 23 | N.V.(<0.5) | 0.21 ± 0.02 | 3 ± 2 | 7.9 ± 1.0 (SKOV3) | 92<sup>h</sup> |
| | | N-terminus<sup>b</sup> | maEGG | 0.15 ± 0.02 | 8.9 ± 0.8 | N.V.(<0.5) | 0.3 ± 0.1 | 8.7 ± 0.8 | N.D. | 92<sup>h</sup> |
| No side chains | Glycine | N-terminus<sup>a</sup> | maGGG | 0.13 ± 0.01 | 5.98 ± 1.14 | 0.06 ± 0.01 | 0.66 ± 0.16 | 29.84 ± 10.83 | 6.12 ± 2.02 (LS174T) | 98<sup>h</sup> |
| | | C-terminus<sup>b</sup> | maGGG | 0.15 ± 0.02 | 3.5 ± 0.3 | 0.21 ± 0.02 | 0.9 ± 0.1 | 2.0 ± 1.5 | 7.7 ± 1.5 (DU-145) | 97<sup>h</sup> |
| | | C-terminus<sup>a</sup> | maGGG | 0.12 ± 0.01 | 8.2 ± 2.9 | 0.42 ± 0.05 | 0.93 ± 0.09 | 2.6 ± 0.9 | 22.6 ± 4.0 (SKOV3) | 97<sup>h</sup> |

N.V. no exact value given; N.D., not determined; Ma, mercaptoacetyl; S, serine; H, histidine; I, isoleucine; K, lysine; E, glutamic acid; G, glycine; data are presented as mean ± standard deviation.

<sup>a</sup>Balb/c nu/nu mice.

<sup>b</sup>NMRI mice.

<sup>c</sup>Data for intestines with content (estimate of hepatobiliary excretion) as presented as percentage injected activity per whole sample.

<sup>d</sup>Organ uptake in mice without a tumor.

<sup>e</sup>Quantitative data obtained from supplementary data of Wallberg et al.97
well-known locations of cysteines present in proteins, site-specific labeling of cysteines increases labeling homogeneity. This leads to a well-defined stoichiometry, increasing batch-to-batch reproducibility of radiolabeled targeted protein drugs.

In addition to lysines and cysteines, chelators can be conjugated to oligosaccharides. Conjugation of DOTA to oligosaccharides of an ephrin type B receptor 4 targeting antibody (hAb47) did not reduce its affinity. In mice, it did promote hepatic accumulation and reduce blood levels of copper-64-labeled hAb47. It was suggested that high hepatic uptake might be due to the fact that DOTA conjugation to the sugar chains could reveal galactose residues from the CH2 domain of antibodies. These could then be recognized by the intrahepatic carbohydrate-recognition systems. At 48 hr after injection, conjugation of DOTA to oligosaccharides of hAb47 also resulted in a tumor uptake that was lower than when DOTA was conjugated to lysines or cysteines. Tumor uptake was, respectively, 9.5 ± 0.6% ID/g, 11.8 ± 2.1% ID/g, and 18.1 ± 1.7% ID/g.

3.1.4 Chelate/protein ratio

Like chelator type and the site of chelator conjugation, the number of chelators attached to protein drugs, expressed in chelate/protein ratios, could also alter in vivo behavior. Increasing the c/p ratios increases the amount of radioactivity that can be labeled to a specific amount of protein drug (specific activity). It reduces the amount of radioactively labeled protein drug that is needed to inject in order to provide good quality PET or SPECT scans. This is especially of importance in preclinical animal studies, as often small amounts of radioactively labeled protein drugs are injected into small animals. However, increasing c/p ratio can also increase the chance that randomly labeled radiolabels attach in or in close proximity of the antigen-binding domains of protein drugs, potentially reducing target affinity and tumor uptake. In addition, increasing c/p ratio can increase charge differences as compared to parental proteins, potentially affecting the biodistribution of protein drugs. In order to determine the optimal c/p ratio, both specific activity and the potential effect on biodistribution and tumor uptake should be taken into account.

In Table 6 we summarized the effect of c/p ratio on the level of antibodies and a F(ab′)2 fragment in blood, liver, kidney, spleen, and tumor. A c/p ratio as low as 5.5 altered the biodistribution of DTPA-conjugated, mesothelin targeting, MORAb-009 in mice. Increasing the c/p ratio of DTPA-MORAb-009 from 2.4 to 5.5 resulted in higher 111In-MORAb-009 levels in the liver and spleen, and decreased tumor uptake. DTPA conjugation also altered the biodistribution of a 111In-labeled antibody-targeting human serum albumin (HSA), in mice. Although exact values were not given, increasing c/p ratio from 2–3.2 to 11.2–16 gradually increased hepatic uptake at 24 hr after injection, while uptake in other organs remained unchanged. Contradictory, increasing the c/p ratio from 3.2 to 5.6 decreased uptake in all studied organs, except for the liver in which levels did not change. As aggregates were removed prior to administration, ex vivo aggregation did not cause the increase in hepatic uptake. The largest increase in hepatic uptake was observed between the c/p ratios of 5.6 and 11.2. This increase in c/p ratio also resulted in the largest difference in isoelectric point (respectively, 6.5 and 5.9), suggesting that the increase in hepatic uptake is influenced by a difference in overall charge.

Similar to DTPA conjugation, DOTA conjugation can also alter the biodistribution of protein drugs. Increasing the c/p ratio (from 3 to 5) of a 177Lu-labeled F(ab′)2 targeting a L1 cell adhesion molecule, decreased its accumulation in the kidneys (from ~35% ID/g to ~5% ID/g) and tumor (from ~15% ID/g to <5% ID/g), while it increased accumulation in the liver (from ~10% ID/g to ~35% ID/g) and spleen (from ~5% ID/g to ~10% ID/g). The labeling of DOTA to the full antibody, targeting the same target, also affected its biodistribution. However, the lowest tested c/p ratio was 7. Increasing the c/p ratio from 7 to 12 to 15 gradually increased both clearance rate and hepatic uptake. In addition to these two studies, DOTA conjugation also altered the biodistribution of a 111In-labeled Sjögren syndrome type B antigen-targeting antibody (DAB4) and its 111In-labeled isotypic control in mice. Increasing the c/p ratio from 5 to 20 of 111In-DAB4 and its 111In-labeled isotypic control promoted the uptake in liver and spleen. Tumor uptake was only affected by c/p ratio for 111In-DAB4 in target positive tumors, suggesting that this is due to the observed decrease in antigen affinity.
TABLE 6  Effect of increasing chelation ratio on biodistribution and tumor uptake

<table>
<thead>
<tr>
<th>Chelator (increase in c/p ratio)</th>
<th>Time after injection</th>
<th>Blood</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies DTPA (2.4–5.5)(^{105})</td>
<td>1 day</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MAG3 (2.9–9.5)(^{110})</td>
<td>40 hr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BAT (4.3–8.3)(^{109})</td>
<td>72 hr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DOTA (5–20)(^{108})</td>
<td>48 hr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DOTA (7–12)(^{107})</td>
<td>24 hr (c/p ratio of 7) and 48 hr (c/p ratio of 12)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>N.D.</td>
</tr>
<tr>
<td>MAG3 (9.5–12.8)(^{110})</td>
<td>40 hr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F(ab')(_2) DOTA (3–5)(^{106})</td>
<td>24 hr</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Red arrow = decrease in protein drug levels; blue arrow = increase in protein drug levels; horizontal black line = no significant change in protein drug levels; BAT, 6-(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; N.D., not determined.

Increasing the c/p ratio of a BAT chelated lymphoma targeting antibody had a similar effect on the biodistribution as above described DOTA or DTPA chelated antibodies.\(^{109}\) The tested c/p ratios were 2.1, 4.3, 8.7, and 11.4. Finding the optimal ratio was triggered by the fact that copper-67 was used as an electron-emitting therapeutic radioisotope, which is contaminated with nonradioactive copper. At 72 hr after injection, tumor uptake significantly decreased at c/p ratios >4.3 and liver levels increased gradually with increasing c/p ratios. The increase in c/p ratio did not affect antibody uptake in other organs. It did affect clearance, which at c/p ratios of 2.1, 4.3, 8.7, and 11.4 were, respectively, 4.0, 5.5, 2.6, and 2.8 days.

The fact that increasing c/p ratios result in higher hepatic uptake of radiolabeled protein drugs, suggests that increasing the c/p ratio promotes hepatic excretion. Indeed, increasing the c/p ratio (from 7 to 10) of a MaGGG-conjugated antibody E48 not only increased hepatic uptake at 24 hr after injection, it also increased the radioactive content (estimation of hepatic excretion) in the ileum and colon of mice.\(^{110}\) At the same time blood levels decreased. Increasing the c/p ratio from 2.9 to 9.5 and from 9.5 to 12 also decreased blood levels at 40 hr after injection. This decrease in blood levels additionally resulted in lower organ uptake. Despite the promotion of hepatic excretion, the increase in c/p ratio reduced the uptake of rhenium-168-labeled MaGGG-E48 in the well-perfused liver, as observed 40 hr after injection.

The above-mentioned studies have been performed in mice, in which high c/p ratios are often used to obtain high-quality scans. However, lower c/p ratios are generally used in nonhuman primate and clinical studies.\(^{111,112}\) Such differences can hamper extrapolation of preclinical results to the clinical setting.

3.1.5 Conclusion

By radiolabelling antibodies, antibody fragments, and non-Ig scaffolds, these can be traced in vivo. However, radiolabeling can also alter their biodistribution and tumor uptake. Such an impact may hamper a sound interpretation of imaging data, as the data are often used to predict the biodistribution of nonlabeled protein drug candidates or protein
drugs. When interpreting imaging data, one should consider the fact that biodistribution depends on the radiolabeling method, type and site of chelator conjugation, and c/p ratio. Generally, indirect labeling results in higher organ and tumor uptake as compared to direct labeling, as it results in a more efficient intracellular accumulation of radiocatabolites over time. Furthermore, the use of cationic chelating amino acid sequences generally increases hepatic and renal accumulation, as seen with affibodies. Increasing hydrophilicity of these amino acid sequences tends to increase the uptake of affibodies in the spleen and liver. These findings may be translational to other small-sized proteins. In contrast to affibodies, switching between chelators has a less pronounced effect on antibodies. However, increasing the c/p ratio can decrease antibody levels in blood and tumor and promote accumulation in spleen and liver. Finally, site-specific labeling can prevent a reduction in tumor uptake of protein drugs that is caused by chelation to sites in or in close proximity of antigen-binding domains.

3.2 Fluorescent labeling

Similar to radioactive labeling of antibodies, antibody derivatives, and non-Ig scaffolds, these proteins can be labeled with nonionizing fluorescent dyes to enable optical imaging. This emerging imaging modality can support researchers to study normal tissue and tumor distribution of these proteins, which could complement the PET/SPECT imaging modalities. Moreover, optical imaging may enable real-time tumor visualization in an intraoperative setting or visualization of tumor margins in excised tissues.113,114 Furthermore, reactive oxygen species generating fluorescent dyes can be used to load tumor-targeting proteins.115 Such labeled proteins possess antitumor activity.115

Several near-infrared (NIR) fluorescent dyes have been used to label proteins. An often used NIR dye is IRDye 800CW, as it can be produced under good manufacturing practice conditions, allowing a fast translation of fluorescent-labeled proteins to clinical studies. NIR fluorescent dyes often have a hydrophobic core with negatively charged groups. Labeling could therefore affect local charge and hydrophobicity of proteins upon conjugation, which may subsequently alter their biodistribution. Furthermore, labeling may induce protein aggregation and albumin binding.116,117 A comparison between the distribution of dual-labeled (99Zr and IRDye 800CW) anti-CA19.9 antibody (5B1) with 99Zr-labeled 5B1 demonstrated that conjugation of IRDye 800CW can increase liver uptake and reduce levels in blood and other organs at 120 hr after injection.118 The decrease in splenic uptake was most pronounced, which decreased from 19.8% ID/g to 5.7% ID/g. An uptake of 19.8% ID/g is relatively high for antibodies. According to the authors, the observed decrease in splenic uptake might be the result of a reduced formation of aggregates. Unfortunately, the formation of aggregates was not studied after tacer production or postinjection. Therefore, the amount of aggregation at the time of injection is unknown.

Although optical imaging holds great promise as a strategy to visualize tumor tissue both in vivo and ex vivo, it is challenging to get sufficient high signals from tumor tissue. In order to overcome this challenge, the administered dose of fluorescent-labeled proteins can be increased. However, the use of high-dose fluorescent-labeled proteins should be evaluated for safety pharmacology and toxicology. A safety pharmacology study has been performed with therapeutic doses of IRDye 800CW labeled cetuximab in cynomolgus macaques.115 Cetuximab-800CW was well tolerated and all significant treatment effects were due to cetuximab and not to the IRDye 800CW. The only exception was a slightly higher and more persistent prolongation of the corrected QT interval after injection of cetuximab-800CW, as compared to nonconjugated cetuximab. This resulted in cardiac monitoring of patients that were injected with cetuximab-800CW, in a terminated clinical phase 1 study (NCT01987375).

3.2.1 Dye/protein ratio

Instead of increasing the total dose in order to obtain sufficient high signals from tumor tissue, one could increase the dye/protein ratio (d/p ratio). However, fluorophores can have the tendency to self-aggregate and to quench each other when placed in near proximity to each other.120 Therefore, increasing the d/p ratio may result in quenching of fluorescent signal and may increase the tendency of labeled proteins to aggregate. Aggregation has been observed with panitumumab conjugated with a NIR fluorescent cyanine dye (FNIR-G-765) at a d/p ratio of 5 and with an antimesothelin antibody that was conjugated with IRDye 800CW at a d/p ratio of 4.111,116 For both constructs a d/p ratio of 2 did not
result in visible precipitation of labeled antibodies. Aggregation can be observed directly after conjugation, however, it may also occur in vivo. Incubation of an IRDye 800CW labeled anti-EpCAM antibody in human serum promoted aggregation of the labeled antibody.\textsuperscript{117} This antibody was labeled with a d/p ratio of 2.6 $\pm$ 0.9. Aggregation increased from $\sim$10% at start of incubation to $\sim$25% at 96 hr of incubation. Interestingly, incubation in human serum also triggered albumin binding, which was maximal after 24 hr of incubation ($\sim$10%) and remained stable up to 96 hr of incubation.

As compared with \textsuperscript{125}I-labeled 8C2, an antibody targeting an unspecified target, IRDye 800CW labeled 8C2 showed higher liver exposure, shorter serum half-life, and lower organ exposure in all other studied organs.\textsuperscript{121} The d/p ratio of 8C2-800CW was 1.2–1.4. It should be noted that the serum half-life of 8C2-800CW was determined by using enzyme-linked immunosorbent assay, while the serum half-life of \textsuperscript{125}I-8C2 was determined by using gamma counting. In mice, increasing the d/p ratio from 2 to 5 also increased hepatic accumulation and decreased tumor uptake of IRDye 800CW labeled cetuximab and panitumumab.\textsuperscript{122} After quantification of ex vivo fluorescent images at 24 hr after injection, increasing the d/p ratio seemed to increase liver-to-tumor ratio of cetuximab-800CW with a factor 5.6 and decrease tumor-to-background ratio with a factor 3.4. Similar results were observed with panitumumab-800CW. Increasing the d/p ratio increased liver-to-tumor ratio with a factor 5.6 and decreased tumor-to-background ratio with a factor 3.5. d/p-dependent tumor-to-background ratio was not observed with FNIR-774-panitumumab and FNIR-774-cetuximab. In contrast, liver-to-tumor ratios increased with a factor 1.4–1.6 after increasing d/p ratio from 2 to 5. These results suggest that FNIR-774 labeling has less impact on tumor and hepatic accumulation of cetuximab and panitumumab as compared to labeling with IRDye 800CW.

Another approach to study the effect of d/p ratio on the biodistribution of proteins is by dual labeling these proteins with both fluorescent dyes and radionuclides. This approach has been used to study the effect of d/p ratio of IRDye 800CW labeled cetuximab and bevacizumab in mice.\textsuperscript{123} Both antibodies were first conjugated with desferal using a c/p ratio of 0.5 and subsequently with IRDye 800CW using d/p ratios of 0–2, whereafter the constructs were labeled with \textsuperscript{89}Zr. Fluorescent labeling with a d/p ratio of 1 promoted liver uptake and decreased blood levels of cetuximab at 72 hr after injection. Increasing the d/p ratio to 2, significantly increased liver uptake and reduced blood levels of both antibodies at 24 and 72 hr after injection. In line, Oliveira et al. also observed extremely high liver uptake of cetuximab-800CW ($\sim$80% ID/g) using a d/p ratio of 1.4.\textsuperscript{124} Similar preclinical findings were reported in another biodistribution study with MN-14 (anti-CEA), girentuximab, and cetuximab dual labeled with \textsuperscript{111}In and IRDye 800CW.\textsuperscript{125} All antibodies were conjugated with DTPA using c/p ratios ranging 1.9–4.1 and with d/p ratios of 0–3. Increasing d/p ratios gradually increase liver uptake in mice, which was significantly increased for all antibodies at a d/p ratio of 3. The same d/p ratio significantly decreased tumor uptake of \textsuperscript{111}In-MN-14-800CW and \textsuperscript{111}In-cetuximab-800CW, but not \textsuperscript{111}In-girentuximab-800CW. Although IRDye 800CW conjugation of cetuximab by using a d/p ratio of 1–2 increased liver uptake and decreased blood levels in mice, the half-life of therapeutically dosed cetuximab-800CW in cynomolgus macaques (2.5 days) is comparable with that of nonlabeled cetuximab when using a d/p ratio of 1.8.\textsuperscript{119} Similarly, conjugation of lgG with Cy5 or Cy3, using a c/p ratio of 1–2, did not affect serum half-life in macaques.\textsuperscript{126} The macaques were co-injected with 50 mg/kg Cy5-labeled lgG and 50 mg/kg Cy3-labeled lgG, resulting in a total protein dose of 100 mg/kg labeled lgG.

### 3.2.2 Site-specific labeling

As discussed in the chapter concerning radiolabeling, labels that are conjugated to proteins may interfere with their antigen binding. In order to prevent such interference, labeling can be performed site specifically at locations distant from antigen-binding domains. Additional advantages of site-specific labeling have been discussed in Section 3.1.3. Site-specific labeling may need modification of protein structures prior to the conjugation of fluorescent dyes. In the case of VHHs, for example, a C-terminal cysteine can be introduced. Such a cysteine is able to react with thiol-reactive NIR fluorescent dyes. As discussed earlier, random labeling of an anti-HER2 VHH (11A4) with IRDye CW800 reduced target affinity of 11A4 a 1000-fold.\textsuperscript{102} This decrease in affinity resulted in a drastic decrease in tumor uptake. In contrast to site specifically labeled 11A4-800CW, tumor uptake of randomly labeled 11A4-800CW was not visible at 4 hr after injection. Similar findings have been published with IRDye 680RD labeled anti-HER2 VHH (2Rs15d) in mice.\textsuperscript{127}
Compared to site-specific labeled 2Rs15d-680RD, random labeling decreased tumor uptake and, except for the kidneys, increased the levels in blood and normal organs, at 24 hr after injection. Like for 11A4, site-specific labeling of 2Rs15d was performed by using cysteine as a conjugation partner of the NIR fluorescent dye. Although the use of cysteine tagging of VHVs may prevent affinity loss after labeling, potentially resulting in higher tumor uptake, cysteine tagging of VHVs currently results in a significant reduction of production yields. This may hamper large-scale production, which is necessary for clinical application. Furthermore, cysteines can form intermolecular disulfide bridges and reduction of disulfide bridges may reduce crucial intramolecular disulfide bridges.

In contrast to site-specific labeling of VHVs, site-specific labeling of huA33, an A33 targeting antibody, with $^{89}$Zr and Dye 680 may have less impact on the distribution. No difference in immunoreactivity was found between random or site-specific labeling of $^{89}$Zr-huA33-800CW. Although the authors concluded that in vivo behavior of both constructs is comparable, data about organ-specific biodistribution and serum half-life are missing.

### 3.2.3 Zwitterionic NIR fluorescent dyes

NIR fluorescent dyes often are negatively charged. Conjugation of these charged dyes to proteins may therefore alter the biodistribution of proteins. In order to prevent a change in total protein charge, zwitterionic NIR fluorescent dyes have been developed (e.g., ZW800-1, FNIR-Z-759, and FNIR-G-765). These dyes have a net charge of +1, which is the same net charge of the terminal amine of lysines they replace. As a result, conjugation of a zwitterionic NIR fluorescent dye to lysines does not change overall protein charge. The labeling with such a zwitterionic dye may therefore have less impact on the charge-dependent biodistribution of proteins as compared to, for example, anionic fluorescent dyes. Labeling of a secondary antibody, used to stain for α-methylacyl-CoA racemase or HER2, with ZW800-1 resulted in less nonspecific cell binding as compared to the same antibody that was labeled with IRDye 800CW or Cy5.5. Nonspecific binding increased when d/p ratio increased from 1.2 to 2.5. The same study showed that labeling a cyclic peptide consisting of Arg-Gly-Asp-D-Tyr-Lys with ZW800-1 resulted in tumor uptake with lower background signal than was observed when was conjugated with IRDye 800CW or Cy5.5. Although total protein charge is not altered after conjugation with zwitterionic dyes, small structural changes in NIR fluorescent dyes can alter whole body distribution of antibodies. This has been observed with panitumumab labeled with the zwitterionic dyes FNIR-Z-759 or FNIR-G-765. Both dyes have a total charge of +1 but different positively charged groups. At the sites FNIR-Z-759 has trimethyl ammonium groups, FNIR-G-765 has guanidine groups. The most pronounced difference was observed in liver accumulation. Hepatic uptake was highest for panitumumab-FNIR-G-765, as was visible on fluorescent images of injected mice.

### 3.2.4 Conclusion

Labeling of proteins with fluorescent dyes can cause aggregation of fluorescently labeled proteins, which is most pronounced at high d/p ratios. Visible precipitation has been observed at d/p ratios of 4–5. In addition, increasing d/p ratio can alter the biodistribution of proteins. Already at a d/p ratio of 2, fluorescent labeling could affect the biodistribution and pharmacokinetics of proteins. However, the impact seems to be depending on the NIR fluorescent dye and protein. Furthermore, the effect of fluorescent labeling on the half-life of the protein may additionally depend on the protein dose or species in which the pharmacokinetic study is performed.

### 3.3 Drug conjugation

In order to increase the selectivity of cytotoxic drugs, they can be attached to tumor-targeting proteins. Due to the clinical successes and their high potential, an increasing amount of ADCs are in development. In 2000 the first ADC (gemtuzumab ozogamicin) gained FDA approval and due to extensive research over 60 ADCs are in clinical development. These successes led to the interest in the conjugation of cytotoxic drugs to tumor-targeting antibody fragments and non-Ig scaffolds. However, drug conjugation can change overall or local charge/lipophilicity and destabilize proteins, for example, by affecting their intraprotein charge and lipophilic interactions or intraprotein disulfide bonds.
This may consequently alter the biodistribution and pharmacokinetics of proteins. Most experience with drug conjugation has been obtained with ADCs.\textsuperscript{134–136} Currently, three ADCs are FDA and EMA approved, brentuximab vedotin, trastuzumab emtansine, and inotuzumab ozogamicin, while gemtuzumab ozogamicin is FDA approved and is under consideration at the EMA.\textsuperscript{137–140} In addition, over 40 different ADCs are evaluated in the clinical setting.\textsuperscript{141} Drugs that have been conjugated to antibodies target tubulin (e.g., maytansinoids and auristatins), DNA (e.g., calicheamicin), or RNA (e.g., amanitin).\textsuperscript{142}

Each time an antibody is loaded with a cytotoxic drug, the biodistribution and tumor uptake potentially changes. Due to the increasing amounts of available linkers, cytotoxic drugs, and protein sites that can be attached to antibodies, the impact of drug conjugation on the distribution of antibodies is potentially different for each new ADC. The impact of drug conjugation is likely higher when antibodies are loaded with relatively high amounts of cytotoxic drugs. High DARs might be beneficial for situations in which tumors marginally express antigens. Current techniques allow the loading of antibodies with drugs using DARs as high as 20, without compromising in antigen affinity and with increased tumor toxicity in mice.\textsuperscript{143} However, such high DARs may change biodistribution in patients and may increase the uptake in normal organs, potentially increasing toxicity.

These drugs can be conjugated to multiple sites of antibodies. Lysines are often used as conjugation sites for cytotoxic compounds. However, mAbs contain ~90 accessible lysines.\textsuperscript{144} Therefore, this strategy results in highly heterogeneous ADCs. When aiming at a DAR of 2–4, \textit{1} \times \textit{10}\textsuperscript{6} different species are statistically possible. This results in a highly heterogeneous mixture of ADCs. The different structures will likely possess different efficacies, toxicities, and pharmacokinetics, as has been demonstrated with a monomethyl auristatin E labeled anti-CD30 antibody (cAC10).\textsuperscript{145} In vitro efficacy and pharmacokinetics in SCID mice were compared between ADCs with a DAR of 2, 4, or 8. Efficacy directly correlated with DAR (8 > 4 > 2), while the ADC with a DAR of 8 cleared threefold faster than with a DAR of 4 and a fivefold faster than with a DAR of 2.

Heterogeneity can be reduced by targeting cysteines instead. Reducing the four available disulfide bindings in antibodies, an antibody to drug ratio of 2–4 will statistically result in ~15 different species.\textsuperscript{144} Although the disulfide bridges in antibodies covalently interconnect the heavy chains and connect heavy chains with the light chains, reducing these disulfide bindings does not affect the chemical structure of antibodies. This is due to the many noncovalent interactions that primarily determine the chemical structure of antibodies.\textsuperscript{145,146} Similar to cysteine conjugation, other conjugation methods can be used to reduce heterogeneity as well. These include chemoenzymatic loading of cytotoxic drugs and the conjugation of cytotoxic drugs to glycans and incorporated cysteines or unusual amino acids.\textsuperscript{142,147}

To study in vivo behavior of ADCs, they can be radiolabeled and traced in vivo using molecular imaging. However, radiolabeling might decrease the stability of ADCs. To circumvent potential problems with stability of the radiolabeled ADC, its naked antibody counterpart can be radiolabeled instead and used to provide insight in its biodistribution. In order to predict the in vivo behavior of an antimesothelin ADC (DMOT4039A), its \textit{89}Zr-labeled naked antibody counterpart (MMOT0530A) has been studied in cancer patients.\textsuperscript{6} This study revealed normal antibody distribution of \textit{89}Zr-MMOT0530A and heterogeneous uptake between tumor lesions. Tumor uptake differed a 2.4-fold within patients and a 5.3-fold between patients. Tumor uptake of radiolabeled naked antibody counterparts of ADCs can have predictive value. It has been demonstrated for trastuzumab emtansine (T-DM1) in the ZEPHIR study.\textsuperscript{148} By combining pretreatment \textit{89}Zr-trastuzumab PET/computed tomography imaging with early \textit{[18}F\textit{]}2-fluoro-2-deoxy-D-glucose PET/computed tomography imaging, patients that benefit from T-DM1 could be distinguished from patients that did not benefit from T-DM1 treatment. This combination resulted in positive and negative predictive values of 100%.

However, ADCs can have a different biodistribution pattern as their naked counterparts. It has been observed in rats with an antibody-targeting six-transmembrane epithelial antigen of prostate 1, conjugated with monomethyl auristatin. Hepatic uptake of the \textit{111}In-labeled ADC was twofold higher than the \textit{111}In-labeled parental antibody at 120 hr after injection.\textsuperscript{149} Drug conjugation only marginally increased spleen uptake of the parental antibody. The observed increased uptake in these organs did not affect whole blood levels. A more pronounced effect was observed after conjugation of a humanized anti-Lewis Y antibody (hu3S193) with calicheamicin in cancer patients.\textsuperscript{150} As determined after \textit{111}In labeling, drug conjugation increased both clearance and hepatic uptake (4.5-fold, at 24 hr after injection), while it decreased maximum tumor uptake (nearly 20-fold).\textsuperscript{150}
The impact of drug conjugation on the pharmacokinetics of antibodies could depend on the site of drug conjugation. This has been demonstrated with the conjugation of maytansine to lysines on trastuzumab (T-DM1, DAR = 3.4) and to three other enzymatically created sites in trastuzumab. By enzymatically converting cysteines into aldehydes in specific inserted amino acid sequences (CXPXR, where X is usually serine, threonine, alanine, or glycine), maytansine could site specifically be conjugated to trastuzumab through Hydrazino-iso-Pictet-Spengler ligation. Maytansine was conjugated to trastuzumab in either the light chain, the CH1 domain, or at the heavy-chain C-terminus. This resulted in ADCs with DARs of ~2. The site at which maytansine was conjugated did impact the half-life of the ADCs. Conjugation of maytansine at the C-terminus resulted in the ADC with the longest half-life in BALB/c mice, being 7.8 ± 0.5 days. The ADC half-lives were shorter when maytansine was conjugated to incorporated aldehydes in the light chain (5.2 ± 0.2 days), the CH1 domain (5.7 ± 0.3 days), or to lysines (T-DM1, 5 ± 0.3 days).

In addition to the enzymatic introduction of aldehydes, aldehydes can be created in an oxidizing environment. Such an environment can affect the pharmacokinetics of ADCs. One of the strategies to site specifically conjugate cytotoxic drugs to antibodies is by oxidizing carbohydrate residues in the native glycans at the Fc-domain of IgG antibodies, in order to create aldehydes. These aldehydes can then be targeted with activated cytotoxic drugs. However, overoxidation could oxidize methionine residues that are located in close proximity of the FcRn binding site of antibodies, which can negatively affect FcRn binding and subsequently serum half-life. Oxidation was also used in the process to site specifically conjugate monomethyl auristatin E and D to modified trastuzumab. Zhou et al. posttranslationally introduced sialic acid into native N297 glycans of trastuzumab. Subsequently, mild oxidizing conditions were used to form aldehyde-functionalized trastuzumab. In addition, these conditions partially oxidized methionine residues close to the FcRn-binding region, roughly 40% of Met-252 and <10% of Met-428 was oxidized, resulting in a ~15% reduction of FcRn binding. Oxidation and reduced FcRn is not likely to majorly affect half-life. Partial oxidation of (~40%) of Met-252 on trastuzumab reduced its FcRn binding by ~25%, which only had a minimal effect on half-life in human FcRn transgenic mouse. However, a higher degree of oxidation (80%) of Met-252 in trastuzumab has been shown to negatively affect both FcRn binding (by ~60%) and serum half-life (by ~80%).

Drug conjugation does not always alter the biodistribution of antibodies. It has been demonstrated in preclinical biodistribution studies with an N-methyl-N-[4-mercapto-4-methyl-1-oxopentyl]-L-alanine ester of maytansinol that was conjugated to lysines of an anti-CanAg antibody (DAR 3.5–4.0), toxic tubulysin analog TUB-OMOM conjugated to lysines of trastuzumab (DARs of 2 and 4), and auristatin conjugated to p-acetylphenylalanine (an unnatural amino acid) that was site specifically incorporated into the heavy chains of an anti-HER2 IgG1 antibody (DAR ∼2.0). The pharmacokinetics of these radiolabeled ADCs were comparable with that of the radiolabeled parental antibodies in nontumor bearing mice or rats. Due to the use of nontumor-bearing mice or rats, it is not known if drug conjugation altered tumor uptake of these antibodies.

Similar to tumor-targeting antibodies, both tumor-targeting antibody fragments and non-Ig scaffolds can be conjugated with cytotoxic drugs. Protein backbones that have been used to target anticancer drugs include diabodies, Fab fragments, and designed ankyrin repeat proteins. The effect of drug conjugation has been described for an anti-CD30 diabody that was engineered with two cysteine mutations for site-specific drug conjugation. The diabody was conjugated with monomethyl auristatin E or F (DAR ∼4), which negatively altered the clearance rate of the diabody by roughly a fivefold. The reason for the observed decrease in diabody clearance is unclear. As the increase in size was small, from ∼38 to 47 kDa, the increase in size is not likely the reason. Remarkably the major route of clearance changed from renal to hepatobiliary excretion. The reason for the change in clearance route was not studied and is therefore unknown. However, the fact that the lipophilicity of the diabodies increases upon conjugation with both drugs may have caused the change in clearance route. As the half-lives between the diabody and the diabody–drug conjugate were similar, the difference in clearance rate might be due to a difference in distribution and distribution rate. Unfortunately, no biodistribution study was performed that could give an answer to that question. Interestingly, drug conjugation of an anti-CD30 an IgG1 antibody (DAR ∼4) in the same mouse models did not significantly alter the clearance of the antibody.
In conclusion, drug conjugation can increase hepatic uptake, clearance from circulation, and decrease tumor uptake of antibodies and decrease the clearance of diabodies. Such an impact should be taken into account when interpreting imaging data that are obtained with radiolabeled ADCs or diabody–drug conjugates.

### 3.4 Glycosylation

When protein drugs are produced in eukaryotic cell systems, they typically undergo the process of glycosylation. Glycosylation of protein drugs can affect their conformation, solubility, stability, in vivo activity, and immunogenicity. In addition, glycosylation may also alter the biodistribution and tumor uptake of protein drugs.

Glycosylation is a complex posttranslational modification by which glycans are enzymatically attached to side chains of asparagine (N-glycosylation), serine, or threonine (O-glycosylation) of protein drugs. This process strongly depends on the expression system of the protein drug. In yeasts, glycans contain many mannose sugars, plants consistently add α1,3-fucose and β1,3-xylose sugars and although *Escherichia coli* bacteria are not capable of adding glycans to proteins, a glycosylation machinery can be incorporated in their DNA. Mammalian cells can add a variety of glycans to proteins, often resulting in highly heterogeneous glycosylated proteins. Switching expression system can therefore change the glycosylation status. Subsequently, it can alter the biodistribution of protein drugs as has been demonstrated with a 124I-labeled anti-HER2 diabody (C6.5db) in mice. Production of C6.5db in yeasts (*Pichia pastoris*) resulted in the addition of branched mannose glycans, while production in *E. coli* resulted in nonglycosylated C6.5db. In mice, mannose glycosylated 124I-C6.5db cleared faster from the blood as compared to nonglycosylated 124I-C6.5db, resulting in ~two-fold lower tumor uptake at 24 hr after injection. The observed increase in clearance of glycosylated 124I-C6.5db may be due to the recognition of mannose sugars by mannose receptors on intrahepatic cells present in mice and humans. Contradictory, at 2 hr after injection hepatic uptake of mannose glycosylated 124I-C6.5db was lower than was observed with nonglycosylated 124I-C6.5db. It was suggested that this was due to mannose receptor-mediated hepatic clearance that likely occurred within 2 hr after tracer injection. In contrast to C6.5db, glycosylation without mannose but with fucose, hexose, N-acetylhexosamine, and sialic acid containing glycans, moderately increased circulation time of a bispecific single-chain diabodies (scDb) targeting CEA and CD3. Except for renal uptake, glycosylation had a minor effect on the biodistribution of this scDb labeled with iodine-131 (131I). Glycosylation did decrease renal uptake from ~25% ID/g to ~5% ID/g at 2 hr after injection.

In order to increase the antibody-dependent cellular cytotoxicity of therapeutic antibodies, glycosylated antibodies can be defucosylated. By removing fucose from the glycans of RG7116, a HER3 targeting mAb, its antibody-dependent cellular cytotoxicity improved. A study with 89Zr-RG7116 in mice demonstrated a normal antibody biodistribution profile, except for extremely high splenic uptake of >50% ID/g for all dose groups tested. Its 111In-labeled naturally glycosylated IgG control showed much lower splenic uptake in mice. The fact that splenic uptake of 89Zr-RG7116 in SCID mice was higher than 111In-IgG might be due to the glycosylation status of RG7116. Glycoengineering of RG7116 increased the affinity to Fc-gamma receptor IIIA. As SCID mice express a murine homolog of this receptor on monocytes and macrophages, both present in the spleen, glycoengineering of RG7116 might have enhanced splenic uptake 89Zr-RG7116. Although a high splenic uptake could be due to the glycosylation status of RG7116, high splenic uptake (>80% ID/g) was also observed with nonglycoengineered 111In-labeled antimesothelin antibody and 89Zr-pertuzumab at, respectively, 24 hr and 7 days after tracer injection in mice. High splenic uptake might be mouse strain-specific instead, as in the above three studies only SCID mice were used. Indeed splenic uptake of 89Zr-pertuzumab was much lower in athymic nu/nu mice (2.7 ± 1.7% ID/g). In addition, extremely high splenic uptake of 89Zr-RG7116 was not observed in patients. Four days after injection, the concentration of 89Zr-RG7116 in the spleen, quantified as maximum standardized uptake value, was lower than its concentration in the blood pool.

In conclusion, unexpected organ and tumor uptake might be the result of the glycosylation status of proteins. Most relevant organs in which protein uptake can be affected by glycosylation status are the kidneys, liver, and spleen. Both defucosylation and glycosylation, especially mannosylation, can change the biodistribution of proteins.
glycosylation status depends on the expression systems, its effect on biodistribution might be relevant in the search for an optimal system.

3.5 Humanization

Protein drugs can elicit antidrug antibodies (ADAs) in patients. Formation of ADA–drug complexes can subsequently increase drug clearance via the liver or spleen. Humanization of targeted proteins reduces the chance of being recognized by the human body as “foreign” and potentially reduces the chance of ADA production. As a result, humanization may affect the biodistribution of protein drugs. Although humanization may reduce the chance of ADA production in humans and in cynomolgus monkeys, both humanized and fully human antibodies can still induce ADA production. This may partly be due to factors other than humanization, for example, related to clinical use, product formulation, and patient characteristics. These factors may be responsible for the variability in ADA production after administration of drugs and drug candidates. By linking variable domains of murine antibodies to human constant regions, murine antibodies can be humanized. To further enlarge the human content of antibodies, mouse complementarity-determining residues (CDRs) can be grafted into human antibodies. Current technology additionally allows the production of fully human antibodies. Despite being fully human, these antibodies can still elicit ADAs. In addition to the humanization of antibodies, VHVs can be humanized as well. Given a high homology with the heavy-chain variable domain of human antibodies, humanization of VHVs is rather straightforward.

Similar to antibodies, other human proteins have been used to develop drug candidates, including anticalins and adnectins. Although these drug candidates have been derived from human proteins, they still can elicit an immune response. For example, an anticalin (PRS-050) targeting vascular endothelial growth factor A, derived from human lipocalins, seems to be nonimmunogenic in cancer patients, while a vascular endothelial growth factor receptor 2 targeting adnectin (CT-322) still provoked ADA production in 31 out of 38 (82%) cancer patients.

Humanization does affect the half-life of antibodies in humans (Figure 2). Generally, half-lives of antibodies in humans correlate with the degree of humanization, in the order of “fully-rodent” < chimeric < CDR-grafted

![Diagram of humanization](image)
human "fully human." In line, humanization can decrease the clearance rate in nonhuman primates. Humanization, using a CDR-grafting method, of an antitumor necrosis factor-alpha murine antibody increased its half-life to 40–90 hr (a two- to threefold increase) in cynomolgus monkeys. 188 In contrast, humanization of antibodies can increase clearance rate in mice. This has been observed with an astatine-211-labeled humanized antibody (Rebmab200) and murine antibody (MX35) targeting sodium-dependent phosphate transporter. 189 At 24 hr after tracer injection in tumor-bearing mice, slightly lower blood levels were observed for astatine-211-labeled Rebmb200 as compared to astatine-211-labeled MX35 (respectively, ~15% ID/g and ~20% ID/g). No differences were observed in organ and tumor uptake. In contrast to the astatine-211-labeled antibodies, no differences in both blood levels and tissue levels were observed between when both antibodies were labeled with 125I instead of astatine-211.

In rare cases, humanization of antibodies can introduce off-target binding to, for example, complement component 3 as observed with a humanized antifibroblast growth factor receptor 4 antibody in mice. 190 This humanized antibody cleared faster than its native chimeric antibody, due to complement component 3 binding of the humanized antibody in plasma. It resulted in reduced target-specific liver accumulation, which decreased from ~80% ID/g to 35% ID/g. This interaction was only present in mice and absent in cynomolgus monkey and human plasma, suggesting that complement component 3 binding and the observed decrease in half-life are species specific.

Humanization of an anti-CEA VH (NbCEAS) marginally decreased antigen affinity. 191 The effect of humanization has been studied in mice by labeling both the humanized and parental form of the VH with 99mTc. Humanization did majorly affect clearance rate and uptake in lung, muscle, liver, and tumor at 1 hr after injection.

In conclusion, humanization of antibodies generally increases their half-life in humans an can increase half-life in cynomolgus monkeys, while it can decrease the half-life in mice. Furthermore, humanization can introduce off-target interaction. Understanding these potential effects of humanization may help to interpret imaging data and pharmacokinetic of protein drug candidates or protein drugs. The effect of humanization on the in vivo behavior of proteins has not extensively been studied for proteins other than antibodies.

### 3.6 Albumin binding

Albumin, the most abundant protein in human and murine plasma, displays an extraordinary ligand-binding capacity. The large size of albumin (67 kDa) prevents fast renal filtration and its FcRn affinity further enhances circulation time. As a result, albumin has a serum half-life of ~19 days in humans. 192 Because of its long half-life, albumin can perfectly serve as a carrier in order to extend the half-life and thus target exposure of targeted proteins. Half-life extension can be achieved by conjugating targeted protein drugs to albumin or by introducing high-affinity domains that bind to albumin in vivo. With the introduction of these high-affinity domains, often antialbumin affibodies or VHHs, the size of proteins also increases. As mentioned earlier in this review, an increase may also affect biodistribution of proteins. However, due to the fact that antialbumin affibodies and VHHs are small in size, the effect of increasing the protein size by introducing antialbumin domains is not discussed in this section. HSA conjugation and subsequent biodistribution studies have been performed with affibodies targeting EGFR (177Lu-DO3A-HSA-Z_EGFR:1907) and HER2 (111In-DOTA-HSA-ZHER2:342) in mice. 193,194 At 4 hr after injection blood levels were, respectively, 19.07 ± 1.05% ID/g and 20.11 ± 3.5% ID/g, which are much higher compared to radiolabeled affibodies that do not bind albumin in vivo, typically <2.4% ID/g (Table 5). High blood levels of both HSA-conjugated affibodies resulted in high uptake in normal organs, especially in the liver. High hepatic uptake has been observed with 177Lu-DO3A-HSA-Z_EGFR:1907 (54.93 ± 4.05% ID/g) and 111In-DOTA-HSA-ZHER2:342 (15.56 ± 1.84% ID/g).

Conjugation of a CEA targeting scFv (T84.66) to albumin resulted in a comparable biodistribution pattern in mice. 195 Although exact values were not given, blood levels of 111In-labeled albumin-bound T84.66 were much higher than that of 111In-labeled T84.66 diabody, respectively, ~25% ID/g and ~5% ID/g at 4 hr after injection. Hepatic uptake of 111In-albumin-T84.66 increased in time, plateauing between 18 and 72 hr after injection (~11–12% ID/g). Similarly, tumor uptake increased in time with a maximum uptake of 125I-albumin-T84.66 at 18 hr after injection (22.7 ± 6.0% ID/g). In contrast, maximum tumor uptake of 125I-T84.66 was observed 0.5 hr after injection in the same tumor model (4.9% ID/g).
In addition to the conjugation of protein drugs to albumin, introducing albumin affinity can also extend their half-life. For that reason, a bivalent anti-EGFR VHH has been conjugated to an albumin-binding VHH (αEGFR-αEGFR-αalb).\textsuperscript{197} Biodistribution studies in mice revealed that blood levels of $^{177}$Lu-αEGFR-αEGFR-αalb were higher than $^{177}$Lu-αEGFR-αEGFR. At 6 hr after injection these levels were, respectively, 21.2 ± 2.5% ID/g and 0.06 ± 0.02% ID/g. At 24 hr after injection, in vivo albumin-binding additionally increased tumor uptake (from 3.2 ± 0.6% ID/g to 35.2 ± 7.5% ID/g) and organ uptake. The increase in organ uptake was most pronounced in highly perfused lungs, heart, liver, spleen, kidneys, and skin.

Similarly, conjugation of an albumin-binding VHH (Hle2) to a nontargeted VHH (Irr3) increased the half-life of Irr3 in cynomolgus monkeys.\textsuperscript{198} Introducing albumin-binding capacity increased the half-life from 0.080 days (Irr3) to 4.9 days (Hle2-Irr3), which was estimated to be in the same range of serum albumin in cynomolgus monkeys. The half-life of serum albumin in cynomolgus monkeys was estimated to be ~5.2 days, as was based on a weight of 2.4 kg and the formula: albumin half-life (days) = 3.75 × body weight (kg)$^{0.368}$.\textsuperscript{199}

The above studies have been performed in mice. Given the fact that albumin and HSA half-life in mice (respectively, ~1.5 days and ~5.2 days) differ from that in humans (~19 days), translating these results to a clinical setting is difficult.\textsuperscript{192,198,200,201} Due to the difference in albumin half-life, the effect of albumin binding on the clearance of protein drugs might be more pronounced in humans than in mice. This may also count for biodistribution and tumor uptake, as these parameters are affected by the clearance of protein drugs. However, despite the long half-life of albumin in humans, the half-lives of drugs and drug conjugates that are conjugated to albumin generally do not exceed 5 days in humans.\textsuperscript{202}

In conclusion, albumin binding can increase protein levels in blood, organs, and tumors. The largest increase can be expected in the liver. The effect of albumin binding on the biodistribution of protein drugs may well be more pronounced in humans than in mice, as albumin is excreted less efficiently in humans. These findings may facilitate the interpretation of imaging data obtained with albumin-binding proteins.

### 3.7 PEGylation

Although small protein drugs hold great promise as anticancer agents, they are cleared fast and therefore provide relatively low target exposure. In order to increase circulation time and tumor exposure, drugs can be PEGylated. PEGylation successfully increased the half-life of antibodies, antibody fragments, and non-Ig scaffolds.\textsuperscript{14,185,203–206} In addition, PEGylation can increase solubility, decrease antigenicity, and decrease proteolysis.\textsuperscript{207,208} To date, PEGylation has been used in the FDA- and EMA-approved cancer drugs, including PEGylated doxorubicin liposomes, PEGfilgrastim, PEGasparaginase, PEGintron, and pegaptanib.

The effect of PEGylation on circulation time depends on different PEG characteristics, including chain length and shape. For example, increasing chain length increased the half-life of a Fab targeting an unspecified antigen, as studied in cynomolgus monkeys.\textsuperscript{209} PEGylation with 25 kDa PEG chain resulted in a plasma elimination half-life of 147.3 hr, while PEGylation with a PEG length of 40 kDa resulted in a plasma half-life of 188.9 hr.\textsuperscript{209} Increasing chain length can additionally decrease target affinity, potentially preventing high antigen-specific tumor uptake.\textsuperscript{210,211} However, a reduction in target affinity does not necessarily decrease tumor uptake of PEGylated proteins.\textsuperscript{212}

Like chain length, the shape of PEG chains can influence the effect of PEGylation on circulation time of PEGylated proteins. Despite the fact that total PEG length was similar, the use of branched PEG chains (2 × 20 or 4 × 10 kDa) increased the serum half-life of tumor necrosis factor-alpha targeting VHH more than PEGylation with linear PEG chains (1 × 40 kDa), as studied in mice, rats, and cynomolgus monkeys.\textsuperscript{213}

PEGylation generally promotes uptake in well-perfused organs, except for the kidneys and liver.\textsuperscript{14,204,206} PEGylation can enlarge protein size, decreasing renal excretion. At 2 hr after injection of a $^{125}$I-labeled anti-CEA/CD3 scDb in mice, renal levels of $^{125}$I-scDb were higher than of $^{131}$I-PEG-scDb.\textsuperscript{14} However, PEGylation increased renal uptake of this scDb at later time points (24, 48, and 96 hr after injection), which is likely due to the increase in blood levels and the strong perfusion of the kidneys. At all time points studied (2–96 hr after injection), PEGylation did increase uptake of this scDb in the well-perfused liver. In contrast, PEGylation reduced hepatic uptake of an anti-EGFR antibody
**TABLE 7** Effect of protein modifications in murine models and humans

<table>
<thead>
<tr>
<th></th>
<th>In mice</th>
<th>In nonhuman primates</th>
<th>In human</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EPR effect</strong></td>
<td>Likely more prominent due to faster growing tumors with less mature vasculature</td>
<td>No data available</td>
<td>Likely less present due to slower growing tumors with more mature vasculature</td>
<td>17</td>
</tr>
<tr>
<td><strong>Effect of antibody humanization on its half-life</strong></td>
<td>Humanization may increase clearance rate</td>
<td>Humanization decreases clearance rate significantly</td>
<td>Half-lives correlate with the degree of humanization, in the order of: &quot;fully-rodent&quot; &lt; chimeric &lt; CDR-grafted human &lt; &quot;fully human&quot;</td>
<td>187–189</td>
</tr>
<tr>
<td><strong>Complement component 3 binding of humanized antibodies</strong></td>
<td>Observed in mice for humanized antifibroblast growth factor receptor 4 antibody. Binding reduced serum half-life and target-specific organ uptake</td>
<td>Binding is absent in plasma of cynomolgus monkeys</td>
<td>Binding is absent in human plasma</td>
<td>190</td>
</tr>
<tr>
<td><strong>Half-life of albumin</strong></td>
<td>1.6 days, effect of albumin binding on half-life of proteins is likely less pronounced than in cynomolgus monkeys and humans</td>
<td>~5.2 days for cynomolgus monkeys as based on a weight of 2.4 kg and the formula: albumin half-life (days) = 3.75 × body weight (kg)^0.368; Effect of albumin binding on half-life of proteins is more pronounced than in mice but less compared to human</td>
<td>~19 days, effect of albumin binding on half-life of proteins is likely more pronounced than in mice and cynomolgus monkeys</td>
<td>192,199–201</td>
</tr>
</tbody>
</table>

(Continues)
**TABLE 7** (Continued)

<table>
<thead>
<tr>
<th></th>
<th>In mice</th>
<th>In nonhuman primates</th>
<th>In human</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life of human serum albumin</td>
<td>21 hr in NMRI mice, the effect of conjugation to human serum albumin on half-life of proteins is less pronounced than in cynomolgus monkeys and humans</td>
<td>5.5 days in cynomolgus monkeys, the effect of conjugation to human serum albumin on half-life of proteins is more pronounced than in mice but less compared to human</td>
<td>~19 days, the effect of conjugation to human serum albumin on half-life of proteins is more pronounced than in mice and cynomolgus monkeys</td>
<td>192,219</td>
</tr>
<tr>
<td>Antidrug antibodies</td>
<td>Mice used in oncology research often are immunodeficient. These mice are incapable of producing antidrug antibodies</td>
<td>Nonhuman primates may develop antidrug antibodies after repeated exposure. ADA response can be different than in patients</td>
<td>Human may develop antidrug antibodies after repeated exposure and may present preexisting PEG recognizing antibodies</td>
<td>177,218,220</td>
</tr>
<tr>
<td>Splenic uptake of antibodies</td>
<td>Nonspecific antibody uptake in the spleen of SCID, but not athymic nu/nu, mice can be extremely high (&gt;50% ID/g). Defucosylation could further increase the splenic uptake of antibodies, as observed with 89Zr-RG7116</td>
<td>Nonspecific antibody uptake in the spleen is not expected to be extremely high in cynomolgus monkeys</td>
<td>Nonspecific uptake of 89Zr-RG7116 in the spleen of patients is not extremely high</td>
<td>112,172,375</td>
</tr>
<tr>
<td>Conjugation ratio</td>
<td>Relatively high, potentially resulting in increased tracer levels in liver and spleen and decreased tracer levels in the tumor and blood pool</td>
<td>Relatively low, indirect labeling likely has less impact on biodistribution and pharmacokinetics</td>
<td>Relatively low, indirect labeling likely has less impact on biodistribution and pharmacokinetics</td>
<td>111,112</td>
</tr>
</tbody>
</table>
PEGylation of $^{111}$In-C225 with antibody:PEG ratios of 1:10 and 1:30 decreased liver uptake in mice with, respectively, 38% and 45%. This might be due to the fact that PEG chains potentially mask sites that could be recognized by scavenger receptors in the liver.

Like in well-perfused organs, PEGylation can also increase tumor uptake of protein drugs. This might be the result of both an increased circulation time, resulting in increased exposure to intratumoral antigens, and the EPR effect in tumor tissue. In mice, tumor uptake of a bispecific anti-CEA/CD3 scDb increased upon PEGylation. Tumor uptake of $^{125}$I-PEG-scDb increased in both CEA positive and negative tumors as compared to $^{131}$I-scDb. The fact that $^{125}$I-PEG-scDb uptake was also higher in CEA negative tumors is likely due to an increase in circulation time and due to the EPR effect. In contrast, PEGylation can also decrease tumor uptake as has been observed with the humanized A33 antibody, targeting the A33 antigen in colon cancer. Although PEGylation did not affect half-life or decrease target binding by more than 50%, it negatively affected tumor uptake in mice. Ex vivo staining of these tumors for human IgG demonstrated that PEGylation of A33 antibodies reduced the speed of tumor penetration. This suggests that a decrease in tumor penetration also prevented high tumor uptake.

Although PEGylation generally increases the half-life of proteins, some studies show that PEGylation decreases the half-life of proteins. Conjugation of $^{124}$I-Fab targeting murine tumor-associated glycoprotein-72 with negatively charged branched maleimide-PEG (2887 Da) or neutrally charged maleimide-PEG (4229 Da) decreased its half-life in mice. Interestingly, PEGylation using larger negatively charged maleimide-PEG (4473 Da) increased its half-life. PEGylation of a $^{111}$In-labeled cetuximab also decreased the level of radioactivity in serum of mice. It was suggested this is due to the detachment of $^{111}$In-PEG from cetuximab. Remarkably, a similar study from the same group demonstrated that PEGylation of cetuximab increased blood levels in mice at 48 hr after injection. In both studies DTPA conjugation and subsequent $^{111}$In labeling was performed similarly at the end of the PEG chain.

As reviewed by Verhoef and Anchordoquy, several mechanisms have been suggested that could cause the PEG-dependent increase in serum clearance. The binding to proteins that interact with receptors on cells of mononuclear phagocyte system may promote the clearance of PEGylated proteins by these cells. In addition, the innate immune system may recognize PEG, as this system is known to recognize pathogen-specific repeating structures. Furthermore, human may develop PEG-specific antibodies after repeated exposure to PEG as has been shown in a clinical study with PEG asparaginase. The results of that same study also indicate that 25% of the study population had preexisting PEG recognizing antibodies prior to the study. According to the authors this could be the result of repeated exposure to PEG in cosmetic products that were likely used by the patients.

In conclusion, when interpreting molecular imaging data with radiolabeled PEGylated proteins, one should take into account that PEGylation generally decreases clearance rate, increasing protein drug levels in blood, tumor, and most organs. Both renal and hepatic uptake can either increase or decrease upon PEGylation. For renal uptake, this can be time dependent. The use of branched PEG chains has a more pronounced effect on biodistribution than the use of linear PEG chains. Although counterintuitive, several studies have demonstrated that PEGylation may increase serum clearance. Furthermore, PEGylation may reduce target affinity and tumor penetration resulting in a decrease in tumor uptake.

### 4 CONCLUSIONS AND FUTURE PROSPECTS

To date tumor-targeting antibodies, antibody derivatives, and non-Ig scaffolds have been modified to enhance their therapeutic effect, tumor exposure, and safety profile. Molecular imaging and biodistribution studies have shown that protein modifications, including radiolabeling of protein drugs, can intentionally and unintentionally alter their biodistribution and tumor uptake. Protein modifications often alter the levels of tumor-targeting antibodies, antibody derivatives, and non-Ig scaffolds in blood, liver, spleen, kidneys, and tumor. The impact of protein modifications on the in vivo
behavior of these proteins depend on the type of protein and should therefore be determined for each type of protein drug separately.

Currently, the distribution and tumor uptake of clinically studied protein drugs is increasingly being assessed using molecular imaging. For example, there are currently 24 known recruiting and active nonrecruiting clinical trials in which the biodistribution and tumor uptake of $^{89}$Zr-labeled antibodies is studied (www.clinicaltrials.gov). These clinical studies are often based on preclinical imaging studies. Given dissimilarities, the impact of protein modifications on in vivo behavior of protein drugs can be species or strain specific. For example, labeling, humanization, and albumin binding, as discussed in this review, can affect the biodistribution of protein drugs differently in preclinical mouse models as compared to nonhuman primates and humans (Table 7). As the majority of studies included in this review were performed in the preclinical setting in mice, further research is warranted to determine to what extent the described effects can be extrapolated to the clinical setting. Further research may additionally clarify which preclinical models best approach the clinical setting and which preclinical models should be avoided to study biodistribution and tumor uptake of protein drugs.

Ideally, the effect of protein modifications (including radiolabeling and fluorescent labeling) on the biodistribution of tumor-targeting antibodies, antibody derivatives, and non-Ig scaffolds is known before molecular imaging is implemented in clinical trials. This benefits a scientifically sound interpretation of the data. Such interpretation of the data can be difficult, raising the need for a comprehensive overview of specific protein properties and modifications that can affect biodistribution and tumor uptake of protein drugs. This review may facilitate in addressing this need by providing such an overview. In order to further increase the knowledge about the effects protein modifications have on the biodistribution and tumor uptake of proteins, molecular imaging can be implemented in more clinical trials. Generally, the implementation of molecular imaging in clinical trials can be further optimized. Especially for nuclear imaging it is critical to take into account the need for standardized scanning methods and the costs of molecular imaging.221,222 Furthermore, a good collaboration between academia and industry will expand the incorporation of molecular imaging in clinical trials.

ACKNOWLEDGMENT
Supported by CTMM grant (MAMMOTH) and ERC Advanced grant (OnQview).

ORCID
Frank-Jan Warnders  http://orcid.org/0000-0003-1278-6146
Jos G. W. Kosterink  http://orcid.org/0000-0001-8358-765X

REFERENCES


AUTHOR’S BIOGRAPHIES

Frank-Jan Warnders is a pharmacist and is currently finalizing his PhD at the Department of Clinical Pharmacy and Pharmacology of the University Medical Center of Groningen (Groningen, The Netherlands). The doctoral research focuses on the radiolabeling and fluorescent labeling of non-IgG protein drugs in order to support drug development.

Marjolijn N. Lub-de Hooge is a Hospital Pharmacist and associate professor at the Department of Clinical Pharmacy and Pharmacology, and at the Department of Nuclear Medicine and Molecular Imaging of the University Medical Center Groningen. She obtained her PhD on pharmaceutical aspects and molecular imaging of new receptor targeted drugs. She is an expert in the development, evaluation, and translation of new radiopharmaceuticals and optical tracers to the clinical setting.

Elisabeth G. E. de Vries is Professor of Medical Oncology at the University Medical Center Groningen, Groningen, The Netherlands. She is involved in patient care, teaching, and research. Her focus is on interdisciplinary, translational research, aiming for personalized medicine. Her research lines are aimed at increasing the sensitivity of tumors to anti-cancer drugs, and she uses imaging techniques to support this.
Jos G. W. Kosterink is Professor of Hospital and Clinical Pharmacy at the Department of Clinical Pharmacy and Pharmacology of the University Medical Center Groningen, Groningen, The Netherlands. His scientific interests include translational research of biopharmaceuticals (monoclonal antibodies and peptides), targeted and personalized medicine, and investigating the added value of pharmacotherapeutic and pharmaceutical innovations.