ENGINEERING ANTIBODIES FOR IMPROVED TARGETING OF SOLID TUMORS

by

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ABSTRACT

Monoclonal antibodies have emerged as an important class of cancer therapeutics due to their ability to specifically bind tumor-expressed antigens. Unfortunately, attempts to treat solid tumors with these drugs are often limited by an inability of the antibodies to fully penetrate the tumor tissue, leaving large regions of untargeted and viable cells. The goal of this thesis is to understand the transport phenomena that contribute to poor antibody distribution in tumors, and engineer novel antibody variants with improved targeting properties. Previous studies identified a core set of parameters that impact tumor uptake including antibody size, binding affinity, plasma clearance rate, and cellular catabolism. Here we probe each of these parameters and its effect on tumor penetration using a combination of computational modeling and protein engineering.

In the first part of this thesis, we characterize the cellular internalization kinetics of a series of anti-carcinoembryonic antigen (CEA) antibodies and antibody fragments. We demonstrate that internalization is independent of antibody affinity, stability, and valency, and that the measured rates can be used to mathematically predict antibody penetration distance in tumor spheroids. Next, we examine the effect of antibody size and affinity by developing a computational model of in vivo tumor targeting that incorporates size-dependent trends for capillary permeability, interstitial diffusion, available volume fraction, and plasma clearance. The model predicts that intermediate size antibody fragments (MW ~30 kDa) have the lowest tumor uptake with greater accumulation of small and large proteins. To probe size effects experimentally, we engineered a novel 79 kDa ds(Fv)-Fc antibody fragment that is approximately half the size of an IgG but retains its binding and Fc salvage activity. In mice, the ds(Fv)-Fc fragments are cleared from the plasma more rapidly than IgGs but have similar tumor uptake levels at 24 hours, likely due to higher capillary permeability. In the last section, we develop a series of matrix metalloproteinase (MMP) activatable antibody fragments that bind their target antigen up to 300 times faster following cleavage by the tumor expressed protease MMP-2. We believe that MMP dependent binding should prevent targeting of antigen depots in healthy tissues and further improve tumor specificity.

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Dedicated to my grandfather George Patrick March
who taught me a love of learning and showed me the power of
education to improve your life and the lives of others
# Table of Contents

Abstract............................................................................................................................................. 3

Chapter 1: Introduction ....................................................................................................................... 8
  1.1. Antibodies as cancer therapeutics......................................................................................... 8
  1.2. Engineering antibody properties....................................................................................... 11
  1.3. Poor antibody penetration in solid tumors........................................................................ 13
  1.4. Tumor targeting parameters............................................................................................... 17
  1.5. Thesis overview.................................................................................................................. 23
  1.6. Works cited........................................................................................................................ 24

Chapter 2: Anti-carcinoembryonic antigen (CEA) antibody internalization................................. 33
  2.1. Introduction ........................................................................................................................ 33
  2.2. Methods ............................................................................................................................. 35
  2.3. Results .................................................................................................................................. 40
     2.3.1. scFv production............................................................................................................. 40
     2.3.2. scFv binding and stability........................................................................................... 42
     2.3.3. Net internalization rate constant (ke) measurements............................................... 44
     2.3.4. Antibody surface decay.............................................................................................. 47
     2.3.5. CEA downregulation and surface antibody levels....................................................... 49
     2.3.6. Metabolic turnover of CEA......................................................................................... 51
  2.4. Discussion............................................................................................................................. 52
  2.5. Works cited........................................................................................................................ 57

Chapter 3: Modeling the effects of antibody size and affinity on tumor uptake............................ 61
  3.1. Introduction ........................................................................................................................ 61
  3.2. Methods ............................................................................................................................. 62
  3.3. Results .................................................................................................................................. 68
     3.3.1. Relationship of molecular size to transport parameters ............................................ 68
     3.3.2. Predicted maximum tumor uptake............................................................................. 70
     3.3.3. Time dependence of tumor uptake............................................................................ 73
     3.3.4. Affinity dependence of tumor uptake......................................................................... 76
     3.3.5. Non-binding mediated uptake: The EPR effect......................................................... 78
     3.3.6. Predicted uptake in human tumors........................................................................... 80
  3.4. Discussion............................................................................................................................. 81
  3.5. Parameter definitions........................................................................................................... 85
  3.6. Works cited........................................................................................................................ 86

Chapter 4: Tumor targeting properties of ds(Fv)-Fc antibody fragments.......................................... 90
  4.1. Introduction ........................................................................................................................ 90
  4.2. Methods ............................................................................................................................. 92
  4.3. Results .................................................................................................................................. 98
     4.3.1. Protein production........................................................................................................ 98
     4.3.2. Cell-surface binding titrations.................................................................................... 100
     4.3.3. Radiolabeled antibody biodistribution....................................................................... 101
Chapter 1 – Potential and limitations of antibodies as cancer therapeutics

1.1 – Antibodies as cancer therapeutics

Despite significant improvements in our understanding and treatment of the disease, cancer remains a leading cause of mortality around the world. In 2008 alone, there were 12.4 million cases causing over 7 million deaths (1). Much of the difficulty in treating cancer stems from the fact that the standard, first-line treatments of surgical resection, external beam radiation, and chemotherapy all have significant limitations. Surgery and radiation are often effective at destroying or removing the primary tumor; however, they are typically unable to eliminate metastatic disease that has disseminated throughout the body. In contrast, systemically administered chemotherapeutic agents can target both primary tumors and micrometastases. Unfortunately, most chemotherapeutic agents act fairly non-specifically by inhibiting cell division, thereby causing toxicities in healthy replicating tissues such as hair follicles and the digestive tract.

An ideal cancer agent would therefore be a drug that can be administered systemically but then specifically target and destroy cancer cells only, both in the primary tumor and micrometastases. One approach for increasing tumor specificity is to target proteins or other molecules expressed or overexpressed in the tumor tissue but not on healthy cells. Due to the cellular deregulation involved in neoplastic transformation, cancer cells often exhibit a distinct molecular profile compared to normal tissues. A number of tumor-associated antigens have been identified including epidermal growth factor receptor-1 and -2 (EGFR and HER2), carcinoembryonic antigen (CEA), and A33 among others (2-5). While some of these molecules such as EGFR and HER2 have proliferative or survival roles that can be directly targeted to
prevent tumor growth, others simply act as markers for delivering cytotoxic effectors to the tumor tissue.

The idea of using a molecule with specific affinity for foreign or diseased cells but not healthy tissue to deliver cytotoxic modalities dates back to the 19th century and Paul Ehrlich’s vision of a “magic bullet” (6). Over the last 100 years, this idea has come largely to fruition with the development of monoclonal antibodies. Antibodies are a class of proteins produced by B cells of the immune system that bind foreign cells and antigens with high affinity and specificity. In 1975, Kohler and Milstein developed hybridoma technology that allowed for the isolation and production of single antibody clones (7). Today, monoclonal antibodies with tailored pharmacokinetic and pharmacodynamic properties can be produced with high affinity binding to a diverse array of tumor targets.

In their most common format, the IgG, antibodies are 150 kDa globular proteins consisting of two binding arms and a constant domain (Figure 1.1). Each binding arm includes a variable domain with 6 peptide loops referred to as complementarity determining regions (CDRs) that form the site of molecular interaction with the antigen. There is extensive sequence and structural diversity between the CDRs of different antibody clones, allowing for specific binding to a diverse array of targets. The constant (Fc) domain of the antibody includes binding sites for the neonatal receptor FcRn, FcγR receptors, and Clq (8,9). Fc binding to FcRn mediates salvage from endothelial degradation and reduces antibody clearance from the plasma. Interactions with FcγR receptors and Clq induce recruitment of immune effector cells and drive target cell killing through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (6).
Figure 1.1 – Schematic of IgG antibody structure. Each 150 kDa IgG molecule is a disulfide locked tetramer comprised of two 25 kDa light chains and two 50 kDa heavy chains. The tetramer forms two Fab binding arms, each consisting of a variable domain (Fv) which includes the antigen binding site, and a constant domain (CH1) which provides stability and modularity. The CH2 and CH3 domains comprise the Fc and are the sites of FcRn, FcγR, and C1q binding.

To date, there are nine FDA-approved monoclonal antibodies for cancer treatment with dozens more in clinical trials (10,11). The approved drugs are listed in Table 1.1 along with their target antigen and approved indication. Six of the antibodies are administered as naked molecules, two as radiolabel conjugates, and one as a toxin fusion. While most of the molecules target the cancer cells directly, avastin is an anti-angiogenic agent that reduces blood vessel growth to prevent the transport of oxygen and nutrients to the tumor.

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade name</th>
<th>Target</th>
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<td>Herceptin</td>
<td>HER2</td>
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1.2 - Engineering antibody properties

While antibody drugs are based on naturally occurring proteins, recombinant protein engineering approaches can be used to tailor the physical and functional properties of the molecules. Early therapeutic antibodies were raised in mice and were often immunogenic in human patients. In clinical trials, a significant fraction of subjects developed human anti-mouse antibodies (HAMA) leading to toxicity and poor efficacy (12). To make antibodies less immunogenic, mouse-human chimeras and CDR grafts have been created in which the variable domain or CDR loops of a mouse antibody are fused to the constant domain and framework regions of a human antibody (13,14). Similarly, mouse antibodies can be resurfaced by mutating surface residues in the variable domain framework to their human counterparts (15).

Alternatively, human antibodies can be directly selected from transgenic mice or non-immune human libraries in phage or yeast (16-18).

Early therapeutic antibodies also suffered from low affinity for the target antigen. As discussed below, low affinity can limit antibody retention in the tumor, particularly for small, monovalent antibody fragments. In order to engineer tighter binding antibodies, in vitro affinity maturation approaches have been developed in which an antibody is mutagenized and variants with improved binding properties are selected. During selections, the antibody is displayed on a scaffold such as phage, yeast, or mRNA that links the selected phenotype to the genetic sequence encoding the antibody (19-21). Through multiple rounds of mutagenesis and selection, antibodies can be engineered to monovalent dissociation constants in the pM or even fM range.

While the IgG remains the most commonly used therapeutic antibody format, its large size, slow plasma clearance, and expensive manufacturing in mammalian cells can pose problems for certain applications. As a result, a number of alternative binding formats have been
engineered with unique pharmacokinetic and structural properties. These include fragments of the full IgG such as scFvs (MW ~ 27 kDa), Fabs (50 kDa), minibodies (80 kDa), scFv-Fcs (105 kDa), and various scFv and Fab based multimers (22,23). Additionally, a number of non-immunoglobulin binding scaffolds have been described including DARPinss (14 kDa), Fn3 domains (10 kDa), affibodies (6 kDa), and anticalins (20 kDa), each with unique physical properties (24-27). In particular, DARPin, affibody, and Fn3 domains all lack disulfide bonds, allowing for intracellular activity and high-yield protein production in bacteria. To extend plasma lifetime, these small scaffold proteins and antibody fragments can be fused to Fc domains or linked covalently or non-covalently to albumin (28,29). Antibodies have also been conjugated to nanoparticles (radius = 10-100 nm) for delivery of a variety of cytotoxic and imaging modalities. The pharmacokinetics of the IgG molecule itself can be tuned by mutating the Fc domain to increase or decrease pH dependent binding to FcRn, and thereby increase or decrease serum persistence (30).

While some antibodies such as anti-EGFR IgGs can directly inhibit proliferative signaling by preventing ligand binding or receptor dimerization, most therapeutic antibodies rely on recruiting immune effectors or delivering cytotoxic agents to the tumor. The IgG Fc domain interacts with FcγR receptors expressed by natural killer (NK) cells, neutrophils, mononuclear phagocytes, and dendritic cells to initiate antibody-dependent cellular cytotoxicity (ADCC) responses. By altering the glycosylation structure or amino acid sequence of the antibody Fc domain, FcγR interactions and ADCC can be increased (31,32). Alternatively, antibodies can be conjugated to biological toxins, radiometals, or synthetic drugs (33-35). Direct conjugation of radiometals to the antibody can lead to significant bone marrow toxicity. As a result, pretargeting strategies have been developed in which a bispecific antibody with affinity for the antigen and a
hapten is administered first, followed by dosing of a radiolabeled hapten at a later time point (36). For diagnostic imaging applications, a variety of radiometal and near infrared dyes can be conjugated to antibodies (37).

1.3 - Poor antibody penetration in solid tumors

Despite significant advances and potential for antibody based cancer therapeutics, there remain numerous challenges. In particular, antibody treatment of solid tumors has proven difficult with lower response rates typically observed compared to treating blood cancers or small micrometastatic disease (38). One of the key factors contributing to poor treatment of solid tumors is limited penetration of antibody drugs into the tumor tissue. Several experimental models have demonstrated heterogeneous antibody distribution in bulk tumors with accumulation around the blood vessels and tumor periphery but largely untargeted areas in the center (Figure 1.2). With the exception of long range radioisotopes and other cytotoxic effectors that elicit a bystander effect, these untargeted areas remain viable leading to repopulation of the tumor and potential development of drug resistance.

Figure 1.2 – Examples of heterogeneous antibody targeting in solid tumors. Left: Anti-HER2 scFv (red) penetrates only several cell layers away from a capillary (yellow). 100 µg of scFv was injected into nephrectomized mice with SKOV-3 xenografts (39). Right: Anti-HER2 IgG
was injected into mice with F2-1282 tumors at a dose of 10 mg/kg. The antibody (green) accumulates around blood vessels (red) with large portions of the tumor left untargeted (28).

Antibody targeting of solid tumors is a complex process involving distribution and clearance in the plasma, extravasation across the capillary wall, diffusion and binding in the tumor tissue, and internalization and catabolism by tumor cells. Due to overexpression of VEGF and other pro-angiogenic factors, tumors have abnormal vasculature with tortuous structure, poor blood flow, and regions of high permeability (40). The high permeability leads to plasma protein leakage, which combined with a lack of functional lymphatics, produces high interstitial fluid pressure (IFP) (40). This elevated interstitial fluid pressure in turn eliminates convective flow out of the blood vessels such that antibody extravasation is driven primarily by diffusion (41). The only significant interstitial convection occurs at the tumor periphery where there is an outward oozing flow into the surrounding normal tissue.

Due to the limited convection, antibody extravasation is slow and typically the rate limiting step for tumor uptake. Mathematically, the extravasation rate can be defined as:

\[
\frac{d[Ab]_{tumor}}{dt} = \frac{2PR_{cap}}{R_{Krogh}^2} [Ab]_{plasma}
\]

where \([Ab]_{tumor}\) is the overall antibody concentration in the tumor, \(P\) is the capillary permeability, \(R_{cap}\) is the blood vessel radius, \(R_{Krogh}\) is the tissue radius surrounding each capillary, \([Ab]_{plasma}\) is the antibody concentration in the blood stream, and \(t\) is the time. This equation is based on reference (42), modified for consistency with the definition of \(P\) from references (43,44).

Similarly, the rate of free antibody diffusion within the tumor tissue can be defined as:

\[
\frac{d[Ab]_{tumor}}{dt} = \frac{D}{R_{Krogh}^2} \varepsilon [Ab]_{plasma}
\]
where $D$ is the interstitial diffusivity and $\varepsilon$ is the available volume fraction in the tumor. A ratio of these two rates is defined as the Biot number:

$$Biot = \frac{2PR_{cap}}{De} = \frac{Extravasation\_rate}{Diffusion\_rate}$$

Biot numbers for most antibodies are on the order of 0.005 indicating that interstitial diffusion is ~200 times faster than extravasation, and extravasation is the rate limiting step for tumor uptake. The amount of antibody at the extravascular surface of the tumor can be estimated as $[\text{Ab}]_{\text{surf}} \approx Bi \cdot [\text{Ab}]_{\text{plasma}}$.

Rapid antibody binding also contributes to poor tumor penetration due to a “binding site barrier” phenomenon (45). For most antibodies, binding is much faster than diffusion such that antibody molecules entering the tumor will bind to the first free antigen they encounter. As a result, the only way to penetrate deeper into the tumor tissue is to first saturate all antigen in the preceding layer of cells. It is this phenomenon that produces the sharp boundaries between fully targeted and fully untargeted cells in Figure 1.2.

While slow extravasation and rapid binding reduce antibody penetration, they alone are not sufficient to produce the heterogeneous distribution observed in Figure 1.2. If given enough time and a constant supply of antibody in the blood stream, the binding front will continue to move through the tumor tissue until all antigen is fully saturated. Rather, the incomplete targeting is due to antibody loss from the system through two distinct routes. First, following iv administration, antibodies are cleared from the blood stream, generally through the kidneys for small fragments or liver for full IgGs. As the plasma concentration drops, there is a decrease in the diffusive gradient for antibodies entering the tumor. Second, after binding cell-surface antigens, antibody molecules are internalized and degraded by the tumor cells at a characteristic
rate. At the same time, new antigen molecules come to the surface to bind additional antibodies, providing a continual metabolic sink for antibody loss.

In effect, the extent of antibody penetration in the tumor is determined by a competition between the rates of transport (capillary extravasation, diffusion, and binding) compared to the rates of loss (plasma clearance and cellular internalization). The time scales of these competing processes can be described mathematically by defining a pair of dimensionless groups: a Thiele number ($\varphi^2$) that compares the rate of transport to the rate of loss due to cellular catabolism and a clearance number ($\Gamma$) that compares the rate of transport to loss due to plasma clearance. The terms are defined as:

$$\varphi^2 = \frac{k_c R_{Krogh}^2 \left( \frac{[Ag]}{\varepsilon} \right)}{D \left( [Ab]_{surf} + K_d \right)} = \frac{\text{endocytic rate}}{\text{transport rate}}$$

$$\Gamma = \frac{R_{Krogh}^2 \left( \frac{[Ag]}{\varepsilon} \right)}{\left( \frac{A}{\alpha} + \frac{B}{\beta} \right) D \left( [Ab]_{surf} + K_d \right)} = \frac{\text{plasma clearance rate}}{\text{transport rate}}$$

where $K_d$ is the dissociation constant, $k_c$ is the net cellular internalization rate, $A$, $\alpha$, $B$, and $\beta$ are terms describing biexponential plasma clearance, and the remaining parameters are defined as above (46). In order for saturation to occur out to a distance $R_{Krogh}$, both the Thiele number and clearance number must be less than one (i.e. transport must be faster than both routes of loss). Importantly, both terms must be satisfied in this model, such that antibody loss by either plasma clearance or cellular catabolism can be limiting.

A series of experimental studies have been performed in \textit{in vitro} tumor spheroids to validate the Thiele number predictions. Thurber et al. showed that anti-CEA antibody fragments
fully penetrated tumor spheroids at a concentration sufficient to achieve the $q^2 < 1$ criteria (47). Ackerman et al. demonstrated that the penetration distance for anti-CEA and anti-A33 antibodies could be predicted as a function of antigen concentration $[\text{Ag}]$ and internalization rate ($k_i$) (48). Additionally, a number of more detailed mathematical models have been developed which reproduce many of the same predictions using more rigorous approaches (41,45,49).

1.4 - Tumor targeting parameters

One of the core strengths of the simple time scale model described above is that it clearly defines a set of parameters describing both the tumor and antibody that are predicted to influence tumor penetration. Some of the key parameters are highlighted below, along with a discussion of how they can be manipulated by target selection, dosing regiments, or protein engineering for improved tumor uptake.

**Antibody dose ($[\text{Ab}]_{\text{plasma}}$)**

The simplest approach for increasing antibody uptake and penetration in solid tumors is to administer a higher dose of the drug. Mathematical and experimental studies indicate that the total amount of antibody in the tumor scales linearly with the administered dose at subsaturating concentrations (50-53). This linear dose dependence for total uptake corresponds to a constant value when reported as %ID/g. At doses above saturation, antibody uptake will start to plateau due to a lack of available binding sites and the %ID/g will decrease (52,53). The dose at which saturation occurs depends on a number of parameters and can vary from 15 $\mu$g to several hundred $\mu$g for mouse xenograft models (52-54). Increasing the antibody dose also increases antibody penetration and homogeneity in both *in vitro* spheroids and *in vivo* xenograft models.
models \((47,55,56)\). Mathematically, the penetration distance is expected to scale with the square root of the dose.

Although increasing antibody dose can improve tumor uptake and penetration, there are limitations due to non-specific toxicity and high background signal. While naked antibodies can be administered at very high doses with low toxicity \((57)\), the addition of cytotoxic effectors such as toxins, drugs, or radionuclides significantly lowers the maximum tolerated dose and narrows the therapeutic window. High doses of radiolabeled antibodies, for instance, can induce significant bone marrow toxicity \((58)\). For imaging, the high doses produce background signal in the blood and other well perfused tissues that make it difficult to distinguish the tumor \((22)\). Even the cost of monoclonal antibodies can be limiting at very high doses \((59)\).

\textbf{Antigen concentration \([Ag]\)}

High antigen concentrations reduce antibody penetration into the tumor tissue by creating a larger binding and metabolic sink. In \textit{in vitro} studies, anti-A33 antibodies penetrate significantly further into low A33 antigen expressing SW1222 tumor spheroids compared to high expressing LS174T spheroids \((48)\). In some cases, it may be possible to actively downregulate antigen levels with crosslinking antibodies or chemotherapeutic agents \((60,61)\). While low antigen density leads to greater tumor penetration, it also lowers the degree of antibody accumulation per cell. This may not be a problem for highly toxic molecules such as alpha emitting radionuclides \((62)\), but it can lead to insufficient cell killing with less potent effectors.

Although not specifically addressed in the time scale model, antigen expression in non-neoplastic tissue can pose additional problems by causing non-specific toxicity or altering antibody pharmacokinetics \((63,64)\). Binding to these healthy tissue depots may be reduced by
pre-administering a ‘cold’ dose of antibody lacking the therapeutic moiety to saturate the antigen (65,66). Alternatively, MMP activatable antibodies can be developed as discussed in Chapter 5.

**Permeability (P)**

As defined by the Biot number, transport across the capillary wall is typically the rate limiting step for tumor uptake. Due to high interstitial fluid pressure, antibody extravasation is driven by diffusion through capillary pores. Diffusive permeability is a function of molecular size such that small antibody fragments and scaffolds extravasate more rapidly across the vascular wall than full IgGs (44). Due to the negative charge of the capillary endothelium, positively charged macromolecules also have higher rates of transcapillary flux (67,68).

Another approach for increasing antibody extravasation is to co-administer a second agent that alters the vascular properties of the tumor to increase diffusive permeability or convective flow. For instance, systemic administration of IL-2 peptides fused to tumor targeting antibodies specifically increases the permeability of the tumor vasculature and improves uptake of subsequently administered drugs (69,70). Similarly, pharmacological agents that increase blood pressure or reduce interstitial fluid pressure can produce an increase in perfusion and convection in the tumor (71-73). These effects lead to increased tumor uptake, although the effect is generally transient. In a different mechanism, anti-angiogenic drugs induce vascular “normalization” in which the tumor blood vessels take on a more normal physiology with reduced IFP, increased vessel perfusion, and increased transcapillary convection (74). Vascular normalization can increase the penetration and efficacy of subsequently administered small molecules, although the effect on uptake of larger antibodies is still somewhat unclear (75,76).
Antibody extravasation can also be increased by utilizing molecules that are actively transcytosed across the blood vessel wall through specific interactions with endothelial receptors. An anti-lung caveolae antibody, for instance, is rapidly and specifically transported into the lung following systemic administration (77). Similarly, there is evidence that albumin is actively transcytosed through interactions with endothelial receptors (78,79), which may explain the impressive tumor uptake of albumin formulated Taxol and antibody fragments fused to albumin binding peptides (28,80). C-end peptides that bind neuropilin-1 also extravasate and penetrate tumors effectively, although the mechanism for this effect is somewhat unclear (81).

**Void fraction (ε), Diffusivity (D)**

Both the interstitial void fraction and diffusivity are a function of the physiological structure of the tumor, in particular the extracellular matrix (ECM), as well as the size and charge of the targeting molecule (82). Small targeting molecules have faster rates of diffusion and higher available volume fractions as they are better able to fit through the extracellular matrix network (83,84). Charge can also impact these parameters as negatively charged species have lower available volume fractions and slower diffusivity due to electrostatic repulsion from the negatively charged ECM (85,86). Tumor treatment with collagenase or other enzymes that degrade the extracellular matrix can lead to increased diffusion and penetration of IgGs (87).

**Affinity (Kd, kon, koff)**

Following extravasation, antibodies typically bind to the first free antigen they encounter as binding is faster than diffusion. Once bound, antibodies will be irreversibly immobilized if their dissociation rate (k_{off}) is slower than internalization (k_e), as is generally the case for bivalent
IgGs and other high affinity ligands (88). In contrast, low affinity targeting molecules \((k_{\text{off}} > k_e)\) are able to dissociate prior to cellular catabolism and diffuse further into the tumor tissue. As a result, low affinity antibodies typically have a more homogeneous distribution in the tumor as demonstrated both mathematically and experimentally \((39, 49, 55, 89, 90)\).

Although low affinity antibodies are able to penetrate further into the tumor tissue, it comes at the cost of decreased antibody loading per cell and potentially decreased tumor retention. For antibodies approaching homogeneous distribution in the tumor, the fractional saturation of antigen on each cell can be estimated at steady-state as:

\[
f = \frac{[Ab]_{\text{surf}}}{k_{\text{off}} + k_e} \frac{k_{\text{on}}}{k_{\text{on}} + [Ab]_{\text{surf}}}
\]

Therefore, the tradeoff is between fully saturating a fraction of cells with a high affinity antibody or saturating a fraction of antigen on all cells with a low affinity antibody. The relative effectiveness of each approach will depend on the potency and range of the cytotoxic effector. Low antibody affinities may also suffer from decreased tumor retention as they are cleared from the tumor by intravasation while in the unbound state. High affinity antibodies often have higher levels of total tumor uptake \((39, 90-93)\), although this effect plateaus at high affinities and is dependent on molecular size as described in Chapter 3.

**Plasma clearance \((A, B, \alpha, \beta)\)**

As discussed above, rapid clearance of antibody molecules from the plasma limits penetration into the tumor by reducing the diffusive concentration gradient. The rate of plasma clearance is largely a function of size as smaller proteins such as antibody fragments and other scaffolds are cleared quickly through the kidneys, reducing tumor uptake. There is no exact
cutoff at which kidney clearance becomes dominant, but it is generally significant for molecules less than 60-70 kDa in size (94). Conjugation of polyethylene glycol (PEG) chains to small antibody fragments has been shown to increase hydrodynamic radius, reduce plasma clearance, and increase tumor uptake in a number of studies (95,96). The increase in size, however, also has negative consequences in terms of reduced permeability, diffusivity, and void fraction as discussed above. A detailed analysis of these tradeoffs is described in Chapter 3.

IgGs have extended serum persistence due to interactions between the Fc domain and FcRn receptors on endothelial cells. Following antibody uptake by pinocytosis, the Fc domain binds to FcRn molecules in a pH dependent manner such that antibodies are trafficked back to the cell surface, escaping lysosomal degradation (97). Due to this slow plasma clearance profile, IgGs typically have higher tumor uptake than similarly sized molecules lacking the Fc. Fc mutations which increase pH dependent binding to FcRn can further increase serum persistence, while those that disrupt the interactions cause faster clearance (8,98). As predicted by the time scale modeling, mutations which drive faster clearance lead to lower tumor uptake (99). Albumin and albumin fusions are also able to bind FcRn in a pH dependent manner (100,101) leading to extended serum persistence and high tumor uptake (28,102).

**Cellular internalization (k_e)**

Cellular internalization and catabolism of bound antibodies is a significant barrier to antibody penetration and retention as it creates a constitutive route of antibody loss within the tumor. Experimental and mathematical studies have demonstrated that antibodies with slower internalization kinetics penetrate significantly further into the tumor tissue and are retained for a longer period of time (47-49). The antibody internalization rate depends primarily on the
trafficking kinetics of the target antigen and can vary from half times on the order of minutes for clathrin coated vesicles to days for tight junction proteins like A33 (103-105). As predicted by the modeling, antibodies against A33 penetrate significantly farther into tumor spheroids than those against more rapidly internalized targets (48). Net antibody internalization can also be reduced by efficient recycling of the antibody back to the cell surface following endocytosis (106). While slow internalization is desired for improved tumor penetration and retention, certain cytotoxic effectors such as toxins may require internalization to kill the cell. These trade-offs between pharmacokinetics and pharmacodynamics have been analyzed by computational modeling (107).

1.5 - Thesis overview

In this thesis, we attempt to understand and manipulate the transport parameters described above with the goal of increasing antibody uptake and penetration in solid tumors. In Chapter 2, we measure the internalization rate ($k_e$) of anti-carcinoembryonic antigen (CEA) antibodies and demonstrate that this rate can be used to predict tumor penetration in in vitro tumor spheroids. In Chapter 3, we develop a computational model to predict how antibody size and affinity impact tumor uptake in vivo. The model analyzes the size-dependent tradeoffs between diffusivity, void fraction, plasma clearance, and capillary permeability. In Chapter 4, a novel 79 kDa, monovalent ds(Fv)-Fc antibody fragment is described, and its in vivo targeting properties compared to an IgG to examine the effects of size and valency. Finally, in Chapter 5, matrix-metalloproteainase activatable antibodies are engineered as a strategy to avoid binding to antigen in healthy tissue.

1.6 - Works Cited


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Chapter 2 – Kinetics of anti-carcinoembryonic antigen (CEA) antibody internalization

2.1 - Introduction

The transport of antibodies into solid tumors is a complex process involving plasma clearance, capillary extravasation, interstitial diffusion, antigen binding, and cellular catabolism. After binding to cell-surface antigens, antibodies are internalized by tumor cells at a constitutive rate and trafficked to the lysozome for degradation. At the same time, newly synthesized antigen molecules refill the surface pool, binding and internalizing additional antibody molecules to produce a constitutive route of antibody clearance. For most cell surface-bound antibodies, endocytic uptake is more rapid than dissociation such that binding is effectively irreversible and antibody catabolism is the dominant route of clearance from the tumor (1).

Theoretical analyses suggest that internalization significantly impacts the penetration and distribution of antibodies in the tumor tissue. According to the timescale model developed by Thurber et al., incomplete penetration will occur if the rate of antibody loss due to internalization is faster than the rate of antibody transport. This situation is defined mathematically as a Thiele number greater than one \((\psi^2 > 1)\) (2). Cellular internalization also influences tumor retention and pharmacodynamics of antibody drugs. Slowly internalized antibodies persist at the cell surface making them ideal agents for ADCC and multi-step pre-targeting therapies. In contrast, rapidly internalized antibodies may be preferred for delivery of therapeutic moieties that act intracellularly such as toxins and siRNA.

In order to select antibodies with appropriate trafficking kinetics for a given therapeutic application, it is important to understand how the physical properties of the antigen and antibody influence the net internalization rate \((k_e)\). In general, the rate of antibody internalization depends primarily on the cellular localization and trafficking properties of the target antigen. Antigens
associated with clathrin coated vesicles or caveolae are internalized rapidly with uptake half times on the order of minutes (3). In contrast, bulk non-specific uptake of cell surface markers occurs with a half time of hours, and tight junction proteins like A33 persist on the surface for days (4,5). Following endocytosis, antibodies can be recycled back to the cell surface, reducing the net-internalization rate (6). Alternatively, binding of multivalent antibody constructs alone or in combination may drive faster antigen internalization through clustering (7).

Carcinoembryonic antigen (CEA) is a 180 kDa GPI linked cell-surface glycoprotein normally expressed in the fetal gut and on the lumenal surface of the adult colon (8). During colorectal carcinoma oncogenesis, CEA loses its polarity and becomes overexpressed throughout the tumor tissue. High levels of CEA expression have also been observed in epithelial tumors in the lung, breast, thyroid, and ovaries (8). Due to this selective tumor overexpression, antibodies against CEA have been investigated as targeting agents for a number of imaging and therapeutic approaches including SPECT and PET imaging, pretargeted radioimmunotherapy, and ADEPT (9-12).

Although CEA is often referred to as a shed or non-internalizing antigen (13-15), there have been sporadic reports that antibodies and immunoconjugates against CEA are in fact slowly internalized by CEA expressing tumor cells (16-18). However, these studies have lacked: (1) quantitative measurement of the bound antibody internalization rate constant (k_e) and (2) systematic study of how antibody properties such as affinity, stability, and valency influence this rate. These limitations are addressed in the current study in which the internalization rates of anti-CEA antibodies are quantified in CEA expressing cell lines using a flow cytometry based internalization assay. Additionally, the internalization rates of anti-CEA agents differing in
affinity, stability to protease degradation, valency, and target epitope are compared to understand whether the physical properties of the antibody impact net uptake.

2.2 Methods

Materials and cell lines

Anti-CEA IgGs M85151a and M111147 were purchased from Fitzgerald (Concord, MA). LS174T cells were obtained from ATCC (Rockville, MD). LIM1215 and SW-1222 cells were the kind gift of the Ludwig Institute. Cells were grown in minimum essential media (MEM) supplemented with 10% FBS and 1% Penicillin-Streptomycin. CEA expressing HT-1080 cells (HT-1080-CEA) were created by transfecting HT-1080 cells with the pIRES-CEA plasmid (kind gift of Dr. G. Prud’homme, St. Michael’s Hospital, Toronto, Canada) using lipofectamine according to manufacturer’s instructions. Transfected cells were selected by growth for 7 days in MEM with 0.75 mg/mL G418.

Antibody fragment production

Secretion vectors for shMFE and sm3E containing a c-terminal His tag have been described previously (19). Disulfide stabilized scFvs were produced by mutating residues R44 and G234 to cysteine using the QuikChange kit (Stratagene) according to manufacturer’s instructions. Mutations were confirmed by sequencing. Plasmids were transformed into the YVH10 strain of yeast using the EZ Yeast Kit (Zymo Research) and plated on SD-CAA media supplemented with 40 µg/mL tryptophan. Individual colonies were grown in 1 L flasks and secretion induced for 48 hours at 37°C as described previously (19). The cleared supernatant was concentrated using Millipore 10 kDa ultrafiltration membranes and the His-tagged proteins purified using BD Talon
metal affinity resin following the manufacturer’s batch-column protocol. Eluted proteins were further purified by anion exchange chromatography on a Hi-Q column equilibrated with 20 mM Tris buffer at pH 8.25 and size exclusion chromatography on a Superdex 75 column (GE Healthcare). Samples were run on a 12% Bis-Tris gel with or without 100 mM DTT and Coomassie stained to check size and purity. Fab fragments were produced from IgG M85151a by papain digestion using the ImmunoPure Fab Preparation Kit (Pierce) according to manufacturer’s instructions. Purified antibody fragments were conjugated to Alexa-488 fluorophores using the Microscale Protein Labeling Kit (Invitrogen) following manufacturer’s instructions. Protein concentration and degree of fluorophore labeling were determined from UV absorbance at 280 and 490 nm as described in the Microscale Kit. The immunoreactive fraction was estimated by incubating scFvs with a five fold molar excess of soluble CEA (Fitzgerald) and determining the fraction of antibody that elutes as a complex on size exclusion chromatography.

**Linker cleavage and protease stability**

The (Gly\textsubscript{3}Ser\textsubscript{3}) linker connecting the V\textsub{H} and V\textsub{L} domains of the scFvs was cleaved by incubating the antibody fragments with 0.02 Units/mL subtilisin in digestion buffer (20 mM Tris-HCl, 5 mM calcium chlorate, pH 7.5) for 90 minutes at 37°C. Digested samples were run on a 12% Bis-Tris gel with or without 100 mM DTT and Coomassie stained. For functional protease stability assays, Alexa-488 labeled scFvs were incubated with increasing concentrations of subtilisin in digestion buffer for 60 minutes at 37°C. Trypsinized LS174T cells were labeled with the digested antibody fragments at subsaturating concentrations for 20 min on ice and mean cellular fluorescence measured on an EPICS Coulter XL flow cytometer (Beckman Coulter, Inc.)
Cell-surface binding

Trypsinized LS174T cells were fixed with Cytofix Buffer (BD Biosciences) for 20 minutes at 4°C to prevent antibody trafficking. For $K_d$ measurements, fixed cells were incubated with Alexa-488 labeled antibodies or antibody fragments at a range of concentrations for 16-20 hours at 37°C. To avoid antibody depletion effects, a 10 fold molar excess of antibody over antigen was maintained throughout. Labeled cells were washed twice with PBS + 0.1% BSA, and the mean Alexa-488 cellular fluorescence measured on an EPICS Coulter XL cytometer. The normalized signal was fit to the equation $MFU = B_{max} ([Ab]/([Ab]+K_d))$ to determine the $K_d$.

For $k_{off}$ measurements, fixed cells were labeled overnight with saturating concentrations of Alexa-488 conjugated scFvs. Cells were washed and resuspended at 37°C in PBS-BSA containing 100 nM unlabeled sm3E to prevent rebinding of dissociated antibodies. At each time point, cells were washed and the mean fluorescence quantified by flow cytometry. Fluorescence values were normalized by the signal at $t = 0$ and fit to an exponential decay to determine the $k_{off}$.

The number of antibodies bound per cell at saturation was determined by labeling cells with saturating concentrations of Alexa-488 conjugated antibodies or antibody fragments and converting the fluorescence intensity to a number of molecules using Alexa-488 calibration beads and the calculated number of fluorophores per antibody.

Net antibody internalization assays

LS174T cells were subcultured into 96 well plates at a concentration of $10^5$ cells per well and allowed to adhere for 12-16 hours. After washing cells once in media, 125 μL of Alexa-488 conjugated antibodies or antibody fragments at a concentration of 20 nM in MEM + FBS was added to each well. Cells were incubated at 37°C in the continuous presence of the labeled
antibodies. At each time point, plates were chilled, wells washed twice in PBS-BSA, and cells lifted by incubating with 200 μL cell dissociation buffer (Gibco) for 10 minutes on ice.

Dissociated cells were transferred to microfuge tubes by pipetting and sedimented at 14000 x g. Cell pellets were resuspended in PBS-BSA with or without 25 μg/mL anti-Alexa-488 quenching IgG (Molecular Probes) for 25 minutes on ice. The mean fluorescence of the surface quenched and unquenched cells were measured on an XL cytometer and used to calculate the relative amounts of surface and internal fluorescence as described previously (6). In brief, surface fluorescence was determined as (unquenched MFU – quenched MFU)/ε where ε is the quenching efficiency determined by comparing the quenched and unquenched signals of cells that had been labeled briefly on ice to prevent internalization. The internal fluorescence was then calculated as Total MFU – Surface MFU. Non-specific internalization of antibodies due to fluid phase uptake was measured using cells where the CEA was pre-blocked with unlabeled antibody and subtracted from the internal signal. The corrected internal MFU values were plotted against the integral of the surface fluorescence determined using the trapezoidal rule and fit to a linear curve, the slope of which is the internalization rate ke (3).

scFv uptake experiments in the LIM1215, SW-1222, and HT-1080-CEA cell lines were performed essentially as described above except trypsin-EDTA was used in place of cell dissociation buffer to lift the cells from the plates at each time point.

Surface decay

LS174T cells subcultured in 96 well plates as above were surface labeled with saturating concentrations of Alexa-488 labeled sm3E, ds-shMFE-M, or M85151a IgG for 1 hour on ice. Unbound antibody was washed from each well and cells were incubated in media at 37°C. At
each time point, cells were chilled and transferred to microfuge tubes as described above. Cells were then surface labeled on ice with PE conjugated secondary and tertiary antibodies to determine the amount of anti-CEA antibody remaining on the surface. Goat anti-mouse PE (1:50 dilution) was used for M85151a and anti-His biotin (1:70 dilution) followed by streptavidin-PE (1:100 dilution) for the scFvs. Cells were analyzed by flow cytometry to measure the 488 signal (total cell associated antibody) and PE signal (surface accessible antibody).

**CEA downregulation**

LS174T cells subcultured into 96 well plates as above were incubated at 37°C in media with or without 50 nM unlabeled anti-CEA antibodies or antibody fragments. At each time point, cells were chilled, washed twice with cold CO₂ independent media, and labeled for 40 minutes on ice with 20 nM of a non-competitive Alexa-488 labeled anti-CEA antibody. Cells were then washed, lifted with cell dissociation buffer, and analyzed by flow cytometry as above. The Alexa-488 signal of cells incubated with unlabeled antibody was normalized by the signal of cells incubated with media alone to determine the degree of antigen downregulation.

**Biotinylated CEA turnover**

LS174T cells were subcultured into 12 well plates at a density of 2 x 10⁶ cells per well and grown for 24 hours at 37°C. Cells were washed and surface biotinylated with 1 mg/mL NHS-SS-biotin (Pierce) in PBS, pH 8.0. The labeling reaction was quenched after 30 minutes by the addition of 100 mM Tris-HCl. The cells were then washed twice in media and incubated at 37°C. At each time point, cells were placed on ice, washed twice with PBS-BSA, and incubated in 500 μL of ice cold lysis buffer for 10 minutes. The cell lysate was cleared by centrifuging at
14000 x g for 15 minutes and biotinylated proteins pulled down with streptavidin resin (Pierce). The bound proteins were washed and eluted by cleaving the disulfide linker with 100 mM DTT. Eluted samples were run on a 4-12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted for CEA using M85151a IgG (1:1000 dilution) and goat anti-mouse-HRP secondary (1:1000 dilution). Band intensities at each time point were quantified using QuantityOne software (Bio-Rad) and normalized by the band intensity at time zero.

2.3 – Results

The internalization rate of cell bound antibodies and antibody fragments is predicted to significantly alter the microdistribution, surface accessibility, and retention time of these molecules in tumor tissue. Therefore, we set out to quantitatively measure the internalization of antibodies and antibody fragments directed against the common tumor target CEA. Furthermore, we examined how antibody parameters such as affinity, stability to protease digestion, valency, and target epitope influence these internalization rates.

2.3.1 - scFv production

As a model system to examine the effect of antibody properties on cellular uptake, we developed a series of anti-CEA single-chain variable fragments (scFvs) with differences in affinity, stability to protease degradation, and valency. scFvs are ~27 kDa antibody fragments consisting of the VH and VL domains of an IgG connected by a peptide linker. Previous affinity maturation work resulted in two scFvs sm3E and shMFE that bind to the same epitope on CEA with a nearly 300 fold difference in affinity (sm3E $K_d = 30$ pM, shMFE $K_d = 8.5$ nM) (19). To make fragments with increased stability, cysteine residues were inserted into the VH and VL
domains of the proteins to form an interdomain disulfide bond. The formation of this interdomain disulfide has been shown to increase the protease and thermal stability of scFvs (20,21). Additionally, the disulfide stabilized scFvs (ds-scFvs) are secreted in yeast as a mix of disulfide locked monomers (ds-scFv-M) and dimers (ds-scFv-D) which can be separated to provide fragments with differences in valency.

Secreted antibody fragments were concentrated and purified at a final yield of 0.5-1 mg/L. All proteins matched expected sizes when run on non-reducing and reducing SDS-PAGE gels and demonstrated purity greater than 99% (Figure 2.1A). The slightly increased mobility of the disulfide stabilized monomers compared to the native scFvs under non-reducing conditions is indicative of interdomain disulfide bond formation, which produces a more compact denatured structure. Formation of the interdomain disulfide was further confirmed by proteolytically cleaving the peptide linker connecting the $V_H$ and $V_L$ domains of the scFvs and assaying protein mobility on SDS-PAGE. Following linker cleavage, the $V_H$ and $V_L$ domains of the disulfide stabilized scFvs are held together under non-reducing conditions by the interdomain disulfide bond (Figure 2.1B).
Figure 2.1 — Antibody fragment production in yeast. A: SDS-PAGE analysis of scFvs under non-reducing (top) and reducing (bottom) conditions. Purified scFvs were run on a 12% Bis-Tris gel with or without 100 mM DTT. All antibody fragments run at their expected molecular weights and are ~99% pure. B: Cleavage of peptide linker suggests that interdomain disulfide bond is formed in ds-scFvs. The peptide linker connecting the V_H and V_L domains of each scFv was cleaved with low concentrations of subtilisin. Following linker cleavage, the two domains of the disulfide stabilized fragments are held together under non-reducing conditions by the interdomain disulfide bond such that the protein runs at ~27 kDa.

2.3.2 - scFv binding and stability

Antibody affinity and avidity was assessed by measuring the binding of Alexa-488 conjugated antibody fragments to fixed LS174T cells (Table 2.1). Both disulfide locked monomers exhibit dissociation constants (30 pM for ds-sm3E-M and 9.2 nM for ds-shMFE-M) similar to those measured previously for sm3E and shMFE by yeast surface display (19). In contrast, the bivalent molecule ds-shMFE-D has a roughly 100 fold higher apparent affinity (85 pM) than its monomeric counterpart due to avidity from bivalently binding to cells. Interestingly, the non-disulfide stabilized shMFE also exhibits a higher affinity than ds-shMFE-M, likely due to the formation of non-covalent dimers in solution. The formation of such
reversible dimers has been observed previously for a closely related scFv MFE-23 (22). sm3E and ds-sm3E-D demonstrate less significant increases in apparent affinity compared to their monomeric counterpart ds-sm3E-M, although this is likely due to the high affinity of the monovalent interaction rather than an absence of bivalent binding. Off-rate measurements in the presence of unlabeled competitor support the conclusion that both disulfide stabilized dimers and native scFvs bind to the cell bivalently as their dissociation constants were ~2-3 fold slower than those of the disulfide locked monomers (Table 2.1). In the absence of competitor, dissociation rates for these bivalent antibody fragments would be significantly slower due to continued rebinding of the two arms (23). The high affinity scFvs were also >95% functional as determined by soluble CEA binding assays as described in the materials and methods (data not shown).

The contribution of the interdomain disulfide to functional scFv stability was assessed by incubating the antibody fragments in increasing concentrations of the protease subtilisin, then measuring binding to LS174T cells at subsaturating concentrations. As expected, the disulfide stabilized proteins displayed significantly higher binding activity following protease treatment than the native scFvs suggesting improved stability (Figure 2.2). As a whole, the in vitro cell binding assays confirmed that the secreted scFvs exhibited the expected differences in affinity, stability to protease digestion, and valency, and were therefore a useful model system for the effects of these parameters on net internalization.
<table>
<thead>
<tr>
<th>scFv</th>
<th>$K_d$ (pM)</th>
<th>$k_{off\text{ compet}}$ (min$^{-1}$)</th>
<th>$t_{1/2\text{ off compet}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sm3E</td>
<td>26 ± 4</td>
<td>7.5 x 10$^{-3}$</td>
<td>153.8 hours</td>
</tr>
<tr>
<td>ds-sm3E-M</td>
<td>30 ± 5</td>
<td>2.4 x 10$^{-4}$</td>
<td>48.1 hours</td>
</tr>
<tr>
<td>ds-sm3E-D</td>
<td>9.6 ± 0.7</td>
<td>9.6 x 10$^{-5}$</td>
<td>120.1 hours</td>
</tr>
<tr>
<td>shMFE</td>
<td>160 ± 24</td>
<td>6.6 x 10$^{-2}$</td>
<td>10.5 min.</td>
</tr>
<tr>
<td>ds-shMFE-M</td>
<td>9300 ± 3300</td>
<td>1.6 x 10$^{-1}$</td>
<td>4.3 min.</td>
</tr>
<tr>
<td>ds-shMFE-D</td>
<td>85 ± 5</td>
<td>8.0 x 10$^{-2}$</td>
<td>8.7 min.</td>
</tr>
</tbody>
</table>

Table 2.1 – Binding constants for Alexa-488 labeled anti-CEA antibody fragments on fixed LS174T cells

Figure 2.2 – The interdomain disulfide bond increases functional protease stability of ds-scFvs. Alexa-488 labeled anti-CEA scFvs with or without the interdomain disulfide bond were incubated with increasing concentration of subtilisin and used to label fixed LS174T cells at subsaturating concentrations. The disulfide stabilized fragment maintains significantly greater binding activity in the presence of the protease.

2.3.3 - Internalization rate constant ($k_e$) measurements

The cellular net internalization rate constant ($k_e$) of each scFv was assayed using a fluorescence quenching protocol, similar to one described previously for Herceptin endocytic trafficking (6). Adherent LS174T cells were incubated at 37°C in the continuous presence of Alexa-488 labeled antibody fragments. At each time point, cells were chilled, lifted, and ½ of the cells surface quenched with anti-Alexa-488 IgG. The efficiency of surface quenching was independently measured for each antibody fragment using cells that had been labeled briefly on ice to prevent internalization, and ranged from 84-91%. The fluorescence intensity of quenched and unquenched samples was quantified by flow cytometry and the surface and internal
fluorescence calculated as described in materials and methods. In general, the surface fluorescence increases over the first hour and then plateaus as antibody binding reaches equilibrium, while the internal fluorescence continues to increase over time as antibodies are endocytosed and the fluorophores retained in the cell (Figure 2.3A). Unlike fluorescein, Alexa-488 fluorescence is not quenched at intracellular pH so there is no significant decrease in signal intensity of endocytosed fluorophores. Non-specific uptake was measured using cells that had been pre-blocked with unlabeled sm3E and was generally found to be low at the experimental antibody concentrations.

Internalization rates were derived from the quenched fluorescence data by plotting the internal fluorescence against the time integral of the surface fluorescence and applying a linear fit (Figure 2.3B). Uptake rates were measured in this manner for all scFvs and are reported as internalization half times in Figure 2.4 (where $t_{1/2} = \ln(2)/k_e$). In general, internalization of the anti-CEA scFvs is slow with half times ranging from 10-16 hours. Surprisingly, neither antibody affinity, stability to protease digestion, nor valency significantly affect the internalization rate of bound antibody fragments in this assay as there is no significant difference in uptake half times among the scFvs.

The fluorescence quenching protocol was also used to measure the uptake rates of a pair of commercially available anti-CEA IgGs M111147 and M85151a. These antibodies bind to different (non-competing) epitopes on CEA from the scFvs and each other (data not shown) and have functional affinities of 70 pM for M111147 and 7 pM for M85151a as measured by cell surface titration. While M111147 was internalized with a half time of 13 hours, similar to the scFvs, M85151a was taken up significantly faster with a half time of 5 hours (Figure 2.4A). A monovalent Fab fragment of M85151a with a functional affinity of 2.5 nM was internalized with
a net uptake rate ($t_{1/2} \sim 14$ hours) significantly slower than the IgG, suggesting that the bivalency of the full antibody is necessary for its faster uptake (Figure 2.4A).

The effect of cell type on anti-CEA antibody fragment internalization was assessed by measuring the internalization of the high affinity scFv ds-sm3E-M in 3 additional cell lines. LIM1215 and SW-1222 are colon carcinoma cell lines that express CEA at lower levels than LS174T, while HT-1080-CEA is a fibrosarcoma cell line transfected with a CEA expression plasmid resulting in antigen overexpression. LIM1215 and SW-1222 internalized ds-sm3E-M with half times of 11.5 and 17.2 hours, respectively, both similar to the 15.3 hour half time measured in LS174T cells (Figure 2.4B). In contrast, HT-1080-CEA cells internalized the scFv at a significantly faster rate with a net uptake half time of 4 hours.

Figure 2.3 - Net internalization assay. A: LS174T cells were continuously incubated at 37°C in the presence of Alexa-488 labeled antibodies. Total cellular fluorescence was measured at each time point by flow cytometry and the internal and surface fractions determined by surface quenching with an anti-Alexa-488 IgG. Non-specific uptake was measured by pre-blocking CEA with a 100 fold excess of unlabeled antibody. Data pictured is for an individual experiment with ds-sm3E-D. B: Derivation of internalization rate $k_e$. Internal fluorescence values are plotted against the integral of the surface fluorescence determined by the trapezoidal rule and fit to a linear curve. The slope of the linear fit is the internalization rate $k_e$. Data points pictured are pooled from 4 separate experiments with ds-sm3E-D.
Figure 2.4 – Net internalization rates. A: Internalization rates were determined for all antibodies in LS174T cells and plotted as a half time for antibody net uptake. With the exception of IgG M85151a, all tested antibodies are internalized slowly with a half time of 10-16 hours. IgG M85151a is internalized significantly faster with a half time of ~5 hours (p < 0.001). Half times are the average of 3-6 individual experiments for each antibody and error bars are SD. B: Cellular internalization rates of Alexa-488 labeled ds-sm3E-M were determined in multiple CEA expressing cell types. Colon carcinoma lines LIM1215 and SW-1222 internalize the scFv at similar rates as LS174T, while HT-1080 fibrosarcoma cells transfected with a CEA expression plasmid internalize the antibody fragment more rapidly.

2.3.4 - Antibody surface decay

Antibody internalization was also measured in the context of a pulse labeling experiment that better simulates the retention phase of tumor targeting. LS174T cells were surface labeled on ice with Alexa-488 conjugated anti-CEA scFvs or IgG, washed to remove unbound molecules, and incubated at 37°C. At each time point, cells were surface labeled on ice with PE-conjugated anti-mouse or anti-His secondary antibodies, and the 488 and PE signals measured by flow cytometry to determine the total cell associated and surface antibody, respectively. The high affinity scFv sm3E exhibits slow decay of both the total antibody and surface accessible fluorescence (Figure 2.5A). The decrease in total signal may be due to
antibody dissociation, antigen shedding, or cellular efflux of degraded fluorophore. The surface signal drops faster than the total signal over the course of several hours, indicating a slow internalization of scFv in rough agreement with the \( k_e \) values measured in the continuous uptake experiments. In contrast to sm3E, the surface level of M85151a IgG drops rapidly over the first 3 hours to approximately 50% of its initial level while the total signal remains virtually unchanged, suggesting a more rapid internalization as observed in the continuous uptake experiments (Figure 2.5B). The slow loss of Alexa-488 signal from the rapidly internalized IgG suggests that intracellular antibody degradation and cellular efflux of degraded fluorophore is relatively slow and the more rapid decrease in total signal for sm3E is primarily driven by scFv dissociation or antigen shedding. The low affinity scFv ds-shMFE-M dissociates completely from the cell surface before any significant internalization takes place (data not shown).

Figure 2.5 - Antibody surface decay. LS174T cells were surface labeled with Alexa-488 conjugated antibodies on ice then chased at 37°C. At each time point, cells were surface labeled with a PE conjugated secondary antibody and the Alexa-488 signal (total antibody) and PE signal (surface antibody) measured by flow cytometry. The difference between the total and surface antibody pools represents internalized antibodies. The high affinity scFv sm3E (A) is slowly endocytosed from the cell surface while IgG M85151a (B) displays a more rapid decrease in surface antibody levels due to faster internalization.
2.3.5 - CEA downregulation and surface antibody levels

Antibodies against EGFR and other cell surface proteins have been shown to downregulate surface levels of their target antigen following binding which may have significant effects on antibody microdistribution and pharmacodynamics (7,24). To determine if anti-CEA antibodies are also capable of downregulating their target antigen, LS174T cells were incubated with saturating concentrations of unlabeled antibodies or antibody fragments at 37°C and the surface CEA concentration determined at each time point by labeling the cells on ice with an Alexa-488 labeled non-competitive anti-CEA antibody. Both the monovalent and bivalent high affinity scFvs (ds-sm3E-M and ds-sm3E-D), as well as the slowly internalized IgG M111147, have no effect on the surface levels of CEA (Figure 2.6). In contrast, incubation with the rapidly internalized IgG M85151a induces a 20% decrease in surface CEA levels that is sustained to 5 hours. This downregulation is not observed when the cells are incubated with a monovalent Fab fragment of the antibody suggesting that the activity is valency dependent.

Antigen concentration and the number of antibody molecules bound per cell at saturation may also influence the tumor microdistribution of antibodies (25,26). To quantify these parameters, the cellular fluorescence of LS174T cells labeled with saturating concentrations of Alexa-488 labeled antibodies or antibody fragments was measured and converted to a number of bound molecules per cell using fluorescent calibration beads. As seen in Figure 2.7A, ~400,000 molecules of the monovalent scFv ds-sm3E-M are bound to each cell at saturation, while the scFv dimer ds-sm3E-D and IgG M111147 saturate the cell with ~40% fewer molecules due to bivalent antigen binding. Interestingly, both the IgG and Fab versions of M85151a bind with approximately twice as many molecules per cell at saturation as the other antibodies of equivalent valency. The greater cell surface binding at saturation for M85151a compared to
M111147 was also observed on HT-1080-CEA cells (Figure 2.7B) while neither antibody binds HT-1080 in the absence of CEA expression suggesting that M85151a’s higher cell labeling is mediated by interactions with CEA and is not due to binding other proteins on the cell surface. The difference in cell binding stoichiometry is also not due to heterogeneous antigen that is partially unreactive with the scFvs and M111147 since >95% of soluble CEA elutes as a complex on size exclusion chromatography when incubated with an excess of any of the tested antibodies (data not shown). One potential explanation for the two fold greater cell binding of M85151a is the possibility that this antibody binds to two different epitopes on each CEA molecule (see discussion).

Figure 2.6 - Effect of antibody incubation on surface CEA levels. LS174T cells were incubated with unlabeled antibodies at 37°C and the relative amount of surface CEA measured at each time point by labeling with an Alexa-488 labeled non-competitive antibody. Incubation with IgG M85151a decreases surface CEA ~20%, while the other antibodies have no effect on surface CEA. All measurements done in triplicate and error bars are SD.
Figure 2.7 – Surface CEA binding stoichiometry. A: LS174T cells were labeled to saturation with Alexa-488 conjugated antibodies and the number of molecules bound per cell calculated as described in materials and methods. Both the IgG and Fab versions of M85151a have approximately twice as many molecules bound at saturation as other antibodies of equivalent valency. All measurements done in triplicate and error bars are SD. B: HT-1080 and HT-1080-CEA cells were labeled with M111147 IgG (grey bars) or M85151a (black bars). The amount of bound antibody was determined with a goat-anti-mouse-488 secondary. Approximately twice as many M85151a molecules bound to the HT-1080-CEA cells as compared to M111147.

2.3.6 - Metabolic turnover of CEA

Finally, we examined the metabolic turnover of the target antigen CEA in the absence of antibody using a biotinylation pulse-chase assay. LS174T cells were surface biotinylated with a NHS-SS-biotin reagent, washed, and incubated at 37°C. At each time point, cells were lysed, biotinylated proteins affinity purified with streptavidin resin, and eluted samples analyzed with anti-CEA western blots. The band intensity of the purified samples decreases over time as biotin-pulsed CEA molecules are catabolized by the cells (Figure 2.8A). Band intensities were quantified and fit to an exponential decay to derive a rate of CEA turnover (Figure 2.8B). Using this assay, the half time for CEA turnover was determined to be 15 hours. This value is approximately equal to the rate of antibody internalization (Figure 2.4), suggesting that the metabolic turnover of CEA drives antibody uptake and that the antibodies themselves do little to modulate this rate - with the exception of IgG M85151a.
Figure 2.8 - Metabolic turnover of CEA. Cell surface proteins were pulsed with biotin using an NHS-SS-biotin reagent and chased at 37°C. At each time point, cells were lysed, biotinylated proteins pulled down with streptavidin resin, and the pulldown blotted for CEA as described in materials and methods. Band intensities were normalized to the signal at time zero and fit to a negative exponential. A: Western blot from a single experiment. B: Pooled data from 3 separate experiments fit to a negative exponential. Each symbol represents a different experiment. CEA is degraded with a half time of 15 hours, similar to the internalization rate of the anti-CEA antibodies.

2.4 – Discussion

Theoretical analyses of antibody transport in solid tumors suggest that cellular internalization and catabolism of bound antibodies significantly retards penetration of these drugs into the tumor. In order to test these predictions using CEA-specific antibodies as a model system, the rates of antibody and antibody fragment internalization by CEA expressing tumor cells were measured. Fluorescence measurements using flow cytometry provided a quantitative and facile method for measuring trafficking kinetics that was higher throughput than imaging approaches and avoided artifacts of incomplete antibody stripping observed with acid washing protocols.
With the exception of IgG M85151a (discussed below), all anti-CEA antibodies and antibody fragments tested were internalized in LS174T cells slowly with uptake half times of 10-16 hours. This time scale is consistent with the metabolic turnover rate of CEA in the absence of antibody (t½ ~ 15 hours) suggesting that the antibodies are taken up passively with the antigen and do little to drive or modulate this uptake. The tested antibodies and antibody fragments have no effect on surface levels of CEA following binding, which is also consistent with a passive uptake mechanism. Since CEA is a GPI linked protein with no cytoplasmic or transmembrane protein domains, the observed uptake is likely the result of bulk membrane turnover rather than specific protein mediated pathways (8). Such non-specific trafficking of CEA is consistent with immunofluorescent microscopy experiments demonstrating that internalized anti-CEA scFvs colocalize partially but incompletely with markers of multiple endocytic pathways (27). Similar slow metabolic turnover has been observed for other antibodies targeting non-receptor cell surface antigens (4).

Slow cellular uptake of the high affinity anti-CEA scFv ds-sm3E-M was also observed in two additional colon carcinoma cell lines, LIM1215 and SW-1222. In contrast, uptake was significantly faster (t½ ~ 4 hours) in a fibrosarcoma cell line HT-1080 transfected with a CEA expression plasmid. It is unclear if this faster uptake is due to greater overall cell surface turnover in this cell line or some function of the artificial CEA overexpression.

Cellular trafficking studies in other systems suggest that the affinity and valency of soluble ligands or antibodies may alter cellular uptake rates by influencing the fraction of molecules that are recycled to the cell surface following endocytosis, or by altering antigen clustering dynamics on the cell surface (28,29). In the case of the anti-CEA scFvs examined here, however, no significant difference in the net uptake constant (kₑ) was observed for
molecules with a range of affinity, stability to protease digestion, and valency. The lack of an affinity dependency may suggest that CEA binding occupancy in the endosome has little effect on the fraction of antibodies that are recycled versus degraded, or alternatively, that both the high and low affinity scFvs are able to maintain antigen binding in the endosome due to the high concentration of CEA. Similarly, the equivalent uptake of the monovalent and bivalent scFvs suggests that crosslinking of two CEA molecules on the cell surface has little effect on the antigen’s distribution or trafficking. This result is consistent with previous observations that bivalent binding of IgGs against folate receptor, another GPI-linked protein, was insufficient to drive receptor clustering on the surface or increase uptake (30).

Although affinity and valency have little impact on the uptake constant \( (k_e) \) of anti-CEA antibodies, they may still influence the total amount of internalized antibody which depends on both \( k_e \) and the amount of antibody bound \( \frac{d[Ab]_{\text{internal}}}{dt} = k_e*[Ab]_{\text{bound}} \). This distinction is clearly observed with the behavior of the low affinity scFv ds-shMFE-M in the continuous uptake and surface decay experiments. While ds-shMFE-M is internalized when continuously incubated with the cells at a 20 nM concentration, it dissociates from the cells prior to internalization in the surface decay assay. Both of these cases are relevant to in vivo tumor targeting. The continuous uptake experiments are similar to the loading phase of tumor targeting where a high plasma concentration maintains a sufficient antibody concentration in the tumor to drive binding and internalization, while the surface decay assay represents the retention phase when antibody clears from the plasma and tumor. In contrast to the low affinity case, the high affinity and bivalent antibodies have dissociation rates \( (k_{off}) \) slower than the internalization rate \( (k_e) \) such that binding will be essentially irreversible in both targeting regimes (4).
Unlike the remainder of the tested antibodies, IgG M85151a exhibited a distinct trafficking profile with a significantly faster net uptake rate (t_{1/2} \sim 5 \text{ hours}) and the ability to downregulate surface CEA. These properties were both valency dependent, as a monovalent Fab fragment of M85151a was internalized slowly and had no effect on surface CEA. Both the M85151a IgG and Fab also bind with approximately twice as many molecules per cell at saturation as compared to other antibodies of equivalent valency. One potential explanation for this two-fold higher cell binding stoichiometry is the possibility that these antibodies bind to more than one epitope per CEA molecule. Monoclonal antibodies capable of binding multiple epitopes per CEA molecule have been reported previously, a phenomenon attributed to the high sequence homology of repeat domains within the antigen (31-33). If M85151a does in fact bind two epitopes per CEA molecule, it may also provide a mechanism for the faster uptake. Bivalent antibodies that bind to a single epitope per antigen can only crosslink two molecules. In contrast, a bivalent molecule that binds more than one epitope per molecule may be able to crosslink larger clusters of antigens. Previous studies have demonstrated that the formation of large clusters of GPI linked proteins can increase antigen localization in caveolae, as well as drive greater antigen internalization and downregulation (7,30,34).

Although the experiments presented here indicate that the internalization of anti-CEA antibodies and antibody fragments in LS174T cells occurs slowly, this rate is sufficient to significantly impact antibody distribution and retention in the tumor. Thurber and Wittrup have shown that anti-CEA scFvs are able to penetrate significantly farther into LS174T spheroids when incubated at 20°C vs. 37°C due to reduced cellular internalization (35). Similarly, Ackermann et al. demonstrated that the slowly internalized IgG M11147 penetrates significantly farther into LS174T spheroids than the rapidly internalized IgG M85151a (26). The
difference in penetration distance can be quantitatively predicted from the $k_e$ values and cell surface binding stoichiometry measured here.

Based on these results and other computational predictions, we suggest that antibodies with slow cellular internalization rates should have advantages for most tumor targeting applications due to their improved penetration and retention in the tumor (with the exception of immunotoxins and antibody-drugs that must be internalized to be cytotoxic (36)). In some cases, it may be possible to engineer more slowly internalized antibodies by either selecting for proteins that are efficiently recycled following endocytosis or by using monovalent antibodies that avoid faster uptake due to antigen clustering. Alternatively, antibody internalization may be reduced by targeting antigens with slower metabolic turnover. One promising target is the colorectal cancer marker A33 which has extended cell surface persistence due to interactions at the tight junction (5).

2.5 - Works Cited


Chapter 3 – Modeling the Effects of Size and Affinity on Tumor Targeting

3.1 – Introduction

To date, IgG monoclonal antibodies have been the dominant format for tumor targeting due to their high functional affinity for the target antigen and favorable pharmacokinetic profile. However, the limitations of these molecules, most notably expensive production in mammalian cell lines and relatively large size, have led to research into alternative targeting agents. Today, there are a number of antigen binding formats with unique physical properties being developed as tumor imaging or therapeutic agents. Initial development of novel targeting agents focused on making smaller fragments of the full IgG that retain antigen binding properties, including 27 kDa scFvs, 50 kDa Fabs, 80 kDa minibodies, and various scFv and Fab based multimers (1). More recently, alternative binding scaffolds including 14 kDa DARPins and 7 kDa affibodies have been engineered that bind antigens with high affinity despite their small size (2,3). At the other end of the size spectrum, nanoparticles and liposomes with molecular radii ranging from 10-100 nm have been developed that incorporate targeting, imaging, and therapeutic functionalities (4). Chemical conjugation approaches such as PEGylation have allowed for further tailoring of molecular size (5).

While these diverse molecules vary in a number of properties including valency, geometry, stability, and surface charge, the most obvious difference is a wide range of molecular radii. Despite several experimental comparisons, however, the exact effects of these size differences on tumor targeting remain unclear. This confusion arises largely from the fact that size influences several distinct transport parameters involved in tumor uptake including permeability across the tumor capillary wall (P), diffusivity within the tumor interstitium (D), available volume fraction in the tumor (ε), and rate of plasma clearance (k_clear) (5-8). These
parameters counteract each other in a manner that makes predicting the effects of size difficult a priori. For instance, small molecules have increased rates of transport across the capillary wall and within the tumor but are also rapidly cleared from the plasma, eliminating the diffusive gradient into the tumor. In contrast, large molecules are cleared from the blood more slowly, but simultaneously suffer from slower rates of transcapillary and interstitial transport.

Due to the complexity of these tradeoffs, computational tools are needed to accurately predict the effects of molecular size on tumor transport. Such models have previously been used to predict antibody microdistribution and macrodistribution within tumors and examine the effect of parameters such as tumor physiology, dose, binding affinity, and antigen turnover (9,10). Here we extend these models to predict the magnitude and specificity of tumor uptake for molecules covering the continuum of sizes from small peptides to liposomes by incorporating derived empirical relationships for the effect of hydrodynamic radius on the parameters \( P, D, \varepsilon \), and \( k_{\text{clear}} \). Our predictions are consistent with published biodistribution studies targeting HER2 expressing xenografts in mice, as well as clinical data from targeting CEA expressing tumors in humans, and shed light on the complex interplay of size and binding affinity in tumor targeting.

3.2 – Methods

Previously reported experimental measurements of capillary permeability (\( P \)), interstitial diffusivity (\( D \)), available volume fraction (\( \varepsilon \)), and plasma clearance (\( k_{\text{clear}} \)) for molecules of various sizes in tumor tissues were collected from the literature (Appendix B). The data sets include studies on proteins, small molecule tracers, dextrans, PEG chains, and liposomes, primarily in mouse xenograft models. Mathematical equations describing the relationship between these parameters and molecular radius (\( R_{\text{mol}} \)) over a broad continuum of sizes were
derived by fitting structural and empirical models of the capillary wall, tumor interstitial space, and renal and non-renal routes of plasma clearance. Implicit in all of these descriptions is the assumption that these are hydrophilic molecules that are not sequestered in membranes or fatty tissue. Fitting was performed using the non-linear least squares method in MATLAB.

The effect of molecular radius on diffusivity ($D$) and available volume fraction ($\varepsilon$) within the tumor can be described by modeling the tumor interstitial space as a series of small and large right circular cylindrical pores. Using this framework, the molecular diffusivity within each pore can be described as $D_{\text{pore}} = D_{\text{free}} \times (D_{\text{pore}}/D_{\text{free}})$, where $D_{\text{free}}$ is the diffusivity of the molecule in solution (cm$^2$/s) and $D_{\text{pore}}/D_{\text{free}}$ is the fractional reduction in free diffusion within the pores (11). $D_{\text{free}}$ can be estimated using the relationship $D_{\text{free}} = (3 \times 10^{-6}$ cm$^2$/s)/$R_{\text{mol}}$, where $R_{\text{mol}}$ is the molecular radius in nm. $D_{\text{pore}}/D_{\text{free}}$ can be solved as:

$$\frac{D_{\text{pore}}}{D_{\text{free}}} = \frac{(1 - 2.105 \lambda + 2.0865 \lambda^3 - 1.7068 \lambda^5 + 0.72603 \lambda^6)}{(1 - 0.75857 \lambda^5)}$$

(3.1)

for values of $\lambda < 0.6$ where $\lambda$ is defined as the ratio of molecular radius ($R_{\text{mol}}$) to pore radius ($R_{\text{pore}}$) (11). For $0.6 < \lambda < 1$, numerical values of $D_{\text{pore}}/D_{\text{free}}$ were determined from previously described lookup tables (12). For $\lambda > 1$, $D_{\text{pore}}/D_{\text{free}} = 0$. To account for diffusion through small and large pores in the tumor, diffusivity over the entire tumor space was defined as $D = (A \times D_{\text{pore small}} + B \times D_{\text{pore large}})$ where $D_{\text{pore small}}$ and $D_{\text{pore large}}$ are the diffusivities in the small and large pores respectively, and A and B are the relative amounts of diffusion that take place through each pore size ($A + B = 1$).

Using the same self-consistent two pore representation of the tumor interstitial space, the available volume fraction can be described using the equation:

$$\varepsilon = V_i (A \times \varphi_{\text{pore small}} + B \times \varphi_{\text{pore large}})$$

(3.2)
where \( V_i \) is the interstitial fluid volume fraction, \( A \) and \( B \) are the ratios of small and large pores, and \( \varphi_{\text{pore\_small}} \) and \( \varphi_{\text{pore\_large}} \) are the partition coefficients of molecules in each pore size defined as

\[ \varphi = (1 - \lambda)^2 \text{ for } \lambda < 1, \text{ and } \varphi = 0 \text{ for } \lambda > 1 \]  

(13). From small molecule tracer studies, \( V_i \) was approximated as 0.5 (7). Since both the interstitial diffusivity and void fraction are described by the same model of the interstitial space, data sets describing each parameter (Appendix B.1, B.2) were simultaneously fit to the respective equations to determine values for \( R_{\text{pore\_small}} \), \( R_{\text{pore\_large}} \), \( A \), and \( B \).

The effect of molecular radius on vascular permeability was similarly modeled using a two-pore representation of the tumor capillary wall. Transport was assumed to be primarily diffusive in nature due to high interstitial fluid pressure in tumors (14), such that permeability across a pore (cm\(^2\)/s) can be modeled as:

\[ P_{\text{pore}} = D_{\text{free}} \left( \frac{D_{\text{pore}}}{D_{\text{free}}} \right) \varphi \]  

(3.3)

where \( D_{\text{free}} \), \( (D_{\text{pore}}/D_{\text{free}}) \), and \( \varphi \) are defined as above (13). Total permeability through small and large pores was defined as \( P = A_{\text{cap}} * P_{\text{cap\_pore\_small}} + B_{\text{cap}} * P_{\text{cap\_pore\_large}} \) where \( A_{\text{cap}} \) and \( B_{\text{cap}} \) are the fractional capillary pore areas per unit membrane thickness (cm\(^{-1}\)) for small and large pores respectively. As above, the model was fit to experimentally determined \( P \) values (Appendix B.3) to estimate \( A_{\text{cap}}, B_{\text{cap}}, R_{\text{cap\_pore\_small}}, \) and \( R_{\text{cap\_pore\_large}} \). While these parameters are similar to those used to describe \( D \) and \( \varepsilon \), here they are describing pores in the capillary wall versus pores in the interstitial space of the tumor.

Due to the various routes and complexities inherent in plasma clearance, there are no simple structural models to describe the size dependency of the clearance term \( k_{\text{clear}} \). Instead a largely empirical model was utilized for the renal and non-renal routes of clearance. For renal clearance, macromolecular filtration can be described as \( Cl_R = GFR * \Theta \), where \( Cl_R \) is the renal
clearance in mL/hr, GFR is the rate of fluid filtration across the glomerular wall estimated at 10 mL/hr in female mice (15), and Θ is the macromolecular sieving coefficient. The sieving coefficient depends on molecular size and can be described as (16):

\[
Θ = \frac{ΦK_{conv}}{1 - e^{-αPe} + ΦK_{conv}e^{-αPe}}
\]  (3.4)

where Φ is the equilibrium partition coefficient, σ is a correction term for the geometry of the glomerular slits approximately equal to 2 for baseline glomeruli, K_{conv} is the solute hindrance factor for convection, and Pe is the Pécel number defined as:

\[
P_e = \frac{(ΦK_{conv})vL}{(ΦK_{diff})D_{free}}
\]  (3.5)

In this description, v is the fluid velocity vector estimated at 0.001 cm/s, L is the membrane thickness approximated at 100 nm in mice (17), D_{free} is the diffusivity in solution discussed above, and K_{diff} is the diffusive hindrance factor. Since there are limited mechanistic models for the effect of size on the hindrance factors K_{conv} and K_{diff}, they, along with the partition coefficient, are defined using empirical terms as reported previously (18):

\[
ΦK_{diff} = \exp(-αR_{mol})
\]  (3.6)

\[
ΦK_{conv} = \exp(-βR_{mol})
\]  (3.7)

where R_{mol} is the molecular radius of the targeting agent and α and β are empirical constants fit to the data (units nm^{-1}). Non-renal clearance was incorporated to account for plasma loss of molecules above the cutoff size for glomerular filtration. With several route of clearance and no structural models a fully empirical description was used with the form:

\[
Cl_{NR} = Cl_{NR,0} - δ\left(\frac{r_{mol}}{r_{mol} + γ}\right)
\]  (3.8)
where $Cl_{NR,0}$ is the non-renal clearance for small molecule tracers (mL/hr), and $\delta$ (mL/hr) and $\gamma$ (nm) are empirical constants fit to the data. While this equation has no physiological significance, it is consistent with experimental observations of decreasing non-renal clearance with increasing molecular size down to a constant level for large molecules (19). $Cl_{NR,0}$ was arbitrarily set to 2 mL/hr to account for the dominance of renal clearance in the size range of small peptides.

The single exponential plasma clearance term $k_{clear}$ (units hr$^{-1}$) was then defined as:

$$k_{\text{clear}} = \frac{Cl_R + Cl_{NR}}{V_{\text{plasma}}}$$

(3.9)

where $V_{\text{plasma}}$ is the plasma volume estimated in mice as 2 mL (20). For predictions of tumor uptake in human patients, the plasma volume was increased to 3 L. This equation was fit to experimental measurements of $k_{clear}$ for molecules of various sizes (Appendix B.4) to determine the constants $\alpha$, $\beta$, $\delta$, and $\gamma$. Although a biexponential description of plasma clearance is more physiologically accurate, the single exponential term is a reasonable approximation that allows us to better describe the broad features of size dependent clearance over the entire continuum of molecular radii using a single parameter.

Tumor uptake of targeting molecules was simulated using a mechanistic compartmental model of antibody uptake in tumors$^1$ (Figure 3.1), in which tumor concentration following a sub-saturating bolus iv injection can be described as:

$$[Ab]_{\text{tumor}} = \left( \frac{2PR_{\text{cap}}}{R_{\text{Krogh}}^2} \right) \left[ \frac{[Ab]_{\text{plasma},0} \left( e^{-k_{\text{clear}} t} - e^{-\Omega t} \right)}{(\Omega - k_{\text{clear}})} \right]$$

(3.10)

$^1$Thurber GM, Wittrup KD. A mechanistic compartmental model for antibody uptake in tumors. Submitted.
where $[\text{Ab}]_{\text{plasma,0}}$ is the initial plasma concentration of the targeting agent (%ID/mL), $t$ is the time, $[\text{Ag}]$ is the target antigen concentration (M), $k_e$ is the rate of endocytic clearance (sec$^{-1}$), $K_d$ is the targeting molecule’s affinity for the antigen (M), $R_{\text{cap}}$ is the capillary radius ($\mu$m), and $R_{\text{Krogh}}$ is the average radius of tissue surrounding each blood vessel ($\mu$m). $P$, $\varepsilon$, and $k_{\text{clear}}$ represent permeability, available volume fraction, and plasma clearance rate, respectively, with values for each sized molecule taken from the fits described above. The diffusivity term $D$ does not appear in the above equations as Thurber et al. demonstrated in the model derivation that antibody uptake from the tumor surface is negligible relative to vascular uptake for most experimentally or clinically relevant tumor types and sizes, and that the permeability term is the rate limiting step in vascular uptake. The diffusivity term is still included in the methods for fitting interstitial pore size, however, as it provides a check for self-consistency with the size-dependent trends in available volume fraction. Size-independent parameter values were estimated from the literature or were varied as described in each simulation. For figures plotted as a function of effective molecular weight, estimates were made from radius using the relationship $\text{MW} = 1.32*\text{R}_{\text{mol}}^3$ (fit from data in reference (21)).

Using the above model, the time of peak tumor uptake following bolus injection can be defined as:

$$t_{\text{opt}} = \frac{\ln\left(\frac{k_{\text{clear}}}{\Omega}\right)}{\left(k_{\text{clear}} - \Omega\right)}$$

(3.12)

where $\Omega$ is defined in Equation 3.11.
3.3 - Results

3.3.1 - Relationship of molecular size to transport parameters

The molecular size of a tumor targeting agent influences four parameters involved in tumor uptake: plasma clearance ($k_{clear}$), capillary permeability (P), interstitial diffusivity (D), and available volume fraction in the tumor ($\varepsilon$). Values of these parameters for molecules of different sizes were collected from experimental studies reported in the literature (Appendix B) and used to fit models of the capillary wall, tumor interstitial space, and renal and non-renal routes of plasma clearance. While these models may not fully represent the physiological phenomena behind each parameter, they provide a reasonable framework for describing experimental trends in these parameters over the range of targeting agent sizes.

The interstitial diffusivity and available volume fraction data sets are best described by a two-pore model of the tumor interstitial space with pore radii of 13.8 nm and 1 µm at a ratio of 9.
to 1 (Figure 3.2A,B). The small pore size is consistent with previous descriptions of size
dependent transport in the tumor interstitial space (11), while the large pores are necessary to
account for the observed diffusion of 2 MDa dextran and liposomes. Similarly, the relationship
between molecular radius and transcapillary permeability was well fit by a two-pore model of the
capillary wall with 4.5 nm and 500 nm radius pores with fractional area to thickness ratios of
17.6 cm\(^{-1}\) and 0.65 cm\(^{-1}\), respectively (Figure 3.2C). These values are again physiologically
reasonable as ~5 nm pores are typical of healthy vasculature (13), and larger pores with 500 nm
radii have been observed in leaky tumor vessels due to overexpression of VEGF and other
hyperpermeability factors (21).

The effect of size on plasma clearance is difficult to model as it is influenced by both
renal and non-renal routes of clearance. As such, a largely empirical model of plasma clearance
was derived with resulting parameters of \(\alpha = 1.6 \text{ nm}^{-1}\), \(\beta = 0.95 \text{ nm}^{-1}\), \(\delta = 1.94 \text{ mL/hr}\), and \(\gamma = 0.20 \text{ nm}\) (Figure 3.2D). While these parameters have no physiological significance, they produce
a fit that closely resembles the trend in the data and is similar to previous empirical descriptions
of size dependent clearance (22). Plasma clearance data for IgGs are displayed separately and
not included in the fit as the Fc domain significantly reduces clearance through interactions with
endothelial FcRn receptors (23).
Figure 3.2 - Size dependent transport parameters. A,B: Relationship between molecular radius and effective diffusivity (D) and available volume fraction (ε) in the tumor. Data points were simultaneously fit to a two pore model of the tumor interstitial space. C: Relationship between molecular radius and effective molecular permeability across the tumor vasculature (P). Data points were fit to a two pore model of the capillary wall. D: Relationship between molecular radius and plasma clearance rate (k_{clear}). Data points were fit to an empirical model of renal and non-renal clearance. IgG clearance is denoted by an open circle and was not included in the fit. All data fitting was performed using a non-linear least squares method. Data points were collected from experimental results reported in the literature and include measurements of proteins (circles), dextran and PEG polymers (squares), small molecule tracers (diamonds), and liposomes (triangles). Additional descriptions of the experimental data are presented in Appendix B.

3.3.2 - Predicted maximum tumor uptake

A previously described compartmental model of tumor uptake was used to predict the peak tumor concentration achieved for radiolabeled HER2 targeting molecules of various sizes following a bolus iv injection. Values for P, ε, and k_{clear} were determined for each size using the relationships derived above. Size-independent model parameters were estimated for a well-
vascularized HER2 expressing xenograft model from values in the literature and are presented in Table 3.1. While molecules bound to HER2 typically undergo net cellular internalization with a half time of ~6-8 hours (ref (24) and unpublished results) endocytic clearance in the context of measured total tumor uptake of radiolabel depends also on the rate at which degraded label is cleared from the cell. Therefore we simulated two different radiolabels, faster-clearing $^{125}$I and residualizing $^{99m}$Tc (25).

<table>
<thead>
<tr>
<th>Table 3.1 Size-independent parameters</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>$[\text{Ab}]_{\text{plasma,0}}$</td>
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<tr>
<td>$[\text{Ag}]$</td>
</tr>
<tr>
<td>$R_{\text{cap}}$</td>
</tr>
<tr>
<td>$R_{\text{Krogh}}$</td>
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<tr>
<td>$k_e$ ($^{125}$I)</td>
</tr>
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<td>$k_e$ ($^{99m}$Tc)</td>
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The simulations predict a complex relationship between peak tumor accumulation and size (Figure 3.3A). In general, predicted tumor uptake is highest for small targeting agents and decreases with increasing molecular radius due to the size-dependent decrease in tumor capillary permeability and available volume fraction reflected in Figures 3.2B and 3.2C. However, this trend briefly reverses in the size range typical of proteins producing a local minimum for tumor uptake at a radius of ~2.8 nm and a local maximum at ~6.5 nm. The existence of the minimum and maximum in this curve can be attributed primarily to the sigmoid dependence of renal clearance on size (Figure 3.2D). Molecules in this size range start to become larger than the kidney filtration cutoff leading to sustained circulation in the plasma which provides increased chances for extravasation into the tumor. Although capillary permeability and available volume fraction are still decreasing in this size range, the decrease in systemic clearance is greater, producing a net increase in tumor uptake. IgGs are predicted to achieve significantly higher tumor uptake than other molecules of equivalent size as a result of their slower plasma clearance.
due to size-independent FcRn-mediated salvage. The use of residualizing $^{99m}$Tc is predicted to increase peak tumor uptake relative to $^{125}$I labeled molecules when used with large molecules.

Model predictions for $^{99m}$Tc labeled targeting agents in the size range typical of proteins (2-500 kDa) were compared to data from experimental HER2 targeting studies in the literature (Figure 3.3B). Each data point represents the highest tumor concentration achieved by a given targeting agent over an experimental time course (Appendix B.5). Since the $R_{Krogh}$ value for intercapillary spacing is the least well-characterized parameter in the model and depends on the extent of vascularization and necrosis within the tumor, simulations were performed for a range of values between 50 and 100 μm. While the computational predictions differ quantitatively from the experimental uptake for several molecules in this size range, the overall trends are consistent. In both the simulations and experimental precedents, intermediate sized proteins (~25 kDa) have the lowest tumor uptake while higher levels of targeting are achieved by smaller or larger agents. Higher predicted IgG uptake in the range of 30-40 %ID/g is also consistent with experimental precedents (Appendix B.5).
3.3.3 - Time dependence of tumor uptake

The time of peak tumor uptake and length of tumor retention also have important implications for imaging and therapy. Therefore, full time course simulations were performed for $^{125}$I or $^{99m}$Tc labeled HER2 targeting molecules ($K_d = 1 \text{ nM}$) ranging in size from 2-1000 kDa. As reported above, peak tumor levels are similar for proteins at the small and large ends of this size range with a local uptake minimum in between (Figure 3.4A,B). The time at which peak uptake occurs, however, differs significantly as small macromolecules reach their maximum tumor level within minutes, while uptake of larger molecules occurs on the time scale of hours to days (Figure 3.4C). Targeting agents labeled with residualizing $^{99m}$Tc are predicted
to achieve peak tumor uptake at later time points and display significantly greater tumor retention than those labeled with $^{125}$I as expected given their differences in cellular clearance. The predictions in Figures 3.4A-C are valid only for molecules lacking Fc domains or other active trafficking as FcRn mediated salvage increases the time until peak tumor uptake by increasing serum persistence.

Computational predictions were compared to published biodistribution time courses for anti-HER2 proteins labeled with $^{99m}$Tc (Figure 3.5) (26-29). In order to more directly compare the time-dependency of the predicted uptake, the magnitude of uptake (%ID/g) was adjusted in each case by fitting the $R_{\text{Krogh}}$ value to the experimental data. For high affinity targeting molecules ($K_d \ll [\text{Ag}]$) as is the case here, the $R_{\text{Krogh}}$ term impacts the height of the curve but has no influence on the shape or time of peak uptake. In all cases, the computationally predicted time course of tumor loading and retention matches the experimental results well. Affibodies and scFvs achieve peak uptake within the first few hours while larger tetramer and IgG molecules achieve tumor uptake more slowly. IgGs in particular have very slow tumor accumulation with peak uptake after days due to the slow rate of plasma clearance driven by FcRn mediated salvage.
Figure 3.4 - Predicted effect of molecular size on time course of tumor uptake. Tumor uptake over time was simulated for $^{125}$I (A) or $^{99m}$Tc (B) labeled non-Fc domain containing HER2 binding molecules ($K_d = 1$ nM) ranging in size from 2-1000 kDa. C: Effect of molecular size on the time of maximum tumor uptake for $^{125}$I (solid line) or $^{99m}$Tc (dashed line) labeled molecules.
Figure 3.5 - Comparison to experimental data. Tumor uptake simulations were performed for affibodies (MW = 7 kDa), scFvs (27 kDa), tetrabodies (130 kDa), and IgGs (150 kDa) and compared to experimentally measured time courses for $^{99m}$Tc labeled HER2 targeting molecules (26-29). $R_{Krogh}$ values were fit to the experimental data for each molecule using the least squares method with results of 57, 80, 101, and 84 μm for the affibody, scFv, tetrabody, and IgG data sets respectively. These values are all in a physiologically reasonable range.

3.3.4 Affinity dependence of tumor uptake

Experimental and theoretical analyses suggest that increasing the affinity of a targeting molecule for its antigen will increase tumor localization up to a point at which tumor levels plateau (9,30). However, the precise affinity at which maximum tumor uptake is achieved depends on the targeting molecule's size. To examine this relationship in more detail, calculations were performed to predict the tumor uptake at 24 hours for macromolecules varying in both molecular size and affinity. For all sizes in the range of 1-1000 kDa, the expected improvement in tumor uptake with increasing affinity was observed up to a plateau at high
affinities (Figure 3.6A). The threshold affinity of this plateau was size dependent, however, as smaller proteins require tighter binding on the order of $10^{-10}$ to $10^{-8}$ M $K_d$ values to maintain significant tumor uptake, while large molecules are able to achieve similar uptake levels at much lower affinities in the $10^{-8}$ to $10^{-6}$ M $K_d$ range (Figure 3.6A, B). IgGs, for instance, require only a $6 \times 10^{-7}$ M affinity to achieve 50% of their peak tumor uptake at 24 hours. The differences in affinity dependence are due to the fact that small, unbound molecules are cleared rapidly from the tumor through vascular intravasation due to their high capillary permeability. As such, small proteins must be anchored to the antigen through high affinity interactions to be retained. In contrast, large, unbound molecules intravasate slowly such that moderate affinity molecules are able to rebind repeatedly and remain in the tumor.

Computational predictions were compared to experimentally reported tumor uptake data for anti-HER2 scFvs ranging in affinity from 15 pM to 320 nM (30). The model accurately predicts the experimental trend in which the three highest affinity scFvs have similar uptake while lower tumor uptake levels are observed for the 16 nM and 320 nM $K_d$ molecules (Figure 3.6C).
Figure 3.6 - Binding and affinity dependence. A: Predicted tumor uptake at 24 hours for $^{99m}$Tc labeled HER2 targeting molecules varying in both size and affinity for the target antigen. B: Affinity necessary to achieve 10% (small dashes), 50% (large dashes), or 90% (solid line) of the maximum tumor uptake at 24 hours as a function of molecular size. C: Comparison to experimental data. The predicted 24 hour tumor concentration for HER2 targeting scFvs (MW = 27 kDa) of various affinities were compared to experimental uptake measurements for affinity variants of the C6.5 scFv (30). Model predictions and experimental data are normalized by their respective uptake values for the highest affinity case.

3.3.5 - Non-binding mediated uptake: size dependence of the enhanced permeability and retention (EPR) effect

Experimental studies have suggested that significant tumor accumulation of large macromolecules may occur even in the absence of tumor-specific binding due to the EPR effect (31). We therefore calculated the uptake of untargeted macromolecules relative to the tumor levels of size-matched molecules that bind the target antigen with a 1 nM $K_d$ (Figure 3.7A). The simulations demonstrate that at early time points uptake is similar for non-targeted
and targeted molecules for all but the smallest peptides. Following this initial uptake phase, unbound molecules are cleared rapidly from the tumor while bound molecules are retained, producing a high level of specificity of targeting at later time points for molecules in the size range of most proteins (radius < 10 nm). In contrast, larger molecules in the size range of liposomes (~50 nm) are predicted to have similar tumor levels of targeted and non-targeted molecules even at later time points, as uptake is dominated by EPR effects. This situation arises as the slow clearance of large, unbound molecules by vascular intravasation occurs at the same rate as clearance of antigen-bound molecules by cellular internalization and degradation. These model predictions are consistent with experimentally measured values of tumor uptake specificity (Figure 3.7B).

![Figure 3.7 - EPR mediated non-specific uptake. A: Predicted tumor concentrations of non-targeted molecules (Kd = 1 M) ranging in radii from 0.5-60 nm were calculated for various times and normalized by the predicted uptake of size matched antigen binding molecules with a Kd of 1 nM (untargeted to targeted uptake ratio). A value of 0 represents fully binding mediated tumor retention, while a value of 1 represents equivalent uptake of targeted and non-targeted molecules. R_Krogh = 100 μm. B: Comparison of non-specific uptake predictions to experimental measurements of specificity. The simulations were performed as described in Figure 4D of the main text using HER2 parameters and size-dependent values for affibodies (R_mol = 1.74 nm), IgGs (R_mol = 4.85 nm), and liposomes (R_mol = 50 nm). Experimental values of specific and non-specific uptake were taken from the literature and reported as a ratio. Non-specific affibody](image-url)
uptake was measured by pre-blocking antigen with an excess of unlabeled affibody and reported in references (28,32,33). Non-specific IgG uptake was measured using a non-HER2 specific control antibody and reported in reference (34). Non-specific liposome uptake was measured with a particle lacking targeting antibodies and reported in reference (35).

3.3.6 - Predicted uptake in human tumors

While comparisons to mouse xenograft studies are a useful validation for model predictions of size dependent trends, the true utility of a model depends on its ability to predict tumor uptake in human patients. Therefore, simulations were performed for tumor uptake of targeting agents of various sizes in human subjects and compared to clinical data for uptake of anti-carcinoembryonic antigen (CEA) scFv, F(ab')2, DFM, and IgG molecules labeled with $^{131}$I (36). $[\text{Ab}]_{\text{plasma},0}$ was reduced from 50 %ID/mL to 0.033 %ID/mL due to the increase in plasma volume from 2 mL to 3 L, and the $[\text{Ag}]$ and $k_e$ values were changed to 300 nM and 9.6E-6 sec$^{-1}$ to reflect the different expression and trafficking properties of CEA (37). All other parameter values were left the same as used in the mouse studies as they should be relatively independent of animal species or body weight in their stated form and few measured values are available for human patients. The predicted max tumor level for molecules ranging in size from peptides to liposomes is presented in Figure 3.8A. The size-dependent trends are identical to those observed in the mouse simulations, while the absolute values are significantly reduced due to the increased plasma volume. The predicted uptake levels in the size range of proteins match closely with the clinically measured tumor concentrations (Figure 3.8B).
Figure 3.8 - Predicted tumor uptake in humans. Simulations were performed as described in Figure 2 except with $V_{\text{plasma}} = 3 \text{L}$, and $[\text{Ag}]$ and $k_{e}$ adjusted for targeting CEA. A: Predicted peak tumor concentrations in humans of CEA binding molecules ($K_d = 1 \text{ nM}$) labeled with $^{125}\text{I}$. IgG uptake was simulated independently and denoted by the solid circle. B: Comparison to clinical data. Peak uptake simulations were performed as above and plotted as a function of effective molecular weight in the size range typical of proteins (2-500 kDa). The predicted uptake trends for $R_{\text{Krogh}} = 50 \text{ mm}$ and $R_{\text{Krogh}} = 100 \text{ mm}$ form the upper and lower bounds respectively of the shaded grey area. The data points represent clinically measured tumor concentrations for scFv, F(ab')$_2$, DFM, and IgG molecules targeting CEA expressing tumors in humans (36).

3.4 - Discussion

The increased development of novel tumor binding agents for applications in cancer therapy and imaging has raised the question of how size differences among these molecules impact their targeting properties. Here we incorporate derived relationships between molecular radius and the transport parameters permeability, available volume, and plasma clearance into a compartmental model of tumor uptake to quantitatively assess the effect of molecular size on the magnitude and specificity of tumor localization. Despite the simplicity of the model, we are able to accurately predict several experimental trends for HER2 targeting molecules in mice and CEA targeting molecules in humans, suggesting that size and affinity alone can largely account for the targeting properties of most macromolecules. The modeling framework presented here can also
be applied to other tumor types and antigens by simply altering the relevant parameters, which can be independently measured.

While several groups have experimentally or computationally compared tumor uptake for small sets of different sized molecules (27,38,39), here we compare molecules across a broad continuum of molecular radii uncovering complex trends of size dependency. In particular, the model predicts that in the size range of most protein agents, there is a local uptake minimum at ~25 kDa while larger and smaller agents achieve higher tumor levels. This prediction is consistent with experimental measurements of HER2 targeting molecules and suggests that small proteins such as affibodies and DARPin{s, along with larger molecules including multimers and PEGylated proteins should be superior targeting agents compared to scFvs. For large molecules, uptake can be further increased by incorporation of Fc or albumin binding domains to actively reduce plasma clearance (23,40).

Although small and large proteins are predicted to have similar peak tumor levels, they differ significantly in the time and affinity dependence of uptake. Small proteins achieve high tumor levels rapidly but require high affinity to be retained, as unbound molecules clear from the tumor rapidly. The rapid uptake of small proteins combined with their efficient clearance from the plasma and normal tissues may make them ideally suited for imaging applications (3). In contrast, large molecules can achieve high uptake at comparatively low affinities but accumulate in the tumor on a much slower time scale. These molecules may be best suited for multi-step pretargeting strategies in which the slow clearance from the plasma can be augmented by clearing agents (41).

Outside the size range of typical protein agents, the model predicts very high tumor uptake for small, hydrophilic, high affinity peptides (low nM or pM $K_d$). Unfortunately,
identifying small peptides that bind tightly is difficult. Most linear or cyclic peptides selected for
\textit{de novo} binding interactions have monovalent affinities on the order of high nM to \(\mu \text{M} \) \(K_d\) (42,43). The formation of multivalent peptide agents or inclusion of synthetic peptidomimetic
domains can contribute to higher affinity (43-45), but these agents may have additional
pharmacokinetic problems including plasma protein binding, biliary clearance, and kidney
localization (46). There are, however, some small natural ligands that bind tightly to tumor
receptors and achieve high tumor accumulation as predicted by the model. Somatostatin
antagonists and glucagon-like peptide analogues have been shown to achieve tumor levels of 61
and 287\% ID/g, respectively, in mouse tumor models (47,48). While these high tumor levels
may be partially attributable to differences among the tumor models or target antigens, they
provide enticing precedents that may motivate future development of targeting agents in this size
range.

At the other end of the size spectrum, the model predicts lower tumor uptake levels on
the order of 1-2 \%ID/g for liposomes and other large targeting agents. There are several caveats
for making general predictions about liposome and nanoparticle uptake including molecular radii
close to the capillary cut-off and greater variation in molecular geometry and chemical structure
relative to proteins. Nevertheless, the model predictions are consistent with experimentally
measured uptake values of 1-8 \%ID/g for a majority of liposomes and nanoparticles (35,49,50).

One of the more intriguing predictions from the model is that for molecules beyond a
certain size there is little to no increase in tumor uptake from antigen targeting. The precise size
at which this targeting independent uptake dominates depends on several parameters, but is
generally predicted to occur in the \(~50 \text{ nm} \) size range typical of liposomes and nanoparticles.
These predictions are consistent with several experimental reports of insignificant differences in
tumor levels of liposomes and nanoparticles with or without targeting ligands (35,49). In contrast, antigen specific targeting may be observed with smaller particles, in tumors with high vascular permeability, or by targeting antigens on the luminal side of the tumor vasculature. For vascular targeting agents in particular, the entire analysis of extravasation and diffusion presented here is irrelevant. It is also important to note that antigen targeting may still improve the therapeutic efficacy of liposomes and nanoparticles even without altering the total tumor concentration by increasing the amount of drug internalized by cells within the tumor (35).

Given the large number of parameters used in the model and the inherent variability in these parameter values due to tumor heterogeneity and experimental error, it is inevitable that there will be some variation or error in the tumor uptake predictions presented here for specific molecules in a given tumor. We believe, however, that the overall trends predicted by the model including predominance of EPR in liposome targeting, and the greater importance of high affinity for small binding molecules should be relatively consistent as they are well supported by published experimental evidence. Better understanding of these trends through the modeling framework presented here should aid in the future design of targeting agents with improved uptake and specificity.

Notes - This chapter was reproduced in large part from Schmidt MM, Wittrup KD. A modeling analysis of the effects of molecular size and binding affinity on tumor targeting. Mol Cancer Ther. 8:2861-71, 2009.
### 3.5 – Parameter definitions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Diffusion coefficient in tumor (cm²/s)</td>
</tr>
<tr>
<td>P</td>
<td>Tumor capillary permeability (cm/s)</td>
</tr>
<tr>
<td>ε</td>
<td>Available volume fraction in the tumor</td>
</tr>
<tr>
<td>(k_{\text{clear}})</td>
<td>Single exponential plasma clearance rate (hr⁻¹)</td>
</tr>
<tr>
<td>(R_{\text{cap}})</td>
<td>Capillary radius (µm)</td>
</tr>
<tr>
<td>(R_{\text{Krogh}})</td>
<td>Radius of tissue surrounding capillary (µm)</td>
</tr>
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<td>([\text{Ab}]_{\text{plasma,0}})</td>
<td>Initial plasma antibody concentration (%ID/mL)</td>
</tr>
<tr>
<td>([\text{Ab}]_{\text{tumor}})</td>
<td>Average concentration of total antibody (bound + free) in tumor (%ID/g)</td>
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<tr>
<td>([\text{Ag}])</td>
<td>Antigen concentration in tumor (M)</td>
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<td>(K_d)</td>
<td>Antibody dissociation constant (M) = (k_{\text{off}}/k_{\text{on}})</td>
</tr>
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<td>(k_e)</td>
<td>Internalization/degradation rate of bound antibody (sec⁻¹)</td>
</tr>
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<td>(D_{\text{free}})</td>
<td>Diffusion coefficient in solution (cm²/s)</td>
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<tr>
<td>(D_{\text{pore}})</td>
<td>Diffusion coefficient in cylindrical pore (cm²/s)</td>
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<tr>
<td>(R_{\text{mol}})</td>
<td>Hydrodynamic radius of the targeting molecule (nm)</td>
</tr>
<tr>
<td>(R_{\text{pore}})</td>
<td>Radius of the pore (nm)</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>Ratio of molecular radius to pore radius</td>
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<tr>
<td>(V_i)</td>
<td>Interstitial fluid volume fraction in tumor</td>
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<tr>
<td>(\varphi)</td>
<td>Partition coefficient in pore</td>
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<td>(A, B)</td>
<td>Relative amounts of diffusion through small and large pores, respectively</td>
</tr>
<tr>
<td>(A_{\text{cap}}, B_{\text{cap}})</td>
<td>Fractional capillary pore areas per unit membrane thickness through small and large pores, respectively (cm⁻¹)</td>
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<tr>
<td>(C_{\text{IR}})</td>
<td>Renal clearance (mL/hr)</td>
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<tr>
<td>(GFR)</td>
<td>Glomerular filtration rate (mL/hr)</td>
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<td>(\Theta)</td>
<td>Macromolecular sieving coefficient</td>
</tr>
<tr>
<td>(\Phi)</td>
<td>Equilibrium partition coefficient at glomerular wall</td>
</tr>
<tr>
<td>(\sigma)</td>
<td>Correction term for geometry of glomerular slits</td>
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<tr>
<td>(K_{\text{conv}})</td>
<td>Solute hindrance factor for convection</td>
</tr>
<tr>
<td>(K_{\text{diff}})</td>
<td>Diffusive hindrance factor</td>
</tr>
<tr>
<td>(P_e)</td>
<td>Péclet number, ratio of convection to diffusion</td>
</tr>
<tr>
<td>(\nu)</td>
<td>Fluid velocity vector (cm/s)</td>
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<tr>
<td>(L)</td>
<td>Membrane thickness (nm)</td>
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<td>(a, \beta)</td>
<td>Empirical constants for describing kidney filtration (nm⁻¹)</td>
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<tr>
<td>(C_{\text{IR}})</td>
<td>Non-renal clearance (mL/hr)</td>
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<tr>
<td>(\delta, \gamma)</td>
<td>Empirical constants for non-renal clearance (units mL/hr and nm, respectively)</td>
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<tr>
<td>(V_{\text{plasma}})</td>
<td>Plasma volume (mL)</td>
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3.6 - Works Cited


Chapter 4 – Tumor targeting properties of monovalent ds(Fv)-Fc antibody fragments

4.1 – Introduction

In Chapter 3, a computational model was developed that describes the effects of molecular size and binding affinity on tumor targeting. The model predicts a complex trend in which two regions of the size parameter space achieve high levels of tumor uptake (Figure 3.3). First, small peptides approximately 1-10 kDa in size accumulate efficiently in the tumor due to rapid capillary extravasation, but require high affinity binding interactions to be retained. Second, proteins approximately 100-300 kDa in size achieve high uptake as they are large enough to escape rapid kidney filtration, while small enough to have reasonable extravasation rates into the tumor. Notably, 150 kDa IgGs have significantly higher uptake than other molecules in this range due to their slow plasma clearance mediated by FcRn recycling (1). A similar effect has been observed for antibodies directly or indirectly fused to albumin (2,3), which also has extended serum persistence due to FcRn binding (4,5).

One area of the size parameter space that has not been fully explored is smaller fragments of the IgG that retain full FcRn mediated salvage activity. As discussed in Chapter 3, most non-Fc containing antibody fragments suffer from rapid kidney filtration that offsets the benefit of faster extravasation and leads to lower total tumor uptake. Renal filtration may be lower for Fc conjugates, however, as there is evidence that the Fc domain can mediate reabsorption of filtered proteins in the kidneys. FcRn receptors are expressed in both podocytes and renal proximal tubular epithelial cells of the kidney, and are believed to contribute to transcytosis of filtered IgG and albumin molecules from the proximal tubules back into circulation (6-8). Perhaps due to this FcRn mediated reabsorption, papain cleaved Fc domains (MW ~50 kDa) have plasma
clearance properties similar to the full IgG (9). If the Fc domain is in fact able to limit kidney filtration and extend serum persistence of antibody fragments, then the greater permeability, void fraction, and diffusivity of these smaller agents may lead to high tumor uptake. To test these possibilities, we engineered a 79 kDa, monovalent ds(Fv)-Fc anti-CEA antibody fragment and tested its targeting properties in mice relative to the full length IgG.

The IgG format consists of two 50 kDa heavy chains and two 25 kDa light chains (Figure 4.1). The pairing of the heavy and light chains forms two binding arms, each consisting of a variable domain (Fv) and constant domain (CH1), connected to a homodimeric Fc domain (CH2-CH3) through a disulfide hinge. The variable domain, comprised of a FvH and FvL heterodimer, forms the antigen binding site, while the CH1 domain provides stability and modularity. Stability can alternatively be introduced by a disulfide bond or peptide linker between the FvH and FvL (10,11). The Fc domain is the site of binding to the FcRn salvage receptor, as well as Fc receptors involved in antibody dependent cellular cytotoxicity (ADCC) and other effector functions (9,12).

To create the 79 kDa ds(Fv)-Fc format, the CH1 domain was removed from the heavy and light chains and replaced with a disulfide bond between the variable domain framework regions to form ds(Fv)\textsubscript{L} and ds(Fv)\textsubscript{H}-Fc subunits. Next, a truncated variant of the heavy chain was constructed consisting only of the hinge and Fc domain. When co-secreted with the modified light and heavy chains, the Fc fragment pairs with the shortened ds(Fv)\textsubscript{H}-Fc to form an Fc heterodimer connected to a single antigen binding arm (Figure 4.1). The final product is a disulfide stabilized trimer consisting of a 13 kDa His-tagged ds(Fv)\textsubscript{L} domain, a 39 kDa ds(Fv)\textsubscript{H}-Fc domain, and 27 kDa FLAG-tagged Hinge-Fc domain (Figure 4.1). Here we demonstrate that anti-CEA ds(Fv)-Fcs can be secreted and purified from Hek cells in high yields, bind to CEA.
expressing cells in vitro with predicted monovalent affinities, and target CEA expressing tumors
in vivo in a comparable manner to full length IgGs.

4.2 – Methods

Plasmid construction

gWiz vectors (Genlantis) encoding the heavy and light chains of anti-CEA IgG clones shMFE and sm3E were obtained from Kelly Davis. The heavy chain sequence for the two clones is identical, while the light chains differ at three amino acids (13). Plasmids encoding the ds(shMFE)-Fc and ds(sm3E)-Fc proteins were constructed using standard molecular biology techniques. Disulfide stabilizing cysteine residues were inserted into each variable domain as described previously (14). Next, a BamHI restriction site downstream of the open reading frame was removed from both plasmids by Quikchange mutagenesis using primers 4.1 and 4.2 (see Table 4.1 for oligonucleotide sequences). For the two ds(Fv)₄ vectors, a N-terminal NheI site was added to the light chain plasmid by Quikchange mutagenesis with plasmids 4.3 and 4.4. The light chain variable domains of shMFE and sm3E were PCR amplified using oligos 4.5 and 4.6 and ligated into the modified light chain backbone digested with NheI and SalI. PCR amplification with oligo 4.6 also added a c-terminal His tag to the ds(Fv)₄ constructs. For the ds(shMFE/sm3E)₄-Fc construct, the heavy chain variable domain was PCR amplified with oligos 4.7 and 4.8, then subcloned back into the heavy chain backbone cut with MluI and NheI. This ligation removed an NheI site between the Fv and CH1 domains and added a new NheI site at the N-terminus. The NheI modified heavy chain was then Quikchanged with oligos 4.9 and 4.10 to remove the CH1 domain. For the truncated Fc construct, the heavy chain leader sequence was replaced by an IL-2 leader sequence by annealing oligos 4.11 and 4.12 and ligating
the insert DNA into the NheI modified heavy chain vector cut with PstI and NheI. The Fc portion of the heavy chain vector, including the hinge, was then PCR amplified using primers 4.13 and 4.14 and ligated into the IL-2 leader sequence backbone digested with NheI and SalI. Oligo 4.14 also introduced a c-terminal FLAG tag into the vector. All mutations were confirmed by sequencing. The final amino acid sequences of all constructs are presented in Appendix A.

Table 4.1 - Oligonucleotides for Chapter 4 constructs

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>4.1</td>
<td>No Bam Ig Top</td>
<td>GCTCTAGACCAGGCGCCTCGATGGGAGATCACTTCTGGC</td>
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<tr>
<td>4.2</td>
<td>No Bam Ig Bott</td>
<td>GCCGAAGTGGATCTCCATCGAGGCCGCCCTGAGCAGGCC</td>
</tr>
<tr>
<td>4.3</td>
<td>LC NheI QC Top</td>
<td>CCAGGGACAGATGTTGAGCCGAAATGTGCTGACCCAATC</td>
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<tr>
<td>4.4</td>
<td>LC NheI QC Bott</td>
<td>GATGGGTAGCAGCACTTTTGGCTAGCACTGGACATGGCTGACCTGG</td>
</tr>
<tr>
<td>4.5</td>
<td>LCL-NB-smK-N</td>
<td>CGATGGTGACTGGTGGTCTAGAGGCCGAAAAATGTCGAGCCAAATCTCCAG</td>
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<tr>
<td>4.6</td>
<td>dsFv-cterm-sall</td>
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</tr>
<tr>
<td>4.7</td>
<td>FabH Miu/Nhe</td>
<td>ATAAAAAGGATGCCGATTGCTGAGCCGATACAAAGTCC</td>
</tr>
<tr>
<td>4.8</td>
<td>E C Avrl</td>
<td>ATATCCCTAGGGCTAGAGCACAGACTAAGGTTCC</td>
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<tr>
<td>4.9</td>
<td>FvFc-short-top</td>
<td>GGACCTTAGGTTCTAGCTCTAGGCAAGGGCAGAAACTCAGACATGGCCACAGGTG</td>
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<td>FvFc-short-bott</td>
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<td>4.11</td>
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<td>4.12</td>
<td>IL2 SP Bott</td>
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<td>CH3-FLAG-C</td>
<td>TATAGTCGACCTAATTAATGGTGTATGGATGTCGGATCTGCCAGCACGTGGCCTTTGACACTTGCTCCAC</td>
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**Protein production in Hek cells**

Plasmids were transfected into DH5α cells and selected on LB + Kan plates. For each plasmid, 400-800 μg of DNA was purified using either 30-60 minipreps (Qiagen) or 1-4 maxipreps (Invitrogen) according to manufacturer’s instructions. Where necessary, the plasmid DNA was concentrated to 0.5-1 μg/μL by ultrafiltration in a Microcon YM-3 device (Millipore) and sterile filtered in a SpinX column (Corning). Hek cells were inoculated at a concentration of 2.5 x 10^5 cells/mL in 40 mL Freestyle 293 Expression Medium (Gibco) in a 125 mL flask at 37°C. After two days, the cells were transferred to a 500 mL flask, and 120 mL of fresh media added. Two days later, the cells were diluted with fresh media to 800 mL, split into a pair of 2 L rolling...
bottles, and grown to a final concentration of 1-1.5 x 10^6 cells/mL. For transfections, either 500 μL of each of the IgG plasmids (light and heavy chains) or 333 μL of each of the ds(Fv)-Fc plasmids (ds(Fv)_L, ds(Fv)_H-Fc, and Hinge-Fc) were mixed with 10 mL of OptiPRO SFM media (Gibco). Simultaneously in a separate tube, 1 mL of sterile PEI (1 mg/mL) was mixed with 10 mL of OptiPRO SFM. After 15 minutes at room temperature, the two tubes were mixed and incubated for an additional 15 minutes. Each transfection mixture was then added to one of the Hek cell roller bottle cultures along with 80 mL of fresh Freestyle 293 media. After an 8 day incubation on rollers at 37°C, the cells were pelleted at 6000 RPM and the cleared supernatant sterile filtered.

Protein purification

The secreted IgGs were purified by Protein-A affinity resin (Pierce) using the manufacturer’s instructions and 1 mL of resin. Proteins were eluted with 100 mM glycine-HCl, pH 3.5, and neutralized with 1/10th volume 1 M TRIS, pH 8.0. For the ds(Fv)-Fc constructs, proteins were first purified using Talon His-tag purification resin (Clontech) according to the manufacturer’s column protocol. Since this step purifies ds(Fv)_2-Fc homodimers in addition to the desired ds(Fv)-Fc species, an additional purification step using M2 anti-FLAG resin (Sigma-Aldrich) was performed. The His elution fractions were directly run over the anti-FLAG resin 2-3 times, and the bound protein eluted with 100 mM glycine-HCl, pH 3.5 as above. Purified proteins were buffer exchanged into PBS by multiple rounds of dilution and concentration using 10 kDa Amicon-ultra 15 ultrafiltration devices (Millipore). Protein size and purity was confirmed by running samples on 4-12% Bis-Tris gels and staining with SimplyBlue SafeStain (Invitrogen).
In vitro binding

Kd values for the purified proteins were measured on fixed LS174T cells. In brief, LS174T cells were trypsinized and fixed with Cytofix buffer (BD Biosciences) for 20 minutes at 4°C. 100,000 fixed cells were incubated at 37°C with unlabeled antibody at a range of concentrations in PBS + 0.1% BSA. At each concentration, a sufficient volume was used to maintain a 10 fold molar excess of antibody over antigen. After 24 hours, cells were pelleted, washed with 1 mL cold PBS-BSA, and labeled with 200 μL Protein A-488 diluted 1:200 in PBS-BSA for 30 minutes on ice. The cells were then pelleted and run on an XL-coulter flow cytometer (Beckman Coulter) to measure the average Alexa-488 fluorescence per cell. The cellular fluorescence of control cells labeled with ProteinA-488 but no primary antibody was measured as non-specific secondary signal and subtracted from the total fluorescence. The corrected fluorescence values were fit to the equation MFU = B_max ([Ab]/([Ab]+Kd)) to determine the Kd.

Protein radiolabeling

800 μg of each protein was diluted to a concentration of 1 mg/mL in PBS, pH 7.4, and mixed with 5 μL of 0.1 M ammonium acetate with 50 mM EDTA at room temperature for 30 minutes. The samples were then diluted in 15 mL 0.1 M Hapes, pH 8.5, and reconcentrated back to ~250 μL using a 10 kDa Amicon Ultra-15 filter unit. The dilution and concentration steps were repeated, and the final protein concentrations adjusted to 2 mg/mL in Hapes. SCN-bz-DTPA (Macrocyclics) was dissolved in 100% ethanol at a concentration of 50 mg/mL and mixed immediately with the protein solutions. A sufficient volume of SCN-bz-DTPA was used for a 20 fold molar ratio of SCN-bz-DTPA to IgG or 10 fold ratio of SCN-bz-DTPA to ds(Fv)-Fc. The reactions were incubated overnight at 4°C. The samples were then diluted with 15 mL 0.1 M
ammonium acetate, pH 6.0, and reconcentrated to 250 μL as before. The concentration and dilution steps were repeated 5-7 times until there was no SCN-bz-DTPA remaining in the flow through as measured by absorbance at 280 nm. Concentrated DTPA-labeled proteins were incubated with ~ 1 mCi $^{111}$InCl$_3$ (Cardinal Health) for 30 min at room temperature. The reaction mixture was diluted with 500 μL saline and concentrated to approximately 50 μL using Vivaspin 5000 MWCO spin columns (Sartorius Stedim Biotech). The dilution and concentration steps were repeated twice, and the $^{111}$In labeled proteins sterile filtered. Greater than 98% of the isotope was associated with the protein as assessed by TLC. The specific activities were 1.045 μCi/μg for sm3E IgG, 1.195 μCi/μg for ds(sm3E)-Fc, 1.08 μCi/μg for shMFE IgG, and 1.39 μCi/μg for ds(shMFE)-Fc.

*Mouse biodistribution*

All animal handling was performed in accordance with Beth Israel Deaconess Medical Center Animal Research Committee guidelines. Xenografts were established in 5-6 week-old male NCR-nu/nu mice (Taconic Farms) by subcutaneous injection of 2-4 x 10$^6$ CEA-positive LS174T cells into the left flank and 0.5-1 x 10$^6$ CEA-negative C6 cells into the right flank of each mouse. After 8-10 days, tumors were 0.1-0.5 g in mass. 100-150 μCi of $^{111}$In-labeled protein was injected retro-orbitally in 100 μL saline. To prevent rapid plasma clearance of the human IgG1 proteins in the nude mice as described previously (15), a bolus of 500 µg non-indium labeled, non-specific human IgG was co-injected with the radiolabeled samples. Blood samples of 10-15 μL were collected from the tail vein at various times and counted on a model 1470 Wallac Wizard (Perken Elmer) 10-detector gamma counter. Mice were euthanized by intraperitoneal
injection of pentobarbital followed by cervical dislocation. Organs and tumors were resected, washed in PBS thrice, weighed, and counted as described above.

*Immunofluorescent tumor microdistribution*

Female NCr nude mice (Taconic), aged 6-8 weeks, were injected subcutaneously with $5 \times 10^6$ trypsinized LS174T cells in the rear flank. The tumors were grown to a diameter of 0.7 – 1 cm, at which time 100 µg of unlabeled antibody in 200 µL PBS was injected via the tail vein. The mice were sacrificed 24 hours post-injection. Tumors were excised, embedded in Optimal Cutting Temperature (OCT) media, and snap frozen in isopentane over liquid nitrogen. Frozen tumors were cryosectioned by the MIT Histology facility at a 5 µm thickness and stored at -80°C until staining. When ready for staining, tissue slices were thawed, air dried for 30 minutes at room temperature, fixed with Cytofix Buffer (BD Pharma) for 10 minutes, and air dried again. The samples were then washed 3 times with PBS and blocked with 5% goat serum for one hour. The blocked samples were incubated with rat anti-CD31 (BD Pharmingen) at a 1:100 dilution in 5% goat serum overnight at 4°C. In the morning, the slides were washed in PBS, then incubated with Alexa-488 labeled goat anti-human Fc IgG (Invitrogen) diluted 1:200, Alexa-555 labeled goat anti-rat IgG (Invitrogen) diluted 1:200, and Alexa-647 labeled mouse anti-CEA IgG (non-sm3E competitive clone M85151a, Fitzgerald Industries) at 20 nM in 0.1% tween-PBS for one hour at room temperature. The samples were washed again in PBS, then mounted with Vectashield including DAPI (Vector Labs) and imaged on a Deconvolution-based high-resolution fluorescence microscope (DeltaVision) with a 10x objective. Multiple image panels were taken covering the entire tumor then stitched together into a single image.
Computational modeling

Tumor uptake time course simulations were performed as described in reference (16). Antibody microdistribution around capillaries was predicted with numeric simulations (17). Values for $A$, $a$, $B$, $\beta$, and $K_4$ were taken from the experimental plasma clearance studies and binding titrations. $R_{Krogh}$ was estimated from the histology slices as 50 $\mu$m. Values for $P$, $D$, and $\varepsilon$ were taken from the size dependent trends described in (16). Non-size dependent parameters were taken from literature descriptions of CEA xenografts as summarized in (16).

4.3 - Results

Mathematical modeling predicts that proteins with small size and slow plasma clearance should achieve high levels of tumor uptake. Therefore, we constructed a 79 kDa, monovalent ds(Fv)-Fc antibody fragment consisting of a single disulfide stabilized Fv domain genetically fused to a heterodimeric Fc. We compare the targeting properties of this novel antibody fragment to the full 150 kDa, bivalent IgG for both low affinity (shMFE, $K_d = 8.5$ nM) and high affinity (sm3E, $K_d = 30$ pM) anti-CEA variable domains (13).

4.3.1 - Protein production

Each ds(Fv)-Fc molecule is a disulfide stabilized trimer consisting of a His-tagged ds(Fv)$_L$ domain, an untagged ds(Fv)$_H$-Fc domain, and a FLAG-tagged Hinge-Fc domain (Figure 4.1). Plasmids encoding each subunit were constructed for both the shMFE and sm3E variable domains and transiently transfected into Hek cells for secretion. After 8 days of secretion, proteins were purified using sequential His-tag and FLAG-tag affinity chromatography.
length IgG variants of shMFE and sm3E were also secreted in Hek cells and purified by Protein-A chromatography.

The ds(Fv)-Fc proteins were purified at a final yield of ~5 mg/L compared to yields of ~10 mg/L for the IgGs. The roughly 50% lower yield of ds(Fv)-Fc molecules is due to the formation of bivalent ds(Fv)_2-Fc tetramers in which two ds(Fv)_H-Fc subunits pair. These species are present at approximately equal concentration with the desired ds(Fv)-Fc trimers after His-tag chromatography, but are efficiently removed during FLAG-purification. The purified IgG and ds(Fv)-Fc molecules match their expected sizes on SDS-PAGE gels and are highly pure (Figure 4.2).

![Diagram of IgG and ds(Fv)-Fc antibodies](image)

**Figure 4.1** – Domain structures of IgG and ds(Fv)-Fc antibodies. In the ds(Fv)-Fc molecule, the CH1 domain is removed and replaced with an interdomain disulfide bond between variable domains. A variant of the Fc domain truncated above the hinge pairs with the ds(Fv)_H-Fc subunit to form a heterodimeric Fc domain fused to a single binding arm.
Figure 4.2 – SDS-PAGE gel electrophoresis of purified proteins. Samples were run on a 4-12% Bis-Tris gel and stained with SimplyBlue SafeStain. As expected, the IgG molecules run at a significantly larger molecular weight than the ds(Fv)-Fc fragments.

4.3.2 – Cell-surface binding titrations

Binding affinities were assessed by $K_d$ titrations on fixed CEA-expressing LS174T cells. The monovalent ds(sm3E)-Fc and ds(shMFE)-Fc molecules bind with $K_d$ values of 22.7 pM and 5.0 nM, respectively (Figure 4.3). These affinities are closely in line with previously measured dissociation constants for monovalent ds(sm3E) and ds(shMFE) scFvs produced in yeast (14). As expected, the bivalent IgGs have significantly greater effective affinities due to avidity. shMFE IgG ($K_d = 44.7$ pM) binds approximately 110 fold higher than the monovalent variant, while sm3E IgG ($K_d = 6.4$ pM) binds 3.5 fold tighter than ds(sm3E)-Fc. The smaller affinity difference between monovalent and bivalent sm3E stems from the fact that monovalent dissociation of this molecule is already extremely slow.
Figure 4.3 – $K_d$ titrations of IgG and ds(Fv)-Fc variants. Unlabeled proteins were incubated with fixed LS174T cells for 24 hours at 37°C, and binding measured by flow cytometry with a Protein A-488 secondary. The measured $K_d$ values are as follows: sm3E IgG = 6.4 pM, ds(sm3E)-Fc = 22.7 pM, shMFE IgG = 44.7 pM, and ds(shMFE)-Fc = 4.96 nM.

4.3.3 – Radiolabeled antibody biodistribution

To compare bulk tumor uptake and biodistribution, antibodies were radiolabeled with $^{111}$In and injected at a 100 µg dose into nude mice bearing CEA positive LS174T tumors and CEA negative C6 tumors. Plasma clearance and tissue accumulation were assessed by gamma counting. Both ds(shMFE)-Fc and ds(sm3E)-Fc are cleared from the plasma with faster kinetics than their cognate IgG molecules (Figure 4.4). The difference is mainly in the beta phase with $\beta$-elimination half times of 28.6 hours for sm3E IgG, 19.4 hours for ds(sm3E)-Fc, 35.3 hours for shMFE IgG, and 11.8 hours for ds(shMFE)-Fc. Faster clearance of ds(Fv)-Fc molecules appears to be mediated largely through increased renal filtration with significant accumulation in the kidneys at 24 hours (Figure 4.5). In an unexpected result, both ds(shMFE)-Fc and shMFE IgG were cleared faster than the corresponding sm3E variants despite sequences that differ at only three residues. Increased shMFE clearance occurred primarily in the alpha-phase and may
reflect some previously uncharacterized difference in stability or aggregation between the two variable domains.

Specific accumulation of all antibodies in the CEA-expressing LS174T xenograft was observed at 24 hours with concentrations of 23.08, 17.82, 12.0 and 11.82 %ID/g for sm3E IgG, ds(sm3E)-Fc, shMFE IgG, and ds(shMFE)-Fc, respectively. When accounting for mouse to mouse variability, there was no statistically significant difference in tumor uptake between the two ds(Fv)-Fc fragments and their corresponding IgGs. Mechanistically, the equivalent tumor uptake of the two formats may be a product of greater capillary permeability and void fraction of the smaller ds(Fv)-Fc fragments offsetting their faster plasma clearance. The lower tumor levels for shMFE IgG and ds(shMFE)-Fc compared to the sm3E variants is likely due to the faster plasma clearance kinetics of these constructs. The lower affinity of the shMFE variable domain may also reduce tumor uptake, although it’s unlikely in this case as shMFE IgG is closer in effective affinity to the sm3E variants than it is to ds(shMFE)-Fc. Uptake in the negative C6 tumor was approximately half that in the positive tumor for all constructs at 24 hours. The significant uptake in the negative tumor is a result of enhanced permeability and retention (EPR) effects, and is in line with previously described predictions for non-specific tumor uptake of IgGs (16).

Computational models simulating total tumor uptake as a function of mechanistic parameters including size and affinity have been described previously (16,17). To compare these model predictions to our experimental results, targeting simulations were performed for all constructs as described in the methods. Parameters were either measured experimentally (A, B, α, β, R_Krogh) or taken from the literature (16). Notably, no parameters were fit to the experimental results. At 24 hours, the model reasonably predicts the relative tumor levels of
each construct with similar uptake for each IgG and ds(Fv)-Fc pair and lower uptake for the two shMFE constructs relative to sm3E (Figure 4.6A). In extended time course simulations, the model predicts that the IgGs will have higher uptake than the corresponding ds(Fv)-Fcs at later time points due to the slower β-phase clearance (4.6B).

Figure 4.4 – Plasma clearance of $^{111}$In labeled proteins in tumor bearing mice. Plasma samples were collected from the tail vain at various times following a 100 µg bolus dose and the radioactivity measured by gamma country. The blood concentrations were fit to the biexponential decay equation $[\text{Ab}](t) = A e^{-\alpha t} + B e^{-\beta t}$ to derive the clearance rate constants.
Figure 4.5 – 24 hour biodistribution of $^{111}$In labeled proteins. Mice containing CEA-positive LS174T and CEA-negative C6 tumors were injected with 100 $\mu$g $^{111}$In labeled antibodies. At 24 hours, the mice were sacrificed, tissues resected, and the radiation in each measured by gamma counting.

Figure 4.6 – Comparison of computational predictions and experimental results. Simulations were performed using a mechanistic compartmental model as described in the methods. A: Experimental values and computational predictions of antibody uptake in LS174T tumor xenografts at 24 hours. B: Computational predictions of antibody tumor uptake over time.
4.3.4 - Immunofluorescent measurement of antibody microdistribution

Antibody microdistribution within the tumor xenografts was assessed by immunofluorescent microscopy. 100 µg of unlabeled antibody was injected into mice bearing LS174T tumors. At 24 hours post-injection, the tumors were excised, cryosectioned, and stained for antibody, blood vessel, and cell nuclei localization (Figures 4.7 and 4.8). Both sm3E IgG and ds(sm3E)-Fc accumulate heterogeneously in the tumor with areas of intense staining around blood vessels and sharp boundaries between targeted and untargeted cells. shMFE IgG also exhibits intense and heterogeneous labeling of perivascular cells, although there are also areas of more diffuse staining distal to the capillaries. In contrast to the other molecules, the low affinity construct ds(shMFE)-Fc is distributed homogeneously with a low level of staining throughout the tumor.

As with the antibody macrodistribution, computational models can be used to predict antibody microdistribution around a capillary (Figure 4.9A). Simulations were performed using parameters measured experimentally or taken from the literature as described in the methods. The model predicts that ds(shMFE)-Fc will have a relatively flat antibody gradient with low levels of accumulation throughout the tumor. In contrast, the model predicts that both IgGs and ds(sm3E)-Fc will have a sharp transition between fully saturated cells near the capillary and fully untargeted cells further into the tumor. The different distributions are a function of affinity as ds(shMFE)-Fc is able to dissociate from the antigen following binding and diffuse further into the tumor, while the three clones with high functional affinity are effectively bound irreversibly.

Image analysis performed on Figure 4.7 shows that the experimental antibody concentrations follow a similar trend to the computational predictions (Figure 4.9B). Both high affinity sm3E variants have high levels of labeling near the capillary but no signal above
background ~200 μm into the tumor. ds(shMFE)-Fc has a flatter gradient with lower labeling intensity near the vessel, but a higher signal at points distal to the capillary. shMFE IgG has a unique trendline in which it follows a similar distribution to sm3E IgG and ds(sm3E)-Fc over the first 100 μm from the capillary but has a significantly higher intensity at distances approaching 200 μm. This result may be partially artifactual given the low number of points at these larger distances, but it may also be due to some monovalent binding of the shMFE IgGs that allows for greater tumor penetration.
Figure 4.7 – Antibody microdistribution in tumor xenografts. 100 μg of unlabeled antibody was injected into mice bearing LS174T tumors. Tumors were excised, sectioned, and stained as described in the methods. The injected antibodies were imaged with anti-Human Fc-488 (green), blood vessels with anti-CD31 (red), and cell nuclei by DAPI (blue). While not shown, CEA antigen was imaged with an anti-CEA antibody and is present throughout each tumor. In general, sm3E IgG, ds(sm3E)-Fc, and shMFE IgG have highly heterogeneous uptake with intense staining around the blood vessels and areas of untargeted cells. In contrast, ds(shMFE)-Fc is distributed homogeneously with a low level of staining throughout the tumor. Antibody distribution was also influenced by variable degrees of cellular necrosis between the different tumor xenografts. In particular, the center of the sm3E IgG tumor and two outer lobes of the shMFE IgG tumor are largely necrotic. Scale bar = 1 mm.
Figure 4.8 – Antibody microdistribution in tumor xenografts. The images are the same as in Figure 4.7 but presented at a higher magnification. The DAPI channel has been removed to make the antibody channel (green) and vessel channel (red) more visible. In general, there are cells and antigen located throughout the presented fields (data not shown). Scale bar = 200 μm.
Model predictions

Experimental distribution

Figure 4.9 – Comparison of experimental microdistribution and computational predictions. The model accurately predicts that ds(shMFE)-Fc will have a flatter gradient of antibody uptake as a function of distance from the capillary compared to the other three constructs. Computational predictions were performed by numeric simulation as described in the methods. For analysis of experimental data, ImageJ was used to convert the capillary distribution images to Euclidean distance maps in which each pixel is assigned a number from 0-255 representing the distance to the closest blood vessel. Necrotic sections and the outer border of each tumor slice were assessed by H & E and DAPI staining and removed from the analysis by masking. The average signal intensity of the antibody channel was calculated for each Euclidean distance using the regionprops method in Matlab. Distances were converted from pixels to microns using the ratio 1 pixel = 1.33 microns.

4.4 – Discussion

IgG antibodies remain the dominant format for tumor targeting due to their high functional affinity, slow plasma clearance, and high tumor uptake. However, the relatively large size of these molecules may limit capillary extravasation, while the high affinity bivalent binding reduces antibody penetration. Therefore, we hypothesized that a smaller, monovalent antibody fragment that maintained slow plasma clearance through the Fc domain may have increased tumor uptake and more homogeneous distribution.
To test this hypothesis, a novel 79 kDa ds(Fv)-Fc molecule was developed consisting of a single disulfide stabilized Fv domain linked to a heterodimeric Fc. The format is similar to a previously described 105 kDa one armed anti-c-Met antibody composed of a full light chain and heavy chain paired with a truncated Fc (18,19). Anti-CEA ds(Fv)-Fc molecules based on the low affinity variable domain shMFE \( (K_d = 8.5 \text{ nM}) \) and high affinity variable domain sm3E \( (K_d = 30 \text{ pM}) \) were produced in Hek cells. Using sequential His and FLAG tag affinity chromatography, the molecules were purified at yields roughly half that of the full length IgG. The reduced yield is due to competitive formation of ds(Fv)\(_2\)-Fc homodimers. It may be possible to improve the yield of the desired trimeric species by inserting mutations into the CH3 domain that encourage heterodimer formation (20,21). The purified ds(Fv)-Fc molecules bind to LS174T cells with monovalent affinities similar to those measured previously for yeast produced ds(scFv) fragments, while the bivalent IgG molecules have significantly higher affinities as expected due to avidity.

To assess the \textit{in vivo} biodistribution of the ds(Fv)-Fc antibody fragments relative to IgGs, the proteins were labeled with \(^{111}\text{In}\) and injected into nude mice bearing C6 and LS174T tumors. For both variable domains, the ds(Fv)-Fc molecules were cleared from the plasma more rapidly than the corresponding IgGs. This faster plasma clearance appears to be driven largely by renal filtration as both ds(Fv)-Fc molecules accumulate in the kidneys at higher levels than either IgG. Although the kidney cutoff is often described as occurring at 60 or 70 kDa (22), these results indicate that larger molecules can still be filtered slowly over time. Unexpectedly, both ds(shMFE)-Fc and shMFE IgG were cleared from the plasma faster than their sm3E based counterparts. This may reflect lower stability or increased aggregation of the shMFE based antibodies. Previous studies using scFv and PEGylated scFv variants of shMFE and sm3E
showed no difference in clearance between the two clones (data not shown), although in those cases clearance may have been too fast to observe different stability effects.

Despite the faster plasma clearance of the ds(Fv)-Fc molecules compared to the IgG, there was no statistical difference in the tumor uptake levels of the two formats at 24 hours post injection for either variable domain. One potential explanation for this result is that greater extravasation of the smaller ds(Fv)-Fc proteins into the tumor offsets the faster plasma clearance. Size-dependent trends in permeability and void fraction suggest that the 79 kDa ds(Fv)-Fc format should extravasate ~25% faster and have a ~20% greater available volume fraction in the tumor compared to the 150 kDa IgG (16). Modeling simulations using the experimentally measured plasma clearance rates and predicted permeability and void fraction values reproduce the experimental targeting results, supporting this hypothesis.

Although we believe that this is the first description of the monovalent ds(Fv)-Fc format, other antibody fragments containing the full Fc domain have been described. In particular, bivalent scFv-Fc molecules (MW ~ 105 kDa) lacking the CH1 domain have been generated as tumor targeting agents against a number of antigens. In one example, an anti-TAG-72 scFv-Fc molecule was described with similar plasma clearance kinetics to the full IgG and significantly higher peak tumor uptake (57.7% ID/g vs. 39% for the IgG) (23). In contrast, an anti-CEA scFv-Fc was cleared from the blood approximately twice as fast as the cognate IgG and had a corresponding decrease in tumor uptake (20 %ID/g vs. 30% ID/g for IgG) (24). Although they do not contain a Fc domain, antibodies linked to albumin either covalently or non-covalently also experience reduced plasma clearance due to FcRn mediated salvage and increased tumor uptake. In one example, an anti-HER2 Fab fragment fused to an albumin binding peptide had similar peak tumor uptake values compared to the full IgG (2). Overall, it appears that the tumor uptake
efficiency of smaller, FcRn binding antibody fragments relative to the IgG varies between constructs and is largely dependent on the rate of plasma clearance. In cases where clearance is similar to the IgG, the smaller molecules have comparable or even improved tumor uptake, likely due to higher permeability.

In microdistribution studies, the three constructs with high functional affinity sm3E IgG, ds(sm3E)-Fc, and shMFE IgG all had heterogeneous uptake with intense perivascular staining, while ds(shMFE)-Fc was distributed homogeneously with less intense cell labeling throughout. Differences in microdistribution as a function of affinity have been well characterized both computationally and experimentally (25-30). In the most analogous case, switching from a bivalent anti-HER2 IgG to a monovalent Fab with an albumin binding peptide significantly increased tumor penetration in mouse xenograft studies (2). While high affinity antibodies including most bivalent IgGs are effectively bound irreversibly, low affinity, monovalent antibodies are able to dissociate prior to internalization and continue diffusing into the tumor (31). At the same time, however, low affinity antibodies suffer from decreased antigen saturation per cell and potentially faster clearance from the tumor. Ultimately, the relevant effectiveness of using high or low affinity antibodies will depend on the cytotoxic effector. Potent, short-range effectors like alpha-emitting radionuclides and drug conjugates may be ideally paired with low affinity agents like ds(shMFE)-Fc, while less potent drugs or molecules with significant bystander effects may be most effective with high affinity antibodies.

Notes – The radiolabeled biodistribution studies were performed by Kelly Davis. The histology studies were performed in large part by John Rhoden.
4.5 - Works Cited


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Chapter 5 – MMP-2 activatable antibodies for improved tumor specificity

5.1 - Introduction

Monoclonal antibodies have become an important class of cancer therapeutics with nine FDA approved drugs and dozens more in clinical trials (1). By specifically targeting antigens overexpressed in tumor tissues, these agents typically have reduced side effects compared to classical chemotherapeutics. However, the presence of low levels of target antigen in healthy tissues can still present clinical problems. Several common tumor targets are present to some degree in healthy tissues including epidermal growth factor receptor-1 (EGFR) expression in the epidermis of the skin and A33 expression throughout the colon (2,3). Other tumor antigens including carcinoembryonic antigen (CEA) are shed into the blood stream where they provide an additional off-target depot (4).

Antibody binding in these healthy tissues can induce toxicity by recruiting immune effectors or blocking receptor activity. Anti-EGFR antibodies, for instance, produce a characteristic papulopustular rash and other side-effects due to disrupted receptor signaling in the epidermis (5). While generally mild, these toxicities lead to dose modifications or suspended treatment in 8-17% of patients (5). Off-target binding may also alter the pharmacokinetics of the antibody, particularly in cases of low antibody dose or high antigen concentration. This function is especially likely for shed antigens that accumulate in the blood due to the highly accessible nature of this compartment for iv administered antibodies (6). By binding to the blood pool of antigen, the amount of free antibody is reduced, which can in turn decrease tumor uptake and penetration. Finally, antibody binding to non-tumor antigen can lead to high background in imaging or diagnostic studies.
An ideal antibody drug would therefore have additional mechanisms to provide specificity for antigen molecules expressed in the tumor tissue versus those expressed by healthy cells. One approach for imparting additional tumor specificity is to exploit matrix metalloproteinases (MMPs), a family of proteases that are highly expressed in a broad range of tumor types. MMPs are synthesized by both tumor and stromal cells, and are involved in cell growth, cell migration, metastasis, and angiogenesis (7). Due to their tumor selective overexpression, MMPs have been used as activating agents for a variety of tumor imaging and therapeutic modalities including toxins, fluorogenic imaging agents, doxorubicin prodrugs, and self-assembling nanoparticles (8-11). More recently, two groups have produced MMP-activatable antibodies and other binding ligands by fusing either a fragment of the target antigen or a synthetic blocking peptide to the antibody through an MMP cleavable linker (12,13). In both cases, antibody masking depends on a specific, molecular interaction between the blocking domain and antigen binding site.

Here we describe an alternative strategy for engineering MMP-2 activatable antibodies in which two halves of a coiled-coil heterodimer are fused to the N-termini of the antibody light and heavy chains through MMP-2 cleavable linkers (Figure 5.1). The coiled-coil forms a clasp over the antibody binding site that sterically blocks antigen-antibody interactions. Following MMP-2 cleavage, the coils are removed and full binding activity is restored.

5.2 - Methods

Plasmid construction

Plasmids encoding all constructs were produced using standard molecular biology techniques including PCR amplification, Quickchange PCR, and digestion/ligation. For the yeast surface
displayed proteins, Fab fragments of the sm3E IgG light chain and heavy chain were PCR
amplified from the gWiz vectors described in Chapter 4 using primers that inserted N-terminal
NheI and BamHI sites. The Fab<sub>L</sub> PCR product was ligated into a prs314 yeast secretion vector
with a C-terminal His-tag, while the Fab<sub>H</sub> product was ligated into a prs316 vector with a C-
terminal cAga2 fusion. Oligonucleotides encoding 3-6 repeats of the K and E coils were
generated by DNA synthesis or PCR amplification and inserted at the N-termini of the Fab
vectors cut with NheI and BamHI. For the soluble, yeast produced ds(Fv) fusions, an
interdomain disulfide bond was introduced by mutating specific variable domain framework
residues to cysteines as described previously (14). The variable domain fragments were then
PCR amplified with primers that introduced an N-terminal MMP-2 cleavable linker or scrambled
linker sequence. The amplified ds(Fv)<sub>L</sub> sequence was ligated into a prs314 vector with a C-
terminal His tag, while the ds(Fv)<sub>H</sub> sequence was ligated into a prs316 vector with a C-terminal
FLAG tag. Primers encoding the VelA1 and VelB1 coils were synthesized and ligated N-
terminal to the linkers in NheI/BamHI cut vectors. N-terminal cysteine residues were inserted by
Quikchange mutagenesis. For the Hek produced Fc fusions, the coil and linker sequences were
amplified from the yeast vectors and inserted at the N-terminus of the ds(shMFE)<sub>L</sub> and
ds(shMFE)<sub>H-Fc</sub> gWiz vectors described in Chapter 4. The final amino acid sequences of all
constructs described in this chapter are presented in Appendix A.

*Yeast surface displayed mmp-antibody binding*

Combinations of K<sub>3-6</sub>-Fab<sub>L</sub> and E<sub>3-6</sub>-Fab<sub>H</sub>-cAga2 fusion constructs were co-transfected into
JAR200 yeast using the EZ-yeast kit (Zymo research) and selected on SD-CAA plates. Colonies
were inoculated and grown in 5 mL SD-CAA liquid media at 30° C overnight. Cells were then
pelleted, resuspended at an OD_{600} \approx 1 in 5\ mL of YPGAL media, and incubated at 20^\circ C for 48 hours. Antibody display was confirmed by labeling cells with Alexa-488 conjugated anti-His IgG (Anaspec) diluted 1:200 and measuring fluorescence by flow cytometry. For binding titrations, cells were incubated with soluble CEA (Fitzgerald Industries) at a range of concentrations in PBS + 0.1% BSA at 37^\circ C. A sufficient volume was used to maintain a 10 fold excess of CEA over surface displayed antibody throughout. After 24 hours of incubation, cells were pelleted and washed with 1 mL ice cold PBS-BSA. The cells were then resuspended in 200 \ \mu L of M85151a (Fitzgerald Industries), a non-competitive anti-CEA mouse IgG diluted 1:1000, and incubated on ice for 30 minutes. After another PBS-BSA wash, cells were resuspended in 200 \ \mu L goat anti-mouse-488 (Invitrogen) diluted 1:200 and incubated on ice for 20 minutes. Cells were pelleted and run on an EPICS Coulter XL cytometer (Beckman Coulter) to measure the 488 signal. The signal was fit to the equation MFU = B_{max} ([Ab]/([Ab]+K_d)) to determine the K_d.

**Secretion and purification of soluble proteins**

Plasmids encoding the coiled-coil-ds(Fv) fusions were transformed into the YVH10 strain of yeast using the EZ-Yeast Kit (Zymo Research) and plated on SD-CAA. Individual colonies were grown in shaker flasks with 125 mL SD-CAA liquid media to an OD_{600} of 5-7, then pelleted and resuspended in YPGAL media at 20^\circ C to induce secretion. After 48 hours, cells were pelleted and the cleared supernatant adjusted to a pH of 7.4. The His-tagged fusion proteins were purified using Talon metal affinity resin (Clontech) according to the manufacturer's batch-column protocol. To remove dimers and other aggregates, proteins were further purified by size-exclusion chromatography with Superdex-200 and Superdex-75 columns.
(GE Healthcare) connected in series using TBS (50 mM Tris, 150 mM NaCl, pH 7.4) as the mobile phase. For secretion of coiled-coil-ds(Fv)-Fc fusions, 333 μg of each plasmid were transiently transfected into Hek cells using PEI as the transfection reagent as described in Chapter 4. Cells were incubated at 37°C on a roller for 8 days, at which time the supernatant was cleared and the proteins purified by sequential anti-His and anti-FLAG chromatography. Aggregates were removed by size-exclusion chromatography as described above.

**In vitro MMP cleavage**

Purified MMP-2 (Anaspec) was activated with 10 mM 4-Aminophenylmercuric acetate (APMA) (Sigma Aldrich) in TBS with 5 mM CaCl$_2$ for 1 hour at 37°C. Coiled-coil masked antibodies at a concentration of 1 μM were incubated with activated MMP-2 at a final concentration of 200 U/mL in TBS + 5 mM CaCl$_2$ at 37°C for 2 - 24 hours. Cleavage was stopped by addition of EDTA to a final concentration of 50 mM and chilling the reactions on ice. Samples were run on 12% Bis-Tris gels and stained with SimplyBlue SafeStain (Invitrogen) to assess the extent of cleavage. To test cleavage specificity, samples were incubated with the MMP-2 inhibitor Galardin (BIOMOL International, Inc.) at a final concentration of 10 mM.

**Soluble protein binding titrations**

CEA expressing LS174T cells were trypsinized and fixed with Cytofix buffer (BD Pharma) for 20 minutes at 4°C to prevent internalization. The fixed cells were incubated with cleaved or uncleaved antibodies at various concentrations in PBS + 0.1% BSA using sufficient volumes to ensure at least a 10 fold molar excess of antibody. For the cleaved samples, the antibodies were incubated with 200 U/mL activated MMP-2 overnight at 25°C prior to mixing with the cells.
Binding incubations were performed at 37°C for 24 hours, after which the cells were pelleted and washed with cold PBS-BSA. The pelleted cells were resuspended in anti-FLAG-phycoerythrin (Prozyme Inc.) diluted 1:500 in PBS-BSA and incubated on ice for 20 minutes. Labeled cells were run on an EPICS Coulter XL cytometer to measure PE fluorescence and the K_d values fit as described above.

5.3 - Results

MMP activatable antibodies have potential for improving the specificity of tumor targeting by reducing binding to antigen molecules expressed in healthy tissue. Here, we describe a method for making MMP-2 activatable antibody fragments by sterically blocking antibody-antigen interactions with a coiled-coil masking domain that is removed by protease cleavage (Figure 5.1).

Figure 5.1 – Schematic of MMP-2 activatable antibodies. In the masked state, the heterodimeric coiled-coil forms a clasp over the binding site that blocks antigen binding. The coil is stabilized by an N-terminal disulfide bond. MMP-2 cleaves the linker sequences (denoted by dashed lines) such that the coils are removed and full antigen binding is restored.
5.3.1 - CEA binding to yeast surface displayed fusions

We first tested the ability of N-terminal coiled-coil domains to sterically block antigen binding using yeast surface displayed anti-CEA antibody fragments (15). Fab fragments of the anti-CEA clone sm3E with or without N-terminal coiled-coil domains were fused to cAga2 and displayed on the surface of yeast. For the coils, we utilized a synthetic system in which the complementary heptamers KVSALEK (K-coil) and EVSALKE (E-coil) were repeated 3-6 times at the N-terminus of the Fab light and heavy chains, respectively (Sequences A.14 and A.15 in the Appendix). By including a range of repeat lengths, we were able to vary the stability of the coiled-coil heterodimer from $\Delta G = -6.41$ kcal/mol for 3 repeats to $\Delta G = -18.08$ for 6 repeats (16). A short linker sequence (GSGGGGS) was included between the coiled-coil and variable domains.

As expected, the addition of heterodimeric coiled-coils to the N-termini of the light and heavy chains significantly reduces the binding affinity of the sm3E Fab for soluble CEA. The addition of 3, 4, 5, or 6 repeat coiled coils produced a 3 fold, 20 fold, 75 fold, or 178 fold decrease in binding affinity, respectively (Figure 5.2). The decrease in affinity is mediated almost entirely through a reduction in the association rate ($k_{on}$), with little to no change in the dissociation rate ($k_{off}$) (data not shown). The trend of decreasing affinity with increased number of repeats suggests that the blocking mechanism is dependent on the size and/or stability of the coiled-coil heterodimer. To directly test the importance of heterodimer formation between the K and E coils, we surface displayed Fabs containing two E5 coils with no K5 coil or one K5 coil with no E5 coil. Both constructs bind only 2-3 fold slower than the unmasked Fab, suggesting that blocking is not due to a specific interaction between either of the coil halves alone with the
antibody binding site and is instead dependent on formation of the coiled-coil heterodimer (Figure 5.2).

Figure 5.2 – Binding titrations for soluble CEA to yeast surface displayed coiled-coil-Fab fusions. A: cAga2 fusion variants of the anti-CEA Fab sm3E with or without N-terminal coiled-coil domains were displayed on the surface of yeast and incubated with soluble CEA. Binding was measured by flow cytometry using a non-competitive anti-CEA secondary and goat anti-mouse-488 tertiary label. The addition of complementary K and E coils to the light and heavy chains, respectively, leads to a significant decrease in binding affinity dependent on the number of repeats. In contrast, minimal blocking is observed with E5 coils fused to both the light and heavy chains, or a single K5 domain fused to the light chain with no corresponding E5 coil on the heavy chain. We were unable to express the K5/K5 pair at sufficient levels to measure binding.

5.3.2 - Soluble protein production and cleavage

To generate soluble, MMP-2 activatable antibody fragments, the heterodimeric coiled-coil sequences were fused to the N-termini of anti-CEA disulfide stabilized variable domains (ds(Fv)s) through MMP-2 cleavable linkers (linker sequence - IPVSLRSG) (17). As a control, constructs were built in which the MMP-2 linker was replaced by a scrambled sequence (IPLSRSVG). The K and E coiled-coils were secreted poorly in this soluble format (data not
shown) so an alternative coiled-coil heterodimer pair VelA1/VelB1 was used instead (18). This coiled-coil has similar thermodynamic properties to the K5/E5 pair with $\Delta G = -11.6$ kcal/mol, but a better balanced charge distribution leading to higher protein expression. Cysteine residues were added at the N-termini of the two coiled-coil halves to form a disulfide bond that further stabilizes the coil and prevents the formation of daisy-chains and other aggregates. To test the ability of the coiled-coil domain to mask both high affinity and low affinity antibodies, soluble constructs were built using two different anti-CEA variable domains: sm3E ($K_d = 30$ pM) and shMFE ($K_d = 8.5$ nM). The sequences are listed in Appendix A, and all VelA1/VelB1-ds(Fv) fusion proteins are hereafter referred to as mmp-ds(Fv) or scr-ds(Fv) for constructs containing the MMP-2 cleavable or scrambled linker, respectively.

Fusion proteins were secreted in yeast cells and purified by a combination of anti-His and size-exclusion chromatography. Final yields ranged from 1-2 mg per liter of secretion. The His-purified heterodimers were >90% monovalent, and the remaining aggregates were efficiently removed by size-exclusion chromatography (data not shown).

To check that the substrate linker was efficiently and specifically cleaved, the coiled-coil masked antibodies were incubated with recombinant MMP-2 at a concentration of 200 U/mL. This enzyme concentration is ~1/2 the level measured in HT-1080 tumor xenografts in vivo (9). The MMP-2 cleavage is efficient as nearly all of a 1 μM protein solution is fully unmasked within 8 hours (Figure 5.3A). The cleavage is also highly specific as the scrambled linker constructs remain fully intact even after overnight incubation with the enzyme (Figure 5.3B).
Figure 5.3 – MMP-2 linker cleavage. A: Time course of MMP-2 cleavage. Mmp-ds(sm3E) was incubated with 200 U/mL recombinant MMP-2 at 37°C and the amount of cleavage assessed on a 12% SDS-PAGE gel. Over time, the 39 kDa fusion product is cleaved into a 27 kDa ds(Fv) domain and 12 kDa coiled coil. The higher molecular weight band seen at intermediate cleavage time points represents fusion proteins in which one but not both of the MMP-2 linkers have been cleaved giving the protein a more open conformation that runs at a larger size. The addition of the MMP-2 inhibitor galardin eliminates all cleavage. B: Specificity of MMP-2 cleavage. Constructs containing either the MMP-2 cleavable or scrambled linker were incubated with recombinant MMP-2 overnight. The scrambled linker constructs remain fully intact.

5.3.3 - MMP-2 dependent binding

After confirming that the fusion proteins were specifically cleaved, we tested the antigen binding properties of the molecules on fixed CEA-expressing LS174T cells. Binding of each construct was measured both before and after complete cleavage with 200 U/mL recombinant MMP-2. mmp-ds(sm3E) binds 300 fold faster following MMP-2 treatment with a $K_d$ shift from 10.6 nM to 35 pM (Figure 5.4A, Table 5.1). The unmasked binding affinity is nearly identical to previous measurements of monovalent sm3E affinity suggesting that full binding activity is restored following cleavage (19). Similarly, mmp-ds(shMFE) binds 272 fold faster following cleavage with a shift from 1.7 μM to 6.4 nM. The post-cleavage $K_d$ is again similar to previous measurements of shMFE affinity (19). In contrast, the two scrambled linker fusions have no significant change in binding affinity after MMP-2 treatment, indicating that the increased binding is dependent on linker cleavage (Table 5.1).
Figure 5.4 – Binding titrations for mmp-ds(sm3E) (A) and mmp-ds(shMFE) (B) before and after MMP-2 cleavage. The titrations were performed on fixed LS174T cells as described in the Methods section. For both variable domains, there is a significant increase in binding affinity following MMP-2 cleavage.

Table 5.1 – Binding affinities for coiled-coil masked anti-CEA ds(Fv)s with MMP-2 cleavable (mmp) or scrambled (scr) linkers before or after cleavage with 200 U/mL MMP-2.

<table>
<thead>
<tr>
<th>Construct</th>
<th>K_d (-MMP2)</th>
<th>K_d (+MMP2)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmp-sm3E</td>
<td>10.6 ± 0.9 nM</td>
<td>35.3 ± 8.1 pM</td>
<td>300</td>
</tr>
<tr>
<td>scr-sm3E</td>
<td>11.0 ± 4.9 nM</td>
<td>5.82 ± 0.8 nM</td>
<td>1.9</td>
</tr>
<tr>
<td>mmp-shMFE</td>
<td>1.74 ± 0.1 μM</td>
<td>6.38 ± 1.6 nM</td>
<td>272</td>
</tr>
<tr>
<td>scr-shMFE</td>
<td>1.83 ± 0.1 μM</td>
<td>2.34 ± 0.1 μM</td>
<td>0.8</td>
</tr>
</tbody>
</table>

5.3.4 - MMP-activatable Fc conjugates

In preparation for future in vivo studies, the constructs were reformatted as monovalent Fc fusions analogous to the ds(Fv)-Fc molecules described in Chapter 4 (Figure 5.5A). The disulfide stabilized VelA1/VelB1 coiled coil was fused to the N-terminus of ds(shMFE)-Fc through either an MMP-2 cleavable or scrambled linker. We used ds(shMFE)-Fc as the antibody domain instead of ds(sm3E)-Fc because the modeling work in Chapter 2 suggested that the affinity switch from pre-cleaved ds(sm3E)-Fc (K_d = 10.6 nM) to post-cleaved (K_d = 35.3 pM)
would have no significant effect on total tumor uptake. In contrast, the ds(shMFE)-Fc switch from 1.74 μM to 6.4 nM was predicted to produce significantly greater tumor uptake after cleavage.

The coiled-coil fusions mmp-ds(shMFE)-Fc and scr-ds(shMFE)-Fc, along with the unmasked fragment ds(shMFE)-Fc, were secreted in Hek cells and purified by sequential His, FLAG, and size exclusion chromatography (Figure 5.5B). In SDS-PAGE analysis, the mmp-ds(shMFE)-Fc molecule shifts down to the size of the unmasked protein following MMP-2 treatment, while the scr-ds(shMFE)-Fc molecule remains unchanged (Figure 5.5C). Cleavage of mmp-ds(shMFE)-Fc leads to increased binding on LS174T cells, while the scrambled linker variant remains blocked (Figure 5.5D).

![Diagram of antibody fragment fusions](image)

**Figure 5.5** – Production and characterization of coiled-coil ds(Fv)-Fc fusions. A: Schematic of mmp-ds(Fv)-Fc with coiled-coil masking domain fused at the N-terminus of the variable domains. B: Coomassie stained gel of antibody fragments demonstrating size and purity. C: anti-FLAG western blot of antibody fusions following MMP-2 treatment. The mmp-ds(shMFE)-
Fc construct is reduced to the size of unmasked ds(shMFE)-Fc following cleavage, while the scr-ds(shMFE)-Fc construct is unchanged. D: Binding of masked constructs following MMP-2 cleavage. The MMP-2 cleavable linker constructs bind to a significantly greater extent following MMP-2 cleavage while the scrambled linker constructs are unchanged.

5.3.5 – Extension to other targets

One of the expected advantages of a steric blocking approach compared to a blocking mechanism dependent on specific interactions between the masking domain and antibody binding site is that the masking domain should be generalizable to a range of antibodies and targets. To test this prediction, we built coiled-coil mmp-ds(Fv) fusions in which the anti-CEA variable domains were replaced by the variable domains of 806, an anti-EGFR antibody, or A33, an anti-A33 antibody. EGFR and A33 were selected as targets as both antigens have significant normal tissue expression in the skin epidermis and colon, respectively.

The fusion constructs were secreted in yeast and purified as above. An additional anti-FLAG purification step was required for the mmp-ds(A33) molecule to remove some light chain dimers. Both the anti-EGFR and anti-A33 fusions were efficiently cleaved by MMP-2 (data not shown). In binding assays on EGFR expressing A431 cells or A33 expressing LS174T cells, both antibody constructs bind the cells to a significantly greater extent following MMP-2 treatment (Figure 5.6).
Figure 5.6 – Binding of coiled-coil masked anti-A33 or anti-EGFR antibody fragments before or after cleavage with 200 U/mL MMP-2. Binding was measured on A33-expressing LS174T cells or EGFR-expressing A431 cells, respectively, by flow cytometry with anti-FLAG-PE secondary. Both constructs bind antigen expressing cells to a significantly greater degree following protease cleavage.

5.4 - Discussion

Antibody binding to antigen depots in healthy tissue can lead to toxicity, altered pharmacokinetics, and high imaging background. Here we describe an approach for avoiding off-target binding and increasing tumor specificity by engineering antibodies that bind their target antigen only after cleavage by the tumor expressed protease MMP-2. In our system, two halves of a coiled-coil heterodimer are fused to the N-termini of the antibody light and heavy chains through MMP-2 cleavable linkers. As the coiled-coil forms, it brings the two halves into position proximal to the antibody binding site in a manner that sterically blocks antigen binding.

We demonstrate that the coiled-coil domain reduces binding of anti-CEA antibodies to CEA-expressing cells up to 300 fold, with full binding activity restored following MMP-2 cleavage. The extent of blocking is nearly identical for low and high affinity anti-CEA clones.
The MMP-2 dependent binding increase is also specific as molecules with scrambled linkers show no difference in affinity following protease treatment. The low level of fusion protein binding in the uncleaved state is likely a function of sufficient linker flexibility and coiled-coil breathing at the linker interface to allow rare instances of antigen binding.

Recently, two groups described the production of MMP activatable antibodies and other binding ligands by attaching a blocking domain with specific affinity for the antibody binding site through a MMP-2 cleavable linker (12,13). While this specific-blocking approach can be effective, we believe that it has two limitations. First, since masking depends on specific molecular interactions between the blocking domain and binding site, a new blocking domain must be selected or engineered for each unique antibody variable domain. Second, since the blocking domain has intrinsic affinity for the binding site, it may continue to function as an inhibitor even after linker cleavage. In contrast, the steric blocking domains described here have no specific affinity for the binding site. As a result, they should be transferable to an array of antibodies with similar blocking activity. In this work, we demonstrate that MMP-2 activatable antibodies can be generated against the target antigens CEA, EGFR, and A33 using the same N-terminal coiled-coil masking domain. Similarly, the coiled-coils should have no inhibitory activity following cleavage.

The steric blocking approach described here is also flexible in regards to the masking domain as two different coiled-coil systems (K/E and VelA1/VelB1) efficiently block antibody binding. It’s likely that other heterodimeric domains such as Fv dimers will also exert a masking functionality. Blocking is also largely independent of the linker sequence as constructs with a Gly-Ser linker (GSGGGGS), MMP-2 cleavable linker (IPVSLRSG), scrambled linker (IPLSRSVVG), and other MMP-2 cleavable sequences (GPLGVRG, data not shown) have similar
masking properties. As a result, we believe that the system should be generalizable to linkers cleaved by other MMPs or tumor proteases such as Cathepsin B, providing an additional mechanism for tuning antibody activation (17,20,21). The only exception to this generality is very short linkers which destabilize coil formation and very long linkers which insufficiently restrict heterodimer location (data not shown).

To test MMP-dependent targeting \textit{in vivo}, we have reformatted the coiled-coil fusion as Fc conjugates based on the ds(Fv)-Fc format described in Chapter 4. In future mouse studies, we will characterize the biodistribution of mmp-ds(shMFE)-Fc, scr-ds(shMFE)-Fc, and unmasked ds(shMFE)-Fc in mice bearing a CEA-positive HT-1080-CEA tumor and a CEA-negative HT-1080 tumor. The HT-1080 cell line was chosen due to its high levels of MMP-2 expression (9,22). Additionally, the mice will have a depot of CEA coated agarose beads in the right shoulder to simulate antigen in healthy tissue (23).

Ideally, the mmp-ds(shMFE)-Fc construct should target the HT-1080-CEA tumor due to the presence of both CEA and MMP-2, while having limited accumulation in the HT-1080 tumor due to a lack of antigen and beads due to a lack of MMP-2. In contrast, unmasked ds(shMFE)-Fc should target both the HT-1080-CEA tumor and antigen coated beads, and scr-ds(shMFE)-Fc construct should have low labeling of all depots. It will also be interesting to assess the microdistribution of these constructs within the tumors. The coiled-coil masking may allow the antibodies to avoid the binding-site barrier and diffuse further into the tumor prior to being uncloaked and binding antigen. Antibody distribution may ultimately depend on the distribution of MMP-2 and the rate of linker cleavage.
5.5 - Works Cited


Appendix A – Amino acid sequences of selected clones

Chapter 2

A.1 – shMFE scFv (yeast produced, non-disulfide stabilized)

EARPASQVKLEQSGAEVKPGASVKLSCASKGNIKNDSYMHWLQPGPQRELEWIGWID
PENGDEYAPKFGQKATFTTTSANTAYLGSSLRPEDTAVYYCNEGTPTGPYYFDYW
GQGTIVTSSGGS GGGS GGSGGGS GGSGSENVLTQSPSSASVGRVTIACSASSSSVPYMH
WFQKPGKPKLLIYSTSGLASVPSFSGSGGDYSLTISSVQPEADAAYYCQQRSSY
PLTGGGKQLEIKAAAGSHHHHHHH

A.2 – ds(shMFE) scFv (yeast produced, disulfide stabilized)

EARPASQVKLEQSGAEVKPGASVKLSCASKGNIKNDSYMHWLQPGPQRELEWIGWID
PENGDEYAPKFGQKATFTTTSANTAYLGSSLRPEDTAVYYCNEGTPTGPYYFDYW
GQGTIVTSSGGS GGGS GGSGGGS GGSGSENVLTQSPSSASVGRVTIACSASSSSVPYMH
WFQKPGKPKLLIYSTSGLASVPSFSGSGGDYSLTISSVQPEADAAYYCQQRSSY
PLTGGGKQLEIKAAAGSHHHHHHH

A.3 – sm3E scFv (yeast produced, non-disulfide stabilized)

EARPASQVKLEQSGAEVKPGASVKLSCASKGNIKNDSYMHWLQPGPQRELEWIGWID
PENGDEYAPKFGQKATFTTTSANTAYLGSSLRPEDTAVYYCNEGTPTGPYYFDYW
GQGTIVTSSGGS GGGS GGSGGGS GGSGSENVLTQSPSSASVGRVTIACSASSSSVPYMH
WLQQKPGKPKLLIYSTSGLASVPSFSGSGGDYSLTISSVQPEADAAYYCQQRSSY
PLTGGGKQLEIKAAAGSHHHHHHH

A.4 – ds(sm3E) scFv (yeast produced, disulfide stabilized)

EARPASQVKLEQSGAEVKPGASVKLSCASKGNIKNDSYMHWLQPGPQRELEWIGWID
PENGDEYAPKFGQKATFTTTSANTAYLGSSLRPEDTAVYYCNEGTPTGPYYFDYW
GQGTIVTSSGGS GGGS GGSGGGS GGSGSENVLTQSPSSASVGRVTIACSASSSSVPYMH
WLQQKPGKPKLLIYSTSGLASVPSFSGSGGDYSLTISSVQPEADAAYYCQQRSSY
PLTGGGKQLEIKAAAGSHHHHHHH

Chapter 4

The leader sequences of Hek secreted molecules are highlighted in italics and removed during secretion.
The heavy chain variable domains for shMFE and sm3E are identical. This is denoted as shMFE/sm3E below for constructs that were paired with both light chains

A.5 – shMFE/sm3E IgG heavy chain (Hek produced, co-secreted with clone A.6 or A.7)
MGWSLILLFLVAVATTROVKLEQSGAEVVKPGASVKLSCASKASGFNIKDSYMHWLRQA
QPRLEWIGWIDPENGDTYEAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYCN
CTGPYYFDYWGQGTLTVCSSASSTKGPSVFPLAPSSKTSTSGTAALGLCKDVDYT
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSVTVPSSSLGTQTYICNVHNKPSN
TVKVEPKSCDKTHCTPCPAPELLGGPSVFLPPKPDTRTPMISRTPEVTCVVD
SHEDPEVKNFYVDGVEVHNAKTKPREEQYNSTYVRVSVLTVLHQLDGWNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREDETJNQVSTLC
VLKVGFYPSIDAVEWESNGQPENNYKTTTVLVDSGSFLLYSKLTVDKS
RWWQQGNVFSCEVMHELHNHYTVQKSSLPGK

A.6 - shMFE IgG light chain (Hek produced, co-secreted with clone A.5)

MRVPAQLLGLLLLWLPGARCENVTQSPSSMSASVGDRVIACSASASSSVPMHFWQQKP
GKSKPKLLYIYSTSNASGVPRSFGSAGSTDYSLTSSVQPEDAAYTCQQRSSYPLTF
GGTKLEIKRTVAAVPFVPFPDEQLKSGTASVCLLNNFYPREAKVQWVDALQSGNSQ
ESVTEQDSKDSTYLSSTLTLKADYEYKRYACEVTHQGLSSPVTSFNRGEC

A.7 - sm3E IgG light chain (Hek produced, co-secreted with clone A.5)

MRVPAQLLGLLLLWLPGARCENVTQSPSSMSASVGDRVIACSASASSSVPMHFWQQKP
GKSKPKLLYIYSTSNASGVPRSFGSAGSTDYSLTSSVQPEDAAYTCQQRSSYPLTF
GGTKLEIKRTVAAVPFVPFPDEQLKSGTASVCLLNNFYPREAKVQWVDALQSGNSQ
ESVTEQDSKDSTYLSSTLTLKADYEYKRYACEVTHQGLSSPVTSFNRGEC

A.8 – Hinge-Fc (Hek produced, co-secreted with several constructs)

MYRMQSSCLASLAVTNSSGGSASKTHCTPCPAPELLGGPSVFLPPKPDTRTPMISRT
PEVTCVVDVSHEDPEVKNFYVDGVEVHNAKTKPREEQYNSTYVRVSVLTVLHQL
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREDETJNQVSTLC
VLKVGFYPSIDAVEWESNGQPENNYKTTTVLVDSGSFLLYSKLTVDKS
RWWQQGNVFSCEVMHELHNHYTVQKSSLPGK

A.9 – ds(shMFE/sm3E)H-Fc (Hek produced, co-secreted with A.8/A.10 or A.8/A.11)

MGWSLILLFLVAVATTROVKLEQSGAEVVKPGASVKLSCASKASGFNIKDSYMHW
LRQPGQCLEWIGWIDPENGDTYEAPKFQGKATFTTDTSANTAYLGLSSLRP
EDTAVYCNCTGPYYFDYWGQGTLTVCSSASSTKGPSVFPLAPSSKTSTSGTA
ALGLCKVDYTVFEPVTVSWNSGALTSGVHTFPAVLQSSGLYSVTVPS
SSLGTQTYICNVHNKPSNTKV
DVKEPKSCDKTHCTPCPAPELLGGPSVFLPPKPDTRTPMISRTPEV
TCVVDVSHEDPEVKNFYVDGVEVHNAKTKPREEQYNSTYVRV
SVLTVLHQLDGWNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSREDETJNQVSTLCVLKVGFYPSIDAVE
WSNGQPENNYKTTTVLVDSGSFL
LYSKLTVDKS
RWWQQGNVFSCEVMHELHNHYTVQK
SSLPGK

A.10 – ds(shMFE)T (Hek produced, co-secreted with A.8/A.9)

MRVPAQLLGLLLLWLPGARCASGSGSENVTQSPSSMSASVGDRVIACSASSSVPMH
FWQQPKPGKSKPLYIYSTSNASGVPRSFGSAGSTDYSLTSSVQPEDAAYTC
QQRSSYPL
TFGCOTKLEIKAAAGSHHHHHHH

135
A.11 – ds(sm3E)L (Hek produced, co-secreted with A.8/A.9)

MRVPAGQLLGLLLLWLPGRARCASGSGSENVLTQSPSSMSVSVDRTIACSASSSVPYMHW
LQQKPGKSKPLLIIYLITLNSLAVPSRFSGSGLSTDYSLTISSVQPAEDAYYCCQQRSSYPL
TFGCGRKLEKIAAAGSHHHHHHH

Chapter 5

A.12 – ds(sm3E)-FabL (Yeast surface displayed, co-secreted with A.13)

LFASGSGSENVLTQSPSSMSVSVDRTIACSASSSVPYMHWLLQQKPGKSPKLLIIYLTSN
LASGVPFRFSGSGLSTDYSLTISSVQPAEDAYYCCQQRSSYPLTFGCGRKLEKATVAAPS
VFIFPPSDEQLKSGTASVCLNNFYPREAKVQWVKDNLQSGNSQESVTEQDSKSTY
SLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNNGESEHHHHHH

A.13 – ds(sm3E)-FabH-cAga2 (Yeast surface displayed, co-secreted with A.12)

EARPASQVKLEQSGAGEVKPGASVLSCKASGFNIKDSYMHWLRQQPGQCLWIGID
PENGDTETAPKFGQKAFITITDSANTAYLGSLRPEDTAVYCYCNEGTPTGPGYYFDYW
GQGTLVSTAGTKGPSVFPLAPSSKSTSKGTAALGCLVKDYFEPPTVSWNGALTSGV
HTFPAVLQSSGLYLSVETVPPSSLGQYTCVNCNHKPSNTKVDDKKEPSKASEQKL
SEELQELTTICEQIPSPTLESTPSLSTTTILANGKAMQGVFEYYKSVTFVSNCGSHPSTT
SKGSPINTQ

A.14 – K3-6-ds(sm3E)-FabL (Yeast surface displayed, co-secreted with A.15; X denotes 3-6 repeats of coiled-coil heptamer KVSALKE)

LFAS(KVSALKE)xGSSGGGSENVLTQSPSSMSVSVDRTIACSASSSVPYMHWLLQQKPG
GKSPKLLIIYLTSANLAVPSRFSGSGLSTDYSLTISSVQPAEDAYYCCQQRSSSYPLTFGCGR
KLEKATVAAPSVFIFPPSDEQLKSGTSVCLNNFYPREAKVQWVKDNLQSGNSQ
ESVTEQDSKSTYSLTSLKADYEKHKVYACEVTHQGLSSLPVTSFNSNGESEHHHHHH
HH

A.15 – E3-6-ds(sm3E)-FabH-cAga2 (Yeast surface displayed, co-secreted with A.14; X denotes 3-6 repeats of coiled-coil heptamer EVSALEK)

EARPAS(EVSALEK)xGSSGGGQVKLEQSGAGEVKPGASVLSCKASGFNIKDSYMHW
LRQQPGQCLWIGIDPENGDTETAPKFGQKAFITITDSANTAYLGSLRPEDTAVYCYCNEGTPTGPGYYFDYW
GQGTLVSTAGTKGPSVFPLAPSSKSTSKGTAALGCLVKDYFEPPTVSWNGALTSGV
HTFPAVLQSSGLYLSVETVPPSSLGQYTCVNCNHKPSNTKVDDKKEPSKASEQKL
SEELQELTTICEQIPSPTLESTPSLSTTTILANGKAMQGVFEYYKSVTFVSNCGSHPSTT
SKGSPINTQ

A.16 – mmp-ds(shMFE/sm3E)H (Yeast produced, co-secreted with A.17 or A.18)
EACGASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSGQVKEQSG
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.17 – mmp-ds(shMFE)\_L (Yeast produced, co-secreted with A.16)

EACGASTTVABLEEKVKTLaENYLKSEVQRSLEEQVAQLGSIPLSRSVLAGQVKL
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.18 – mmp-ds(sm3E)\_L (Yeast produced, co-secreted with A.16)

EACGASTTVABLEEKVKTLaENYLKSEVQRSLEEQVAQLGSIPLSRSVLAGQVKL
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.19 – scr-ds(shMFE/sm3E)\_H (Yeast produced, co-secreted with A.20 or A.21)

EACGASTSVDELQAEVDQLEDENYALITKVAQLRKKVEKLGSIPVSLRSGQVKEQSG
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.20 – scr-ds(shMFE)\_L (Yeast produced, co-secreted with A.19)

EACGASTTVABLEEKVKTLaENYLKSEVQRSLEEQVAQLGSIPLSRSVLAGQVKL
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.21 – scr-ds(sm3E)\_L (Yeast produced, co-secreted with A.19)

EACGASTTVABLEEKVKTLaENYLKSEVQRSLEEQVAQLGSIPLSRSVLAGQVKL
AEVVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.22 – mmp-ds(A33)\_H (Yeast produced, co-secreted with A.23)

EACGASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSGQVMEQSG
GGLVKPGASVKLSCASKAGFNKDSYMHWLRQPGQCLEWIGWIDPENGDTEYAPKFQG
KATFTTDTDTSANTAYLGSSLRPEDTAVVYCNEGTPTGYPYFDYWGQGTLVTVSSDYKD
DDDK

A.23 – mmp-ds(A33)\_L (Yeast produced, co-secreted with A.22)
EACGASTTVAQLEEKVKTLRAENYELKSEVQRLEEQQVAQLGSIPVSLRSGELQMTQSPL
SLSASVGDRTVITTCASEFLNGVSWSFYQQKPGKCPKFIYIGASNLIESGVPSRFSGS
GTDFTLTISSLQPEDVATYYCLCGGYSGSGLTFGGTKVEIKRAAGSHHHHHHH

A.24 – mmp-ds(806)_H (Yeast produced, co-secreted with A.25)

EACGASTSVDELQAEVDQLEDENYALKTKVAQLRKXVEKLGSIPVSLRSGQLQESGSPSL
VKPSQSLSLTCTVTGYSITSDFAWNRWQFPNGKLEWMGYIYSNGNTRPNSLSRISITR
DTSKNQFFQFLNSVTIEDTAYYYCVTAGGRFYWGCGLTVTVDYKDDDDK

A.25 – mmp-ds(806)_L (Yeast produced, co-secreted with A.24)

EACGASTTVAQLEEKVKTLRAENYELKSEVQRLEEQQVAQLGSIPVSLRSGDILMTQSPL
MSVSLGDTVSITCHQDINSNIGWQQRPGKCFKGLIYHTNLDEVPSPRSFGSGSGAD
YSLTISSELEDFAVYYCVQYAYFPWFIGGKTLEIKRAAGSHHHHHHH

A.26 – mmp-ds(shMFE)_H-Fc (Hek produced, co-secreted with A.8/A.27)

MYRMQLLLSLALVTNSGGCGASTSVDELQAEVDQLEDENYALKTKVAQLRKKE
KLGIPSVLRSGGQKLEQSGAEVKPGASVKLCSAGFNKDSYMHWLQGGPQCLE
WIGWIDPENGDEYAPKFGQKATFTTDSANTAAYGLSLRLPEDTAVYYCNEGTPTGY
YFDYWGQGTILTVSTSSDADKTHTTPCPAPELLGPGSFLFPPKDKTLISRTPEVTCVV
VDVSHEDPEVKFNYWVDGVEVHNAKTKPREEQNYSTYRRVSLLVHLQDWLNGKEY
KCKVSNKALPAPIEKTSKAKGQPREPQVYTLPPSQDLETKNQVLKGFYPSDIAY
EWESNGQPENNYKTTPPVLDSDGSFLYKTLVDKSRWQQNVFSCVMHEALHNHYT
QKSLSLSPGK

A.27 – mmp-ds(shMFE)_L (Hek produced, co-secreted with A.8/A.26)

MYRMQLLSLALVTNSGGCGASTTVAQLEEKVKTLRAENYELKSEVQRLEEQQVA
QLGSIPVLSRGGENVTQSPPSMASVGDRTVITACASSSVPPYMHWFQKPGKSPKLIYST
SNLASSGVPSRSFGSGSGDSTVYSSTLISSVQPEDAAATYYCQRRYPLYTLFGCGL
TEIKAAGSHHHHHHH

A.28 – scr-ds(shMFE)_H-Fc (Hek produced, co-secreted with A.8/A.29)

MYRMQLLSLALVTNSGGCGASTSVDELQAEVDQLEDENYALKTKVAQLRKKE
KLGISPLRSVRQVQKLEQSGAEVKPGASVKLCSAGFNKDSYMHWLQGGPQCLE
WIGWIDPENGDEYAPKFGQKATFTTDSANTAAYGLSLRLPEDTAVYYCNEGTPTGY
YFDYWGQGTILTVSTSSDADKTHTTPCPAPELLGPGSFLFPPKDKTLISRTPEVTCVV
VDVSHEDPEVKFNYWVDGVEVHNAKTKPREEQNYSTYRRVSLLVHLQDWLNGKEY
KCKVSNKALPAPIEKTSKAKGQPREPQVYTLPPSQDLETKNQVLKGFYPSDIAY
EWESNGQPENNYKTTPPVLDSDGSFLYKTLVDKSRWQQNVFSCVMHEALHNHYT
QKSLSLSPGK

A.29 – scr-ds(shMFE)_L (Hek produced, co-secreted with A.8/A.28)
MYRMQLSCIALSLAVINGGGCAGASTTVAQLEEVKTLEONAELKSEVQRLEEQVAQLGSIPLSRSVGENVLTQSPSSMSAVGDRVTIACSAASSSVPYMHWFQKPKGSPKLLIYSTSNLASGVPERSGSGSTDSYTISSVQPDAAATYYCQQRSSYPLTFCGTLKLEIKAAGSHHHHHH
Appendix B – Size-dependent parameter data sets

B.1 – Effective diffusivity (D) data

<table>
<thead>
<tr>
<th>Name</th>
<th>MW (kDa)</th>
<th>Radius (nm)</th>
<th>Diffusivity (cm²/s)</th>
<th>Tumor line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-FITC</td>
<td>0.376</td>
<td>0.45</td>
<td>6.40E-06</td>
<td>VX2 carcinoma</td>
<td>(1)</td>
</tr>
<tr>
<td>Na-FITC</td>
<td>0.376</td>
<td>0.45</td>
<td>4.30E-06</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>Na-FITC Average</td>
<td>0.376</td>
<td>0.45</td>
<td>5.35E-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>20</td>
<td>3.2</td>
<td>7.50E-07</td>
<td>VX2</td>
<td>(1)</td>
</tr>
<tr>
<td>Dextran</td>
<td>40</td>
<td>5.0</td>
<td>4.20E-07</td>
<td>VX2</td>
<td>(1)</td>
</tr>
<tr>
<td>Dextran</td>
<td>70</td>
<td>6.5</td>
<td>1.90E-07</td>
<td>VX2</td>
<td>(1)</td>
</tr>
<tr>
<td>Dextran 2000</td>
<td>2000</td>
<td>22.1</td>
<td>2.47E-08</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>Dextran 2000</td>
<td>2000</td>
<td>22.1</td>
<td>4.11E-09</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>Dextran Average</td>
<td>2000</td>
<td>19.2</td>
<td>1.44E-08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>21.5</td>
<td>2.54</td>
<td>1.01E-06</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>Fab</td>
<td>50</td>
<td>3.36</td>
<td>2.70E-07</td>
<td>LS174T</td>
<td>(3)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>1.30E-07</td>
<td>LS174T</td>
<td>(3)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>9.6E-09</td>
<td>HSTS 26T</td>
<td>(4)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>1.9E-07</td>
<td>LS174T</td>
<td>(4)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>1.9E-07</td>
<td>McaIV</td>
<td>(4)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>8.7E-08</td>
<td>U87</td>
<td>(4)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>1.91E-07</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>9.38E-08</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>IgG Average</td>
<td>150</td>
<td>4.85</td>
<td>1.27E-07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>900</td>
<td>8.81</td>
<td>7.7E-08</td>
<td>LS174T</td>
<td>(5)</td>
</tr>
<tr>
<td>IgM</td>
<td>900</td>
<td>8.81</td>
<td>1.05E-07</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>IgM</td>
<td>900</td>
<td>8.81</td>
<td>4.30E-08</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
<tr>
<td>IgM Average</td>
<td>900</td>
<td>8.81</td>
<td>7.5E-08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome</td>
<td>76.2</td>
<td>2.97E-09</td>
<td>2.97E-09</td>
<td>U87/Mu89</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Table B.1 - Effective diffusivity (D) for molecules of various sizes. Data points were collected from experimental studies reported in the literature. Experiments were primarily performed in mouse xenograft models *in vivo* and include studies on small molecule tracers, proteins, and dextrans. All molecules are non-specific meaning they have no affinity for the tumor tissue. Bovine serum albumin (BSA) diffusivities reported in the literature were excluded from the data set as the negatively charged nature of these molecules can significantly influence their interstitial transport. For molecules characterized in multiple tumor types, an average value was determined and used in the fit. When not directly stated in the reference, molecular radii for globular proteins were estimated as $R = 0.912 \times MW^{0.333}$. This relationship was derived from fitting data in supplemental reference (6). Molecular radii for Dextran 40, 70, and 2000 are the average of values given in references (1) and (7). Data values reported in graphical form were estimated using the DataThief program (http://www.datathief.org/).
B.2 – Tumor void fraction (ε) data

<table>
<thead>
<tr>
<th>Name</th>
<th>MW (kDa)</th>
<th>Radius</th>
<th>Available volume fraction</th>
<th>Tumor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran 10</td>
<td>10</td>
<td>2.7</td>
<td>0.26</td>
<td>Rat fibrosarcoma</td>
<td>(8)</td>
</tr>
<tr>
<td>Dextran 20</td>
<td>20</td>
<td>3.2</td>
<td>0.28</td>
<td>Rat fibrosarcoma</td>
<td>(8)</td>
</tr>
<tr>
<td>Dextran 40</td>
<td>40</td>
<td>5.0</td>
<td>0.28</td>
<td>Rat fibrosarcoma</td>
<td>(8)</td>
</tr>
<tr>
<td>Dextran 70</td>
<td>70</td>
<td>6.5</td>
<td>0.1</td>
<td>Rat fibrosarcoma</td>
<td>(8)</td>
</tr>
<tr>
<td>Dextran 2000</td>
<td>2000</td>
<td>22.1</td>
<td>0.055</td>
<td>Rat fibrosarcoma</td>
<td>(8)</td>
</tr>
<tr>
<td>Cationized albumin</td>
<td>66</td>
<td>3.69</td>
<td>0.29</td>
<td>Rat mammary tumor</td>
<td>(9)</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>4.85</td>
<td>0.24</td>
<td>Rat mammary tumor</td>
<td>(9)</td>
</tr>
</tbody>
</table>

Table B.2 - Available volume fraction in the tumor (ε) for molecules of various sizes. Data points were collected from experimental studies reported in the literature. All experiments were performed in rat tumor tissues either ex vivo (8) or in vivo (9). All molecules are non-specific meaning they have no significant affinity for the tumor tissue. Void fraction data for native bovine serum albumin (BSA) were excluded from the analysis as the surface charge of these molecules impacts their tumor distribution (9). Instead, cationized albumin with a pI of 7.6 was used as a tracer. Data points in reference (9) were converted from relative volume fractions to absolute values by dividing by the interstitial volume V_ı estimated as 0.5. When not directly stated in the reference, molecular radii for globular proteins were estimated as R = 0.912*MW^{0.333}. Molecular radii for Dextran 40, 70, and 2000 are the average of values given in references (1) and (7). Data values reported in graphical form were estimated using the DataThief program (http://www.datathief.org/).
B.3 – Capillary permeability (P) data

<table>
<thead>
<tr>
<th>Name</th>
<th>MW (kDa)</th>
<th>Radius (nm)</th>
<th>Permeability ($10^{-7}$ cm/s)</th>
<th>Tumor type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>3.3</td>
<td>1.5</td>
<td>154</td>
<td>LS174T</td>
<td>(7)</td>
</tr>
<tr>
<td>Dextran</td>
<td>10</td>
<td>2.7</td>
<td>32</td>
<td>LS174T</td>
<td>(7)</td>
</tr>
<tr>
<td>Dextran</td>
<td>40</td>
<td>5.0</td>
<td>9.5</td>
<td>LS174T</td>
<td>(7)</td>
</tr>
<tr>
<td>Dextran</td>
<td>70</td>
<td>6.5</td>
<td>9.8</td>
<td>LS174T</td>
<td>(7)</td>
</tr>
<tr>
<td>Dextran 2000</td>
<td>2000</td>
<td>22.1</td>
<td>1.7</td>
<td>LS174T</td>
<td>(7)</td>
</tr>
<tr>
<td>Mouse Fc fragment</td>
<td>25</td>
<td>2.66</td>
<td>3.74</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Mouse Fab fragment</td>
<td>25</td>
<td>2.66</td>
<td>4.61</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45</td>
<td>3.24</td>
<td>5.77</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>104</td>
<td>4.28</td>
<td>1.53</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Mouse F(ab')$_2$ fragment</td>
<td>110</td>
<td>4.36</td>
<td>1.51</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>160</td>
<td>4.94</td>
<td>2.82</td>
<td>LS174T</td>
<td>(6)</td>
</tr>
<tr>
<td>Liposome</td>
<td>45.0</td>
<td>0.2</td>
<td></td>
<td>LS174T</td>
<td>(10)</td>
</tr>
<tr>
<td>Liposome</td>
<td>60.0</td>
<td>0.155</td>
<td></td>
<td>LS174T</td>
<td>(10)</td>
</tr>
</tbody>
</table>

Table B.3 - Effective capillary permeability (P) for molecules of various sizes. Data points were collected from experimental studies reported in the literature. Experiments were performed in mouse xenograft models in vivo by quantifying the extravasation of fluorescent tracers into the tumor. Since extravasation may contain both convective and diffusive components, these are apparent permeability values. All molecules are non-specific meaning they have no affinity for the tumor tissue. The higher apparent permeability values for dextran molecules (7) compared to proteins (6) may be due to geometry effects (linear vs. globular molecules), charge effects, or differences in the experimental assays. Bovine serum albumin (BSA) permeabilities reported in the literature were excluded from the data set as the negative surface charge on these molecules can retard transcapillary flux (11). Molecular radii for Dextran 40, 70, and 2000 are the average of values given in references (1) and (7). Data values reported in graphical form were estimated using the DataThief program (http://www.datathief.org/).
### B.4 – Plasma clearance ($k_{\text{clear}}$) data

<table>
<thead>
<tr>
<th>Format</th>
<th>Eff. MW (kDa)</th>
<th>Rad. (nm)</th>
<th>A (%ID/mL)</th>
<th>B (%ID/mL)</th>
<th>$k_a$ (hr$^{-1}$)</th>
<th>$k_b$ (hr$^{-1}$)</th>
<th>AUC (%ID*hr/mL)</th>
<th>$k_{\text{clear}}$ (hr$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTPA</td>
<td>0.50</td>
<td>0.73</td>
<td>6.79</td>
<td>0.07</td>
<td>1.36</td>
<td>0.03</td>
<td>7.01</td>
<td>7.13</td>
<td>(12)</td>
</tr>
<tr>
<td>DOTAG-Benzone</td>
<td>0.62</td>
<td>0.78</td>
<td>6.91</td>
<td>0.68</td>
<td>1.01</td>
<td>0.13</td>
<td>12.07</td>
<td>4.14</td>
<td>(12)</td>
</tr>
<tr>
<td>DOTAG-FITC</td>
<td>0.99</td>
<td>0.91</td>
<td>8.59</td>
<td>0.09</td>
<td>1.37</td>
<td>0.03</td>
<td>8.97</td>
<td>5.58</td>
<td>(12)</td>
</tr>
<tr>
<td>DOTAG$_2$-Tyr-Lys</td>
<td>1.19</td>
<td>0.97</td>
<td>5.27</td>
<td>0.16</td>
<td>1.25</td>
<td>0.02</td>
<td>12.91</td>
<td>3.87</td>
<td>(12)</td>
</tr>
<tr>
<td>Affibody</td>
<td>7</td>
<td>1.74</td>
<td>49.86</td>
<td>0.08</td>
<td>2.83</td>
<td>0.03</td>
<td>20.19</td>
<td>2.48</td>
<td>(13,14)</td>
</tr>
<tr>
<td>Affibody dimer</td>
<td>15.6</td>
<td>2.28</td>
<td>49.18</td>
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<td>3.10</td>
<td>0.12</td>
<td>22.82</td>
<td>2.19</td>
<td>(13,15)</td>
</tr>
<tr>
<td>scFv</td>
<td>27</td>
<td>2.74</td>
<td>47.00</td>
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<td>3.20</td>
<td>0.11</td>
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<td>(16)</td>
</tr>
<tr>
<td>scFv</td>
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<td>14.40</td>
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<td>21.02</td>
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<tr>
<td>scFv</td>
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<td>49.10</td>
<td>0.54</td>
<td>10.40</td>
<td>0.04</td>
<td>17.52</td>
<td>2.85</td>
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</tr>
<tr>
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<td>3.47</td>
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<td>0.80</td>
<td>2.81</td>
<td>0.05</td>
<td>33.47</td>
<td>1.49</td>
<td>(18)</td>
</tr>
<tr>
<td>scFv dimer</td>
<td>55</td>
<td>3.47</td>
<td>48.15</td>
<td>0.76</td>
<td>1.17</td>
<td>0.05</td>
<td>57.49</td>
<td>0.87</td>
<td>(20)</td>
</tr>
<tr>
<td>scFv dimer</td>
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<td>3.47</td>
<td>43.85</td>
<td>3.04</td>
<td>0.75</td>
<td>0.05</td>
<td>124.43</td>
<td>0.40</td>
<td>(21)</td>
</tr>
<tr>
<td>scFv dimer</td>
<td>55</td>
<td>3.47</td>
<td>33.00</td>
<td>6.42</td>
<td>2.76</td>
<td>0.24</td>
<td>38.71</td>
<td>1.29</td>
<td>(17)</td>
</tr>
<tr>
<td>Minibody</td>
<td>80</td>
<td>3.93</td>
<td>22.92</td>
<td>8.31</td>
<td>0.66</td>
<td>0.07</td>
<td>157.83</td>
<td>0.32</td>
<td>(22)</td>
</tr>
<tr>
<td>Minibody</td>
<td>80</td>
<td>3.93</td>
<td>26.44</td>
<td>7.57</td>
<td>0.48</td>
<td>0.06</td>
<td>189.75</td>
<td>0.26</td>
<td>(22,23)</td>
</tr>
<tr>
<td>scFv dimer-PEG</td>
<td>300</td>
<td>6.11</td>
<td>34.07</td>
<td>16.00</td>
<td>1.15</td>
<td>0.03</td>
<td>669.63</td>
<td>0.07</td>
<td>(19)</td>
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<tr>
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<th>AUC (%ID*hr/mL)</th>
<th>$k_{\text{clear}}$ (hr$^{-1}$)</th>
<th>Ref.</th>
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Table B.4 - Plasma clearance rates for molecules of various sizes. Experimental measurements of plasma concentration over time following a bolus iv administration were collected from the literature for proteins, PEG chains, and liposomes of various sizes. Data points were pooled prior to fitting for identical molecules or closely related affinity variants measured in multiple references. Data points were fit to a bi-exponential clearance model of the form:

\[
[Ab]_{\text{plasma}}(t) = Ae^{-kat} + Be^{-ktb}
\]

where \([Ab]_{\text{plasma}}(t)\) is the macromolecule’s plasma concentration in %ID/mL, \(A\) and \(B\) are the fraction of alpha and beta clearance in %ID/mL, and \(k_a\) and \(k_b\) are the clearance rates for the alpha and beta phases with units h\(^{-1}\). Fitting was performed in MATLAB using the least squares method. For data sets with no \(t = 0\) time point, a plasma concentration of 50 %ID/mL was used for the initial concentration based on the assumption of a 2 mL plasma volume in mice. The plasma area under the curve in units %ID*hr/mL was calculated for each data set as:

\[
AUC = \frac{A + B}{k_a + k_b}
\]

The single exponential plasma clearance term can be calculated as:

\[
k_{\text{clear}} = \frac{1}{\left(\frac{AUC}{[Ab]_0}\right)}
\]

where \([Ab]_0\) is the initial plasma concentration of the targeting agent estimated as 50 %ID/mL (again assuming a 2 mL mouse plasma volume). When not directly stated in the reference, molecular radii were estimated as \(R = 0.912*\text{MW}^{0.333}\) for globular proteins and \(R = 0.44*\text{MW}^{0.65}\) for PEG chains. The PEG relationship was fit from data in supplemental reference (32).
### B.5 – Max tumor uptake data

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<th>Radius (nm)</th>
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<td>$^{99}$Tc</td>
<td>SKOV-3</td>
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<td>(33)</td>
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<td>(18)</td>
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<td>39.8</td>
<td>(22)</td>
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Table B.5 - Peak tumor concentrations for HER2 targeting molecules of various sizes. Data points were collected from mouse biodistribution studies reported in the literature. All studies report the uptake of HER2 binding molecules in HER2 expressing tumor xenografts. For each biodistribution time course, the highest reported concentration of targeting agent in the tumor was included in the data set. Tumor uptake measurements with only a single time point were excluded. Additionally, only data sets using residualizing radiometal labels ($^{99}$Tc, $^{111}$In, and $^{64}$Cu) were included. The slow clearance of these labels from the tumor helps maintain tumor concentrations close to the peak level for longer periods of time and reduces errors due to infrequent sampling. For cases in which multiple affinity variants of a single molecule were tested, only the uptake for the highest affinity molecule was included. When not directly stated in the reference, molecular radii for globular proteins were estimated as $R = 0.912 \times MW^{0.333}$. Data values reported in graphical form were estimated using the DataThief program (http://www.datathief.org/).
Appendix C – Site-specific PEGylation of anti-CEA ds(scFv) molecules

Introduction

Conjugation to polymer chains such as polyethylene glycol (PEG) is a well-characterized method for increasing protein molecular weight. The random conformation of PEG chains and their ability to coordinate water molecules produces a large effective hydrodynamic radius. PEGylation has been shown to reduce renal filtration and increase serum persistence, as well as protect proteins from protease cleavage and immunogenicity (37). As described in Chapter 3, reducing serum clearance can increase tumor uptake, particularly for small targeting agents such as antibody fragments and peptides (38-40).

NHS-activated PEG chains can be conjugated to proteins through random reaction with primary amines on surface lysines. The products are typically heterogeneous, however, and may exhibit reduced activity if PEG chains react near the antibody active site (39). Alternatively, PEG molecules can be site-specifically reacted with a free cysteine residue on the protein, including cysteines native to the molecule or inserted through mutagenesis (41-43). By inserting the cysteine residue distal to the antibody active site, binding activity is typically less significantly impacted (43,44). Here we describe a method for site-specifically reacting maleimide-PEG molecules to a c-terminal cysteine in a disulfide stabilized scFv. We also describe how these PEGylated antibody fragments can be used to make monovalent quantum dot conjugates for imaging single receptors on cells.

Methods

Protein production
Plasmids encoding disulfide stabilized variants of the anti-CEA scFvs shMFE and sm3E plasmids were constructed as described in Chapter 2. QuikChange mutagenesis was performed to insert a free cysteine at the c-terminus of the ds(scFv)s directly preceding the His tag to produce ds(shMFE)-cys and ds(sm3E)-cys. Mutations were confirmed by sequencing. Plasmids were transformed into the YVH10 strain of yeast using the EZ Yeast Kit (Zymo Research) and plated on SD-CAA media supplemented with 40 μg/mL tryptophan. Individual colonies were grown in 1 L flasks and secretion induced for 48 hours at 37°C as described previously (45). The cleared supernatant was concentrated using a 10 kDa ultrafiltration membrane (Millipore) and the His-tagged protein purified with Talon metal affinity resin (BD Biosciences) following the manufacturer’s batch-column protocol. Monovalent scFvs were further purified by size exclusion chromatography on a Superdex 75 column (GE Healthcare) and eluted into PEGylation buffer (100 mM Na₂HPO₄, 500 mM NaCl, 2 mM EDTA, pH 6.5).

Site-specific PEGylation

ds(shMFE)-cys or ds(sm3E)-cys at a concentration of 0.5 – 1 mg/mL was co-incubated with a 5 fold molar excess of 5 kDa or 20 kDa PEG-maleimide (Nektar) and immobilized TCEP reducing gel (Pierce) at a concentration of 150 μL gel per 1 mL reaction (Figure C.1A). Using TCEP resin instead of soluble TCEP or DTT to reduce the c-terminal cysteine prevented reduction of the partially buried disulfide bonds in the protein. The reaction mixture was incubated at 25°C for 5 hours on a rocker. The TCEP resin was then removed by centrifugation and the unreacted PEG by ion exchange chromatography on a Hi-Q column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.2. PEGylated scFvs were separated from unconjugated scFvs on a Superdex 75 column at a flow rate of 0.5 mL/min and eluted in PBS. The PEGylation efficiency and
conjugate purity were assessed by SDS-PAGE electrophoresis on a 12% Bis-Tris gel (Invitrogen). Binding activity of the 20 kDa PEGylated molecules was tested by Kₐ titrations on fixed LS174T cells as described previously (46).

**Monovalent conjugation to quantum dots**

As part of a collaboration with Dr. Mark Howarth and Professor Alice Ting to make monovalent antibody conjugated small quantum dots (sQDs), the 5 kDa PEGylated ds(sm3E) molecules were incubated at varying concentrations with the sQDs for 1 hour in PBS (47). The conjugates were then separated on a 1% agarose gel, visualized under UV light, and the single antibody-sQD band excised and purified. Cell labeling of the conjugates was tested on CHO A7 cells co-transfected with CEA and Blue Fluorescent Protein (BFP). Cells were incubated for 10 minutes at 4°C with 20 nM sQD-ds(sm3E)-PEG₅ and imaged live. CHO A7 cells transfected with AP-LDL instead of CEA were used as a negative control, as well as non-conjugated sQD molecules.

**Results and Discussion**

**Protein production and PEGylation**

The ds(shMFE)-cys and ds(sm3E)-cys proteins were secreted in yeast at similar levels as the non-cysteine containing variants ds(shMFE) and ds(sm3E) with final purified yields on the order of 1 mg/L. The molecules secreted as a mix of disulfide stabilized monomers, dimers, trimers, and higher order aggregates, which could be separated by size exclusion chromatography to isolate the monomeric species. The purified monomers did not react directly with maleimide-PEG, suggesting that the C-terminal cysteine was not reactive and likely in a mixed disulfide with glutathione or some other small molecule. Attempts to reduce the C-terminal cysteine with
soluble DTT or TCEP prior to PEGylation led to the formation of multiple high MW PEG-scFv bands suggesting that the interdomain disulfide bonds were being reduced and reacting with the PEG (data not shown). In contrast, reduction with immobilized TCEP reducing gel (Pierce) during conjugation led to the formation of a single dominant PEG-scFv band at the appropriate size. Presumably, the large size of the agarose resin sterically precludes the TCEP molecules from reaching the internal disulfide bond while maintaining access to the exposed C-terminal cysteine residue. Additionally, since TCEP, unlike DTT, is poorly reactive with maleimide, the reduction and conjugation steps could be carried out simultaneously. The final conjugation efficiency was 60-80% and the desired PEG-scFv molecule could be purified to >95% purity by ion exchange and size exclusion chromatography (Figure C.1B).

---

**Figure C.1 – Site-specific PEGylation reaction.** A: Schematic of the reaction conditions. Immobilized TCEP resin was used to specifically reduce the C-terminal cysteine residue which was then conjugated with maleimide activated PEG. The X denotes an unknown species, presumably glutathione, that forms a mixed disulfide with the cysteine during secretion. B: Coomassie stained Bis-Tris gel showing ds(sm3E)-PEG20 in lane 1 and non-PEGylated ds(sm3E) in lane 2.
Quantum dot conjugation

In a collaborative project with Dr. Mark Howarth and Professor Alice Ting at MIT to create single molecule imaging reagents, 5 kDa PEGylated sm3E variants were conjugated to small quantum dot (sQDs). The conjugation reaction creates a heterogeneous product with varying numbers of antibody fragments per sQD molecules (Figure C.2A). The different species could be resolved and the desired monovalent conjugate purified by 1% agarose gel electrophoresis (Figure C.2B). In contrast, sQD reaction with a non-PEGylated ds(sm3E) molecule produced heterogeneous conjugates that were not sufficiently different in size to be resolved and purified on the gel (data not shown). The ds(sm3E)-PEG$_5$-sQD conjugates retain their binding activity to CEA as demonstrated in live cell imaging studies (Figure C.2C).
Figure C.2 – Production and characterization of ds(sm3E)-PEG₅-sQD conjugates. A: 1% agarose gel showing conjugates formed at a variety of antibody–sQD ratios. As expected, an increase in the relative concentration of antibodies produces molecules with several antibodies per sQD. B: The desired single antibody species can be resolved and excised from a 1% agarose gel. C: The ds(sm3E)-PEG₅-sQD conjugate labels CEA expressing CHO cells. Control experiments using non-CEA expressing cells (AP-LDLR) or non antibody conjugated sQD molecules produce no labeling.

Works Cited


(45) Graff CP, Chester K, Begent R, Wittrup KD. Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. Protein Eng Des Sel. 2004;17:293-304.
