Vascular Targeting of Nanocarriers: Perplexing Aspects of the Seemingly Straightforward Paradigm

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ABSTRACT Targeted nanomedicine holds promise to find clinical use in many medical areas. Endothelial cells that line the luminal surface of blood vessels represent a key target for treatment of inflammation, ischemia, thrombosis, stroke, and other neurological, cardiovascular, pulmonary, and oncological conditions. In other cases, the endothelium is a barrier for tissue penetration or a victim of adverse effects. Several endothelial surface markers including peptidases (e.g., ACE, APP, and APN) and adhesion molecules (e.g., ICAM-1 and PECAM) have been identified as key targets. Binding of nanocarriers to these molecules enables drug targeting and subsequent penetration into or across the endothelium, offering therapeutic effects that are unattainable by their nontargeted counterparts. We analyze diverse aspects of endothelial nanomedicine including (i) circulation and targeting of carriers with diverse geometries, (ii) multivalent interactions of carrier with endothelium, (iii) anchoring to multiple determinants, (iv) accessibility of binding sites and cellular response to their engagement, (v) role of cell phenotype and microenvironment in targeting, (vi) optimization of targeting by lowering carrier avidity, (vii) endocytosis of multivalent carriers via molecules not implicated in internalization of their ligands, and (viii) modulation of cellular uptake and trafficking by selection of specific epitopes on the target determinant, carrier geometry, and hydrodynamic factors. Refinement of these aspects and improving our understanding of vascular biology and pathology is likely to enable the clinical translation of vascular endothelial targeting of nanocarriers.

KEYWORDS: drug targeting · intracellular delivery · nanoparticles · nanomedicine · nanocarriers · endothelium · cell adhesion molecules · ICAM-1 · ACE · PECAM

Objectives and Challenges of Vascular Targeting of Nanocarriers. Drug Delivery Systems (DDS). DDS promise to improve pharmacotherapy by optimizing: (i) drug solubility, (ii) its isolation from the body en route to the target site, (iii) its pharmacokinetics (PK) and biodistribution (BD), (iv) control of activity, (v) permeation through biological barriers, and (vi) targeting (Figure 1).¹⁻⁵ Nanocarriers for drug delivery have diverse chemical contents,⁶⁻¹⁰ morphologies, surface features, geometries, and physical properties.¹¹⁻²⁰ Intricacies of their design have been reviewed elsewhere.²¹⁻²⁴ This article instead focuses on the biological aspects of carrier interactions with target cells.

DDS and drugs are eliminated by the reticuloendothelial system (RES, including liver, spleen, and lymphatic nodes) and other tissues—kidneys, lungs, and the bile tract.²⁵,²⁶ A carrier can "passively" accumulate in a desired sites. For example, particles in the size range ~10⁻200 nm tend to accumulate in tumors and inflammation foci due to enhanced permeability of pathological vasculature.²⁵,²⁷ (Enhanced Permeation and Retention, EPR, Figure 2B). Nonspecific retention of carriers in the microvasculature is another example of "passive targeting."²⁸ (Figure 2C). It is exemplified by perfusion imaging using mechanical entrapment of particles with diameter 20⁻50 μm²⁹ and delivery of plasmid DNA using cationic liposomes that bind to negatively charged vascular cells.³⁰ Delivery is enriched in areas downstream from the site of injection, where released drug is removed by blood. "Passive targeting" provides little, if any, guidance in cellular delivery.

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Active targeting is a more precise approach (Figure 2D). It uses ligands that bind to molecules uniquely present or enriched in a cell, tissue, or pathological structure of interest (target determinants). Antibodies and their derivatives including single chain antigen binding fragments (scFv), nutrients, hormones, receptor ligands, peptides, aptamers, and nucleic acids have been explored as targeting ligands.31/C0

Targeting involves DDS delivery to the target site, initial physical contact, anchoring, residence on the cell surface or internalization, and excretion or storage.36–41

Vascular Endothelium: Drug Delivery Barrier and Destination. Intravascular injection, notwithstanding its downsides, is a preferable route for drug carriers, and their encounters with endothelial cells lining the vessels are involved in practically every conceivable...
Drug delivery paradigm \(^{31,42,43}\) (Figure 3). Carriers designed for prolonged circulation must avoid binding to endothelial cells to minimize carrier’s elimination, danger of impeding blood flow and adverse effects via perturbation of these cells \(^{44,45}\) (Figure 3A), which exert important functions. \(^{46–48}\)

The endothelium controls vascular permeability. \(^{49}\) In the vascular sinuses of the RES organs, interendothelial openings are patent for micrometer-size objects. In lungs, heart, skin, mesentery, muscles, and most of other vascular areas, the endothelium transports particles in the range of \(\sim 50 – 500 \text{ nm}\) via dynamic intercellular gaps and transcellular fenestrae and vacuolar pathways initiated in endocytic vesicles. \(^{50,51}\) These pathways are restricted in cerebral vessels (the blood–brain barrier, BBB) \(^{52}\), where transport occurs via specific receptors and transporters. In conditions such as inflammation, thrombosis and ischemia, vascular permeability increases, mostly due to endothelial contraction widening pericellular gaps. Veins (especially venules \(^{53}\)) and capillaries are more permeable than arteries. \(^{39,54–57}\) Pathological vascular leakiness in tumors and inflammation foci favors DDS transport to these sites, while transcellular pathways support passive extravascular delivery in normal vasculature \(^{54,58,59}\) (Figure 3B).

The endothelium controls the following: (i) blood fluidity and hemostasis; (ii) vascular tone, signaling, and angiogenesis; and (iii) trafficking blood cells. \(^{47,48,60}\) Its abnormalities are implicated in the pathogenesis of ischemia, thrombosis, inflammation, tumor growth and metastases, diabetes, hypertension, stroke, atherosclerosis and other maladies. In these conditions, endothelial cells represent therapeutic targets (Figure 3C) \(^{38,43,61,62}\). Yet, drugs and DDS have no natural affinity to endothelial cells. Conjugation with endothelial ligands enables delivery to, into, or across these cells (e.g., “vascular immunotargeting”, Figure 3C) \(^{63–68}\). Since the late 1980’s, numerous groups have pursued this strategy. \(^{68–77}\)

Endothelium is more accessible to circulating agents than many other targets such as extravascular tumor cells. Yet, endothelial targeting has its own challenges. For example, concerns about side effects are more serious in endothelial relative to tumor targeting. Diverse medical goals require distinct and precise subcellular addressing of cargoes delivered using endothelial targeting. For example, fibrinolytics need to be anchored to the luminal surface, enzyme replacement therapies for storage disorders need to be delivered into the lysosomes and antitumor agents need to go across the endothelial monolayer (Figure 3C) \(^{33,63,68,78}\).

Many chronic conditions involving the endothelium (e.g., hypertension, atherosclerosis, diabetes, and arthritis) do have pharmacological options, tempering the enthusiasm for development of complex targeted DDS that may cause problems when used repeatedly. Acute, life-threatening conditions lacking effective therapeutic options (e.g., sepsis, acute lung injury, ischemia, infarction, thrombosis and stroke) provide a more attractive clinical context for endothelial nanomedicine. We focus on circulation, cellular binding and transport of carriers targeted to endothelial cells, envisioned for use in these acute conditions (and, perhaps, beyond). For the sake of cohesiveness, we present here a discussion of drug delivery, not imaging, applications—despite this being a tremendously important and extensively developed area. \(^{79–89}\)

**Nanocarrier Behavior in the Circulation.** To deliver drugs to endothelial cells, carriers must circulate in the
bloodstream for a period of time sufficient for distribution in the vasculature. This time is less than a minute in animals with a high heart rate and small blood volume, but varies from several minutes to tens of minutes in humans, depending on their health status. Characteristics of carrier design and biological factors control its pharmacokinetics and biodistribution (PK and BD) and influence targeting.

The pace of binding correlates with carrier avidity, concentration, perfusion rate, and the density of binding sites. Avidin-coated particles bind to biotinylated surfaces almost instantaneously due to their extraordinarily high avidity, while antibody-coated carriers perfused over endothelial cells require minutes to hours to achieve sufficient interactions with target cells that allow for firm adherence to the cell. Further, endothelial targets that appear in the pathological sites may require more time for binding due to inefficient perfusion. Conversely, a carrier’s ability to circulate for a prolonged time, find the intended vascular network and bind to newly exposed molecular signatures of vascular pathology would be of great diagnostic and therapeutic utility.

Carrier Circulation in the Vascular System. The intravenous routes bypassing the liver direct the first pass of blood to the lungs. The pulmonary vasculature represents ~25% of the total endothelial surface in the body and collects the entire cardiac output of venous blood from the right ventricle, whereas all other organs share the arterial output of equal volume. A local intra-arterial infusion via a catheter advanced to the conduit vessel favors first-pass carrier interaction with vascular cells in an organ or a vascular area downstream the vessel. Microvasculature (arterioles, capillaries, and venules) is the preferable target for endothelial nanomedicine. Extended luminal surface area, micrometer-scale vessel caliber, and low flow rate favor interaction of particles with endothelium in this vascular segment. Hydrodynamic conditions in arteries (high shear stress and pulsatile flow) are less favorable for particle interactions with endothelium than in veins. A significant (if not predominant) fraction of transport from blood to arterial walls occurs from the vasa vasorum, i.e., the microvascular network in the external adventitia layer.

The nature of the flow and perfusion govern particle behavior in the bloodstream. Laminar flow carries particles with minimal frequency of collisions with vessel walls. At sites of disturbed flow and turbulence, either physiological (vessel bifurcations, heart chambers) or pathological (atherosclerotic plaque, aneurism, coarctation), carriers are more likely to collide with the endothelium. Therefore, carrier delivery to sites involved in or predisposed to pathology is naturally favored. For example, micrometer-sized carriers composed of polymeric nanoparticles that dissociate under high shear stress accumulate in site of vascular stenosis. However, perfusion insufficiency downstream of an obstruction caused by vasospasm, thrombus, or surgical ligatures diminishes carrier delivery to ischemic zones. In addition, blood stasis inhibits delivery to vasculature above the occlusion site.

Carrier Longevity in Circulation: Surface Properties, Stealth Coating, and Ligand Attachment. Particles, similar to dead cells and their fragments, are marked for elimination from the circulation by opsonization, i.e., adsorption of immunoglobulin, complement, and other plasma components that stimulate uptake by phagocytes in the RES and other tissues. Unless protected (see below), carriers are eliminated quickly following their injection. Generally, hydrophobic and charged carriers are opsonized and eliminated more rapidly than their hydrophilic and neutral counterparts.

Coating with hydrophilic molecules (e.g., poly(ethylene glycol), PEG) that form a hydrated shell decelerates opsonization, inhibits a carrier’s interaction with phagocytes and other cells and extends circulation lifetime. However, PEG chains may inhibit interactions of ligand molecules immobilized on the carrier with the target (Figure 4A). Both the masking and stealth effects are proportional to PEG chain length and surface density. Ligand conjugation via PEG alleviates the masking effect. Furthermore, ligand conjugation via PEG provides a flexible spacer that may improve the ligand steric freedom for interactions with target components (unless the spacer is so extended that it can “fold in” hiding the ligand). Conversely, conjugated ligand molecules diminish the stealth features of PEG coat (Figure 4B).

A carrier’s stealth and targeting features may be optimized by conjugating corresponding moieties using linkers responsive to local stimuli of the target environment, such as temperature, pH, or proteolytic activity. For example, PEG conjugated via a cross-linker stable at physiological plasma pH 7.4 but labile at acidic pH will shed from a fully stealth carrier once it arrives at the acidic target site, therefore exposing ligands for binding. Practical implementation of “environmentally” sensitive nanocarriers depends on fine-tuning the dynamic range of their response. For example, unrelated proteases may impede the tissue selectivity of proteolytic transformation of carriers by enzymes preferentially active at target sites, such as a specific metalloproteinase. It is also difficult to fine-tune a carrier’s response to smaller pH changes, such as pH 6.0–6.5 vs <4.0, typical of ischemic and inflamed vasculature vs lysosomal vesicles, respectively.

PEG is generally viewed as a biocompatible compound. Yet, the human immune response to PEG needs to be carefully assessed. PEG antibodies have been shown to form upon repeated administration of PEGylated compounds in animals. This phenomenon has been shown for a variety of carriers,
ranging from proteins to liposomes. One possible mechanism for the accelerated blood clearance of PEGylated carriers upon repeated administration is the initial generation of anti-PEG antibodies (IgM) in the host, which persist in serum and bind to reinjected PEGylated carriers, reducing the circulation time. This initial anti-PEG IgM generation occurs in the spleen and likely involves B-cells, as B-cell deficient mice do not exert accelerated clearance of PEG-coated carriers.

Coating carriers with molecules inhibiting activation of complement, opsonization, and phagocytosis such as sialic acid-containing glycolipids has been also pursued. More recently, specific biologically inspired means have been used to endow natural stealth properties to particulate carriers. For example, cells contain a membrane glycoprotein CD47, which interacts with receptors of macrophages eliciting signals that inhibit phagocytosis. Conjugation of CD47 and CD47-derived peptides to carriers helps to evade clearance via this natural “self-recognition” mechanism. Another approach to increase blood persistence of carriers is coating with fragments of cell membrane. Model PLGA carriers coated with mouse red blood cell (RBC) membranes circulated twice as long as PEGylated PLGA particles.

In a similarly inspired study, leukocyte membrane coating inhibited carrier association with phagocytes and reduced hepatic uptake at short time points (<60 min) in vivo.

Carrier Geometry and Plasticity. Neither too small nor too large particles circulate well. Renal filtration eliminates particles smaller than 10 nm. In addition, they extravasate via transendothelial fluid phase transport pathways, leading to elimination in skin, lungs, mesentery, muscles, and other tissues with extended surface of fenestrated microvasculature. On the other hand, particles larger than ~500 nm are mechanically entrapped in capillaries. Agglomeration mediated by plasma components likely further enhances the latter outcome.

Carrier shape is an important factor of its behavior in the circulatory system and targeting. This is an area of research of astonishing complexity in every aspect: synthesis and quality control of carriers, rheological modeling and analysis, and appraisal of the biological relevance. The fundamental finding, confirmed in animal studies, is that elongated carriers of an appropriate size align with blood flow and have prolonged circulation in the bloodstream. For example, polystyrene elliptical disks with a maximal dimension of up to a few micrometers display a higher level in blood and lower nonspecific uptake in organs than spherical counterparts of a smaller size.

One reason for the prolonged circulation of elongated objects is that they elude uptake by phagocytes. Macrophages internalize opsonized disk-shaped particles contacting the cell by the rounded
end of the particle as effectively as spherical particles but cannot completely internalize disk-shaped particles contacting on their flat face (Figure 5). In support of the notion that nonspherical geometry may inhibit phagocytosis, filamentous particles coated with phagocytosis-promoting ligands were able to avoid phagocytosis by macrophages in cell culture.144

Carrier flexibility also has an important influence on circulation and targeting. Rigid elongated particles (rods, disks, tubes) larger than a few micrometers quickly become entrapped in the microvasculature in vivo. However, carriers with sufficient plasticity to reversibly change shape in response to hydrodynamic factors and vessel narrowing, can pass through the microcirculation, thereby imitating erythrocytes: ~7 μm discs that repeatedly squeeze through capillaries with 1–2 μm lumen and effectively avoid clearance by immune cells. Indeed, red blood cell mimetic particles were shown to have extended in vivo circulation times.Flexibility was tuned via the extent of cross-linking and resulted in plasticity similar to that of red blood cells. Enhancing carrier’s flexibility prolongs its circulation. Follow-up studies with similarly flexible particles varying in size have shown that particles closer in diameter to red blood cells have prolonged circulation half-lives.

Elongated flexible carriers have also been developed. Filomicelles are a distinctive subset of micelles made of amphiphilic block copolymers that assemble in flexible flow-responsive filaments. Filomicelles are able to persist in blood for extended periods of time by taking advantage of both their wormlike shape and cell-like flexibility. Their ability to align with blood flow and avoid immune system clearance enables them to persist in circulation for days, which is up to 10 times longer than their spherical counterparts. Pristine filomicelles exhibit little to no cellular binding, an attractive feature for a long-circulating DDS.

Challenges and Perplexing Issues. Studies of carrier behavior in vivo are challenging and prone to artifacts. Often, researchers bypass them and test in vivo effects of drugs loaded in DDS characterized only with a variable degree of scrutiny in vitro, betting that observation of a desired effect exceeding that of “untargeted drug” provides a mandate to publish “proof of concept” papers. However, effects in animals are of limited value if the mechanism of delivery and effect of the DDS is not known. PK and BD directly influence targeting, and vice versa. It is essential to compare the PK and BD of targeted vs untargeted carriers formulated identically and coated by scrambled or inactive ligand (control IgG of corresponding type is a reasonable control for targeting antibody). Pristine carriers are not a proper control (different size, charge, surface features, etc.). Carriers with ligands conjugated to PEG have shorter PK and different BD vs pristine PEGylated carriers (Figure 4). All these factors may alter the therapeutic/side effects of a ligand-coated vs uncoated carrier by mechanisms distinct from targeting.

Preferential uptake in the target site may originate from mechanisms irrelevant to the ligand, e.g., passive retention of aggregated particles or interactions with blood components that may serve as “endogenous targeting agents” (lipoproteins, transferrin, immunoglobulin, blood cells, etc.). Competitive inhibition of targeting by injected free ligand may be complicated by different PK/BD and avidity of free ligand vs those of ligand-coated carriers, or simply because an effective competitive concentration in blood is above a realistically achievable one.

Thus, a quantitative analysis of PK/BD is essential; however, its implementation is not always feasible. Optical methods reveal nanoparticles in tissues at the microscopic level at post-mortem and at macroscopic level in real time (in sufficiently transparent sites) but do not provide accurate quantitative measurements in the body. Isotope imaging including PET is not limited by tissue penetration and affords real-time longitudinal analysis, but quantitative measurement of signals in organs is convoluted by factors including overshadowing by sites with high basal uptake. The resolution of isotope imaging is insufficient to analyze cellular distribution.

Another issue that is associated with this field is that labeling of labile components of a nanoparticle can lead to artifacts of their dissociation in the body. Labeling ligand moieties is prone to artifacts of tracing detached ligand leading to overestimating targeting. Noncovalent intercalation of hydrophobic labels in carriers is marred by artifacts of their redistribution in cellular membranes and other biological sinks, such as lipoproteins. Ideally, both the cargo and carrier should be stably traced by conjugated labels. A direct stable conjugation of isotopes to polymeric matrix allows...
tracing of the carrier within a reasonable time frame from a few hours to a few days, usually sufficient for vascular targeting.\textsuperscript{159}

A direct measurement of the isotope level in drawn blood samples is arguably the best method for PK studies.\textsuperscript{74,76,90,160–162} Admittedly, extensive experimentation is needed to characterize the kinetics of targeting. Nevertheless, this simple approach affords the most accurate quantitative analysis of preclinical PK/BD. It yields the key parameters of targeting and BD: percent of injected dose (%ID) in tissues, localization ratio (LR, or ratio of %ID per gram of tissue to that in blood), and immunospecificity index (ISi, or ratio of LR for targeted vs untargeted formulations normalized to their blood levels).\textsuperscript{76,163} Injecting in the same animal a mix of targeted and untargeted formulations labeled by different isotopes is a useful maneuver to account for individual variability and significantly reduce the necessary efforts. Carrier PK/BD must be normalized to the injected dose. DDS heterogeneity is a serious problem. A fraction of large particles in a formulation may be eliminated within seconds by mechanical retention and not accounted for by normalizing data to “initial” level detected in blood post-injection.

Further complication in studying the interactions of DDS is that the behavior of nanoparticles in vivo can be drastically different than that in vitro. Nanoparticles bind blood components, aggregate, and dissociate.\textsuperscript{164} Even dense PEG-coating of polymeric carriers does not prevent eventual absorption of plasma components.\textsuperscript{165} Generally, carrier interactions with body components influence desired as well as adverse effects, including accumulation in the target and uptake by off-target cells.\textsuperscript{167,168} Yet, these processes remain poorly understood: there are limited studies on carrier behavior in blood even in vitro (e.g., DLS measurements in plasma are not informative). The methodology of studying nanoparticles in vivo also continues to evolve: a recent study employed a new high-throughput approach to study deposition of plasma proteins on model polymeric particles.\textsuperscript{169} Better analytical techniques are necessary to understand these interactions. For example, the use of citrate anticoagulated plasma in such studies is questionable because many mechanisms are not active in such a specimen, as opposed to serum.\textsuperscript{170–172}

Generally, advanced approaches for characterization of DDS behavior in vivo are needed to provide objective and accurate information on carrier PK/BD. Such data will provide valuable insight about targeting efficacy, kinetics, and specificity. It will also shed light on the mechanisms of cell-specific interactions.

**Carrier Binding to Endothelium.** Anchoring to specific cells is a key step in targeting. It is controlled by ligand affinity and configuration (surface density, interactive freedom, etc.) as well as other features of the nanoparticle (size, charge, PK/BD, geometry, plasticity, etc.).

Equally important are target features: surface density, clustering and accessibility of binding sites, phenotypic characteristics, and the microenvironment of target cells (perfusion, pH, protease activity, etc.). Carrier interactions with target determinants govern the specificity, selectivity, efficacy, fate, and safety of the DDS.

**Endothelial Determinants for Nanocarrier Targeting.** A target determinant should ideally meet the following criteria: (i) it anchors a carrier to endothelium in the area of interest; (ii) it provides desirable subcellular addressing; and (iii) it is not adversely affected by carrier binding in the context of disease treatment.

Selective proteomics of the endothelial plasma-lemma\textsuperscript{64,173} and in vivo phage display\textsuperscript{72} are advantageous for finding new targets, as they identify binding sites accessible from circulation in normal\textsuperscript{174} and pathological vasculature.\textsuperscript{175} The list of endothelial determinants theoretically useful for vascular drug targeting continues to grow.\textsuperscript{75,176–178} Table 1 briefly outlines the main features of the most intensely studied and promising candidates; more details are provided in this section.

**Target Molecule Expression, Localization, And Accessibility.** Not all endothelial target determinants are “endothelial-specific markers”, such as E-selectin. The utility for vascular targeting depends on relative levels in endothelial vs other cells accessible to blood. For example, ICAM-1 is expressed by fibroblasts, epithelial, and muscle cells at levels comparable with those in endothelium. Nevertheless, these extravascular cells are not accessible to circulating macromolecules and carriers and hence do not compete with endothelial targeting. In contrast, cytokine and transporter receptors are abundantly expressed on cells in blood, RES, lymphoid tissues, hepatocytes, and other compartments accessible to circulating DDS and, therefore, provide very modest (if any) endothelial delivery.

Constitutive determinants (e.g., PECAM, ACE, and APP) are suited for prophylactic drug delivery. Some, including APP and ACE,\textsuperscript{27,28} disappear from the endothelium in pathological conditions, which inhibits targeting.\textsuperscript{179,180} Constitutive molecules stably exposed in the lumen, such as PECAM, can be theoretically used for prophylactic and/or therapeutic effects.\textsuperscript{161} In contrast to constitutive molecules, inducible counterparts expressed or exposed to the lumen in pathological sites (e.g., APN, TEM-1, VCAM-1, and selectins) are less likely to find prophylactic utility but seem preferable for diagnostic imaging and therapeutic interventions.\textsuperscript{39,65,68,71,181}

With respect to localization in the vascular system, endothelial surface molecules fulfill the continuum ranging from pan-endothelial to domain-specific determinants (i.e., expressed throughout the vasculature or in certain vascular areas). Pan-endothelial targets (e.g., PECAM) can be used for systemic delivery to treat generalized conditions: sepsis, disseminated...
### Table 1. Endothelial Determinants: Selected Candidate Targets for Drug Delivery

| Target | Vascular Localization | Cellular Localization | Functions | Regulation |
|--------|-----------------------|-----------------------|-----------|------------|
| ACE (CD143) | Ubiquitous, enriched in the lung capillaries | Cell surface, single pass type I membrane protein | Plays a key role in rennin-angiotensin system, regulates blood pressure. Converts Ang I into Ang II and degrades bradykinin. | Catalytic activity is increased by chloride. |
| TM (CD141) | Ubiquitous, specific for endothelial cells | Cell surface, single pass type I membrane protein | Receptor for thrombin, participates in the generation of activated protein C. | Oxidative stress, hypoxia, oxidized LDL, C-reactive protein, phorbol esters, cAMP, and TNF tend to downregulate TM expression. Thrombin, VEGF, retinoic acid, and heat shock upregulate TM. TM level reflects a balance between those opposite forces. |
| PECAM-1 (CD31) | Ubiquitous | Cell junction, lateral border recycling compartment | Leukocyte transendothelial migration; antiapoptotic repulsion signaling from phagocyte | Stable expression |
| ICAM-1 (CD54) | Ubiquitous | Tetraspanin microdomains on cell surface, single pass type I membrane protein | Leukocyte firm arrest and transendothelial migration; immunological synapse formation. Ligand of LFA-1, MAC-1 | Up-regulated in inflammation |
| VCAM-1 (CD106) | Inflamed vascular endothelium | Tetraspanin microdomains on cell surface, single pass type I membrane protein | Leukocyte firm arrest and transendothelial migration. Ligand of VLA-4 | Up-regulated in inflammation |
| E-selectin (CD62E) | Inflamed vascular endothelium | Cell surface, single pass type I membrane protein | Leukocyte rolling | Up-regulated in inflammation |
| P-selectin (CD62P) | Inflamed vascular endothelium | Cytosol: Weibel-Palade bodies of endothelial cells, alpha-granules of platelets | Leukocyte rolling | Upon activation by agonists transported rapidly to cell surface |
| APP | Enriched in pulmonary vascular endothelium. Expressed in heart, liver, kidney | Enriched in caveolae on the luminal surface of endothelial cells; lipid-anchored to cell membrane | Caveolipid that plays a role in inflammatory process; cleaves and inactivates circulating polypeptides such as bradykinin | Unknown |
| PV1 (PLVAP) | Expressed in majority of tissues | Endothelial fenestrations, caveolar membrane | Formation of stomatal and fenestral diaphragms of caveolae, regulation of microvasculature permeability | Up-regulated by VEGF in vitro |
| APN (CD13) | Vasculature of tissues that undergo angiogenesis and in multiple tumor types | Cell surface, single pass type II membrane protein; cytosol | Metabolism of regulatory peptides, processing of peptide hormones (angiotensin III and IV, neuropeptides, and chemokines) | Estradiol and interleukin-8 decrease APN activity in vitro |
| Integrins α5β1, αvβ3, αvβ6, αvβ1 | Enriched in tumor vessels and other types of angiogenesis | Cell surface | Angiogenesis, tumor neovascularization, and tumor metastasis | αvβ3 is up-regulated by bFGF, TNF, unknown |
| TEM1 (CD248) | Tumor endothelial cells | Cell surface, single pass type I membrane protein | Tumor angiogenesis | αvβ3 is up-regulated by VEGF, TGF-α unknown |

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EC, endothelial cells; ACE, angiotensin-converting enzyme; TM, thrombomodulin; PECAM, platelet-endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; APN, aminopeptidase N; APP, membrane-bound aminopeptidase P; TEM-1, tumor endothelial marker-1; PV-1, plasmalemma vesicle-associated protein.
intravascular coagulation, or hypertension. The level of expression and surface density of many pan-endothelial determinants vary between organs and types of vessels. For example, ACE and thrombomodulin are expressed in the pulmonary capillaries at several fold higher level than in other organs, and antibodies to these molecules (anti-ACE and anti-TM) preferentially accumulate in the lungs. Domain-specific molecules, preferentially expressed in certain vascular areas, are attractive for local delivery. For example, inducible molecules APN and TEM-1 are upregulated in endothelium involved in angiogenesis in tumors and inflammation, whereas adhesion molecules VCAM-1 and selectins are expressed predominantly in sites of inflammation. Determinants differ with respect to preferential localization in domains of the endothelial plasmalemma. For example, PECAM and VE-cadherin are localized predominantly in cell–cell borders, VCAM-1 and ICAM-1 are found in tetraspanin microdomains of the cellular apical surface, special types of membrane “rafts” of distinct protein and lipid composition with a diverse array of function, including adhesion, proliferation, and immune cell signaling. Glycoprotein GP85 localizes in the luminal surface of the plasmalemma that belongs to a thin organelle-free part of endothelial cell separating alveoli from blood, and APP and PV-1 are located within caveoli. The location in the plasmalemma dictates the target accessibility, interference in cellular functions, and the fate of cell-bound DDS.

The endothelium is normally accessible to blood cells, lipoproteins, and other particles circulating in the bloodstream. Yet, accessibility to circulating particles varies dramatically among the endothelial surface molecules, depending on their localization in plasmalemma, location of the binding site epitope in the molecule, endothelial phenotype, and vascular conditions. Accessibility limitations are more stringent for carriers relying on multivalent binding and are proportional to the size of carriers. Epitopes located more proximally to the plasmalemma within the same target molecule are less suitable for harboring carriers than distal epitopes. Epitopes masked by the glycocalyx, buried in the intercellular junctions or in plasmalemma invaginations, are less accessible to affinity ligands and ligand-targeted DDS (Figure 6). Of note, shedding of endothelial glycocalyx induced by pathological mediators is implicated in enhanced accessibility of ICAM-1 to targeted carriers and activated leukocytes. On the other hand, adherent leukocytes may mask endothelial surface from blood.

Target Function and Safety. Functions of target determinants are important in the context of drug delivery. Molecules involved in transport from the bloodstream seem attractive to deliver drugs in the corresponding addresses. On the other hand, interference with functions of target molecules must be considered...
in the context of pathological condition(s) to be treated. For example, ACE and APP are peptidases that cleave important peptide mediators (e.g., bradykinin); their unintended inhibition may cause harmful side effects, including vascular edema. Conversely, inhibition of angiotensin II production by ACE may be beneficial in management of hypertension and inflammation.

The issue of adverse inhibition of the target is illustrated by thrombomodulin, an endothelial surface glycoprotein that has been studied for targeting of diverse cargoes to the pulmonary endothelium in mice and rats. However, thrombomodulin controls thrombosis, and its inhibition poses danger of thrombosis. This complication prohibits clinical use of this target, restricting its utility to model animal studies.

Ligand-coated carriers may inflict more potent effects than ligands alone. Cross-linking of target molecules by a multivalent carrier may induce ill-understood or adverse signaling in the endothelial cells, shedding and/or internalization of target determinants, changes in their functionality, or other disturbances of the endothelium. With the exception of targeting anticancer agents to tumor endothelium, endothelial drug delivery should not damage or disturb target cells.

A more general aspect of this issue that should be considered is that biocompatibility of a single component does not guarantee safety of the nanocarrier. Loading a benign agent into a carrier composed of biocompatible materials decorated by innocuous ligands may yield a combination with pro-inflammatory or advautous features. Systemic effects, such as activation of complement, coagulation or platelets, and toxicity toward the clearing tissues (liver, kidney, lungs, etc.), must be meticulously appraised in vivo.

**Endothelial Cell Adhesion Molecules.** Endothelial cell adhesion molecules (CAMs) represent arguably one of the most well-studied and versatile groups of endothelial determinants in the context of targeting nanocarriers. Inhibition of CAM-mediated leukocyte adhesion and signaling is generally viewed as safe in the context of many pathological conditions, except certain infections. Further, targeting to these molecules achieves localization of drugs to diverse endothelial compartments.

**Inducible Endothelial Cell Adhesion Molecules.** Vascular Cell Adhesion Molecule-1 (VCAM-1), P-selectin, and E-selectin are expressed on the surface of activated endothelial cells and facilitate the rolling phase of the vascular adhesion of leukocytes. Pathological factors including cytokines, oxidants, and abnormal flow cause surface exposure of P-selectin from intracellular storage within 10–30 min and induce de novo synthesis and surface expression of E-selectin and VCAM-1. E-selectin and VCAM-1 seem to be expressed by the activated endothelium in arteries and skin microvasculature to a higher extent than in the pulmonary vasculature.

Carriers conjugated with antibodies to these molecules bind to activated endothelium, which favors intracellular delivery into endothelial cells of E-selectin targeted liposomes, drugs, and genetic materials. Inducible adhesion molecules represent excellent determinants for visualization of activated endothelium in inflammation foci by delivery of conjugated isothiocyanates or ultrasound contrast agents.

**Constitutive Cell Adhesion Molecules PECAM and ICAM-1.** PECAM and ICAM (CAMs) are glycoproteins composed of a large extracellular region containing several Ig-like domains, a transmembrane segment, and a cytoplasmic tail mediating signaling. CAMs are present in cell types including platelets (PECAM), epithelial and smooth muscle cells (ICAM), and leukocytes (both), but among the cell types accessible to blood, surface levels of PECAM and ICAM are the highest in endothelial cells.

PECAM and ICAM are expressed on the endothelium throughout the vasculature. PECAM is stably expressed at a level of (0.2–2) × 10^6 copies per cell predominantly in the interendothelial borders. ICAM tends to localize in lipid rafts in the luminal membrane and may exist in either a monomer or oligomer form. Quiescent confluent endothelial cells in static culture express ICAM at very low levels, and treatment with cytokines or thrombin leads to 50- to 100-fold up-regulation. In contrast to such a dramatic difference in vitro, ICAM is expressed in blood vessels constitutively by quiescent endothelium in vivo at levels of ~0.2 × 10^3 binding sites for anti-ICAM per cell, while cytokines and other pathological mediators induce its additional synthesis and upregulate ICAM-1 surface expression roughly twice to the level of (0.5–3) × 10^5 sites per cell in endothelium and other cell types.

CAMs are involved in adhesion and trafficking of leukocytes and in vascular signaling. Their clustering by multivalent ligands initiates signal transduction via their cytosolic domains. Adhesion via these molecules supports leukocyte mobilization in sites of inflammation. Via its extracellular domain, endothelial PECAM engages in heterophilic binding to heparin-containing proteoglycans and integrins of leukocytes, and in homophilic PECAM–PECAM interactions. The extracellular region of ICAM binds ligands including fibrin, certain pathogens, and integrins of activated leukocytes, mediating their firm adhesion to endothelial cells. Therefore, PECAM and ICAM are involved in cellular recognition, adhesion, and migration of leukocytes. Inhibition of these functions may be beneficial in treatment of inflammation.
Affinity Ligands for CAM Targeting. PECAM and ICAM antibodies (anti-ICAM and anti-PECAM) are popular ligands for experimental vascular targeting. These antibodies and antibody-carrying carriers and drug conjugates bind to endothelial cells and accumulate in vascularized organs after intravascular injection. Intravenous administration favors their uptake in the pulmonary vasculature, while local arterial infusions enrich accumulation in the downstream cardiac, cerebral, and mesentery vasculature.

Many groups have devised nanocarriers targeted to CAMs by conjugated antibodies. Designer affinity ligands for CAMs have also been devised, including scFv fragments and affinity peptides selected using a phage display library. Advantages of using scFv include lack of Fc-fragment mediated effects and feasibility of scaled-up GMP production of recombinant constructs. A humanized monoclonal antibody binding to human ICAM with 50-times higher affinity than parental mouse anti-ICAM has been produced as well as multivalent Fab fragments of a monoclonal antibody to human ICAM.

Some of these recombinant proteins have been clinically tested as anti-inflammatory agents and showed generally acceptable safety and toxicity. More recently, a short 17-mer linear peptide derived from one of ICAM’s natural ligands, fibrin, provided endothelial targeting of nanoparticles on par with ICAM antibodies in animal studies.

This type of ligand offers the advantages of a reduced risk of immune reactions and utility in diverse animal species.

Carrier Binding to Endothelium. Targeting is controlled by carrier avidity, defined by ligand affinity, density, and freedom to interact with target. The carrier geometry and plasticity modulate the latter parameter and carrier ability to engage in multivalent binding. The binding is also controlled by target features (surface density, accessibility, and organization of epitopes) and hydrodynamics, which all may change under pathological conditions.

Hydrodynamics. Reviewing the extensive literature on rheological control of interactions of ligand-coated particles with the vessel wall, pertinent to both adhesion of leukocytes and carrier targeting, is beyond the scope of this paper. The majority of these studies involved particles of micrometer size and surfaces coated with immobilized molecules or endothelial cells in vitro. These models allow quantitative analysis under controlled conditions, but revealed trends need in vivo validation in the types of vessels and vascular areas of interest.

For example, several studies have indicated that binding is inversely correlated with shear stress. Computational analysis showed that the shear stress influence diminishes for nanocarriers coated with antibodies at a surface density above a threshold level. Modeling studies considering the contribution of buoyancy, hemodynamic forces, van der Waals, electrostatic, and steric interactions between circulating particles and the endothelium identified a critical diameter of 200 nm for which the margination time is maximal. It was proposed that carriers either larger or smaller than this size may have an advantage anchoring on the endothelium. Studies in a parallel plate flow chamber indicated that particles with a diameter greater than 200 nm undergo the most effective margination due to sedimentation in horizontal capillaries and lateral drift in vertical capillaries with downward flow, enhancing the likelihood of adhesion to the endothelium.

Similar experiments in a flow chamber model evaluated binding of spherical particles ranging from 100 nm to 10 μm coated with endothelial ligand. Binding increased with an increase in diameter from 0.5 to 10 μm at a shear rate of 200 s⁻¹ typical of arteries, whereas a further increase in shear favored binding of 2–5 μm particles. One explanation is that the dragging force of the highest shear stress applied to larger particles prevails over the adhesive force. Carriers experience the hydrodynamic dragging force of blood proportional to their size; hence, the larger the DDS is the more target molecules need to be engaged to anchor DDS on the cell. Spherical carriers (1 μm) coated with ICAM antibody displayed a typical pattern of binding to endothelium in vitro dependent on the antibody surface density: move in the flow at high speed without visible interactions with cells, roll continuously over cells, roll first and then bind firmly to cells, or roll first and then detach thereafter traveling along the cell surface.

In some studies carriers were perfused in the presence of blood components. For example, RBCs are known to occupy the mainstream in high-shear vessels, expelling smaller particles to the marginal level, thereby directing their flow in the glycocalyx-protected layer and interaction with endothelial surface. Indeed, it was shown that addition of RBCs to the perfusion buffer stimulates binding of ligand-coated particles to the walls of target-coated model vessel. Recently, studies focused on the effect of circulating RBCs on carrier margination have shed light on an area known as the cell-free layer, which separates bulk RBC flow and the endothelial wall. Simulated binding, simplified as contact between carrier and endothelium, was performed in both large and small vessels in the presence and absence of RBCs. Due to the interaction of particle and cell, smaller particles are physically pushed by RBCs via volume exclusion. Likewise, an increase in particle dispersion coefficient is attributed to the increased rotation and tumbling of RBCs under increasing shear. These two contributions, increased particle dispersion and volume exclusion,
Carrier Geometry and Plasticity. As mentioned earlier, the carrier’s physical characteristics (e.g., shape) play a critical role not only in its circulation profile, but also in its binding to target cells. Studies with targeted DDS in vivo have shown that while endothelial localization may increase with size into the micrometer range, targeting specificity tends to have a maximum in the hundreds of nanometers range. A study comparing vascular targeting (through measurements of pulmonary uptake) of anti-ICAM coated polystyrene spheres with sizes of 0.1, 1, 5, or 10 μm showed that, while the overall %ID found in the lung increased with size, the targeting specificity decreased about 5 times (the ISI was \( \sim 10 \) vs \( \sim 2 \) for 100 nm and 1000 nm particles, respectively), due to more pronounced mechanical retention of large untargeted carriers coated with control IgG. Similarly, PECAM-targeted protein carriers showed increased pulmonary uptake with an increase in size from 200 to 800 nm, but nonspecific uptake of control nontargeted counterparts increased when the size was >300 nm.

Carrier enlargement aggravates accessibility issues. For example, determinants located in caveoli, invaginations with neck diameter <50 nm, are accessible to relatively small ligands such as antibodies but not to submicrometer carriers. In a similar example, 100 nm spheres targeted by antibodies to well-mapped epitopes of endothelial PECAM failed to bind to the cells when the antibody to the most distal of the plasmalemma epitopes was used, while the antibody itself showed excellent binding.

At a nanometer scale, it has been shown that elongated carriers bind to endothelium more effectively than spherical ones (Figure 7A). Specifically, polystyrene nanorods (aspect ratio \( \sim 3 \)) functionalized with anti-ICAM showed upward of 3-fold attachment to brain endothelial cells in static cell culture. Higher association of rod-shaped particles with endothelial cells was also exhibited in vivo as anti-ICAM coated rods exhibited \( \sim 2 \)-fold higher accumulation in target tissue (lungs) than that of spheres of identical volume. This was further investigated by coating nanorods and spheres with anti-transferrin-receptor monoclonal antibody. Rods exhibited almost an order of magnitude fold enhancement in attachment to brain endothelium.

In a computational model comparing spheres and nanorods, the binding probability of a nanorod under a shear rate of \( 8 \text{ s}^{-1} \) was found to be 3 times higher than that of its spherical counterpart. Anti-ICAM coated polystyrene disks showed enhanced targeting specificity over spheres (immunospecificity index, ISI, of 35.7). Previously discussed long-circulating filomicelles display a unique interaction with their target surfaces: biotinylated worm micelles were shown to “zip up” on avidin-coated surfaces due to their multivalent high affinity interactions. In comparing the in vivo targeting of ICAM-coated filomicelles and spheres, both resulted in similar specificity though the overall targeting was lower for filomicelles.

Flow-induced interactions play an important role in binding of nanoparticles to endothelium. Several devices have been synthesized and mathematical models have been developed to investigate and explain these phenomena. Mathematical modeling has shown that rod-shaped particles are more likely to adhere to endothelium than their spherical
counterparts under the same flow conditions due to the tumbling and rolling motions that rods will undergo near endothelial walls. Numerous studies supported by mathematical models by Decuzzi and Ferrari detail various advantages of nonspherical particles in cellular adhesion under flow: (i) oblate particles adhere more strongly to surfaces under flow and (ii) particles with radii under 100 nm, as is typically the case with elongated particles, are ideal for margination and interaction with the endothelium.

These interesting mathematical predictions are supported by in vitro flow studies designed to mimic in vivo flow. Notably, discoid particles have been shown to marginate to the wall under flow better than their spherical counterparts. BSA coated rod- and disc-shaped particles have been shown to adhere up to 5 times more than BSA coated spheres to anti-BSA microchannels (bifurcation geometry), and anti-ICAM coated rods were shown to adhere twice as well to ICAM-expressing endothelial cells over identically coated spheres under flow.

Further, rigid particles have shown to be greater than their flexible counterparts under the same stability of the bond and reduction of Fab affinity, among other potential factors. The transformational shift of DDS binding, which usually boosts targeting, is from a monovalent or bivalent ligand to a multivalent carrier. For example, an effective avidity of binding to endothelium of anti-ICAM coated carriers is markedly higher than that of free anti-ICAM. Multivalent binding helps retain large particles that experience more powerful detaching forces than a ligand molecule. For example, computational analysis revealed that at least three ICAM antibodies coupled to a spherical carrier with diameter 100 nm should engage simultaneously in binding to an endothelial cell in order to anchor the carrier. The key issue, therefore, is to find an optimal ligand configuration on the carrier that provides multivalent interactions with target molecules.

Generally, higher affinity/avidity is viewed as an advantage for targeting. For example, an increase in ligand density providing higher avidity results in an increase of vascular targeting. Conjugation of a number of ligand molecules per carrier less than a certain minimum leads to a failure of the carrier to bind to the target. Yet, an excessive ligand density beyond one that saturates binding sites may be undesirable due to costs and benefit/risk ratio. Additionally, studies of nanocarrier-based vaccines imply that carriers with high ligand density are likely to cause immune reactions.

Furthermore, in some cases, ligands with higher affinity yield inferior targeting. One scenario where this is the case is when high-affinity nanoparticles fail to penetrate into the target due to their retention at the surface of the target mass, such as tumors or thrombi. Although such a scenario is less likely in endothelial targeting, there are additional complicating considerations. Unlike free ligands, ligand—drug or drug—carrier conjugates require congruency with target molecules for multivalent binding, which does not necessarily correspond to the maximal ligand density. In some cases, as illustrated in Figure 8, an excessive ligand density may inhibit the binding of NCs to target cells. In this phenomenon, “ligand overcrowding” on the surface of a NC could inhibit individual ligand molecules from achieving the optimal orientation or congruency with clustered target molecules favorable for binding. This phenomenon was also observed using nanocarriers targeted with aptamers intended for tumor delivery. Beyond a certain threshold, further elevation of ligand density resulted in a significant decrease in tumor localization.

Defining the optimal surface density of a ligand, which may vary depending on the features of a carrier, ligand, target, and application, is a daunting and important task. Several factors could contribute to the outcome including carrier and ligand properties (size, orientation, density, etc.) and characteristics of the target molecule’s environment (membrane localization, glycocalyx, clustering, etc.). Quantitative
in vivo surface density and clustering of target determinants at a low basal level. Specifi-
cally, lowering the carrier avidity using controlled reduction of anti-ICAM surface density resulted in a marked 2-fold increase in selectivity of uptake of anti-ICAM/carriers in the lungs of animals with endotoxin-induced acute pulmonary inflammation vs lungs of healthy control mice and improved detection of pathology using PET imaging.156

Targeting Vascular Damage. Damaged vascular wall exposes specific markers including normally hidden intracellular endothelial molecules (e.g., von Willebrand Factor) and components of subendothelial layers including collagens, tissue factor, and proteo-
glycans. These compounds activate coagulation and platelets to form a hemostatic plug preventing bleeding. To compensate for deficiency of natural hemostatic mechanisms in patients with bleeding disorders, development of synthetic hemostats has been investigated.295–297

These synthetic hemostats must be specific and not induce clotting at off-target endothelial sites.298 Hemostatic-promoting carriers must (i) circulate in blood without interacting with off target (i.e., non-
damaged) endothelium, (ii) marginate to the endothelial wall at the wound site, and (iii) specifically interact with the damaged endothelium and circulating platelets to form a suitable hemostatic plug. Carrier design parameters become increasingly important if carriers are to perform such specific interactions in terms of circulation and endothelial interaction considering the exposure of multiple binding sites at injured vasculature and the dynamic nature of blood flow. To address the former, a number of spherical carriers have been proposed presenting either fibrinogen295 or fibrinogen-derived peptides,296,299 which bind to markers exclusively present on activated platelets at the wound site. These single-ligand carriers have been successful in providing an ~2-fold increase in survival compared to saline control mice that were subjected to liver blunt trauma wounds.300 Recently, the complexity of multiple ligand presentation following physical vascular damage has been investigated by combining collagen and von Willebrand Factor binding ligands, in addition to fibrinogen-derived peptides, onto a single carrier.297

In case of the latter, the size, shape, and flexibility must be finely tuned in order to perform on-demand hemostasis at a vascular injury site which is undergoing dramatic changes in blood flow and loss. Extensive efforts have focused on mimicking the size, shape, and flexibility of RBCs to better navigate carriers in the vasculature. Likewise, platelet mimetics are receiving much attention. Recently, Doshi et al. have shown the utility of cell-like flexible particles in targeting surfaces coated with markers for damaged endothelium.280 Synthetic platelets, which mimic the size, shape, and flexibility of real platelets, were shown to adhere over
MULTILIGAND TARGETING

Selective targeting to pathological endothelium is an important task. One intriguing idea explored by several laboratories is the strategy of combining on the surface of the carrier affinity ligands that bind to different determinants. This has the potential to boost the selectivity and efficacy of drug delivery. Figure 10 illustrates this principle: one ligand binding to an inducible determinant provides the selectivity of recognition of altered cells, while the second ligand binding to a pan-endothelial determinant supports the anchoring. The idea, in essence, comes from the biology of leukocyte vascular homing and infiltration of the endothelium at sites of inflammation. Leukocytes bind to two different types of adhesion molecules, low-affinity selectins (E/P-selectin) and high affinity Ig-type cell adhesion molecules (PECAM and ICAM), in order to attach, roll, and come to firm adhesion on the endothelium.

In this vein, carriers coated by combinations of antibodies with relatively nonspecific high-density determinants (ICAM, PECAM) and antibodies to inducible adhesion molecules (selectin, VCAM-1, ELAM) have been tested in vitro in models that employ immobilized antigens or cytokine-activated cells. In studies with particles targeted to inflamed vasculature using ICAM-1 and P-selectin, greater binding was achieved with dually targeted particles relative to particles targeted to P-selectin or ICAM-1 alone. Targeting liposomes to E-selectin and either VCAM-1 or ICAM-1 on cultured endothelial cells has also been reported. Maximal binding was observed with equimolar ratios of both ligands. The biological significance of these findings to real situations in vivo remains to be defined. In addition to typical limitations associated with cell cultures and immobilized antigens, expression of binding data in relative scale further convolutes interpreting results of these studies.

However, a dual targeting strategy employing spherical 100–200 nm carriers carrying antibodies to both ICAM and transferrin receptors has recently been tested in vivo and showed promising results: each of the ligands apparently promoted targeting to the vascular area of its destination, i.e., nanocarriers could be directed to the inflamed pulmonary vasculature via ICAM and to cerebral vasculature via transferrin receptor.

The dual targeting paradigm has also been used for molecular imaging: MRI-based NCs were targeted to cerebral vasculature and to cerebral vasculature and to cerebral vasculature via transferrin receptor.
to P-selectin and VCAM to image atherosclerosis in mice. Dual targeted NCs demonstrated approximately a 6-fold higher binding relative to single targeted NCs.306 Another group has used dually targeted particles for imaging of inflammation via microbubble contrast agents for ultrasound. Here microbubbles were targeted to ICAM-1 and selectins and demonstrated that dually targeted bubbles had significantly better adhesion strength to activated endothelial cells relative to single target bubbles.307

**Competitive and Collaborative Targeting.** Monomolecular ligands interact with target determinants either in bivalent (e.g., antibodies themselves) or monovalent fashion (e.g., Fab-fragment conjugates and scFv-fragment fusion proteins). Bivalent binding of an antibody to glycoprotein(s) on the cell surface offers higher affinity yet requires more freedom and congruency of carrier-target interaction. Ligands binding to distinct epitopes on the same target molecule may influence each other, for example, inhibiting binding to adjacent epitopes. The competitive inhibition of binding to overlapping epitopes has been described for antibodies to ACE.308–310

Recently, it has been found, however, that distinct monoclonal antibodies directed to adjacent epitopes in the distal domain of the extracellular moiety of PECAM, stimulate binding of each other, both in cell culture and in vivo.311 The endothelial binding of PECAM-directed mAbs is increased by co-administration of a paired mAb directed to adjacent, yet distinct, PECAM epitopes. The “collaborative enhancement” of mAb binding was affirmed in mice, manifested by enhanced pulmonary accumulation of intravenously administered radiolabeled PECAM mAb when co-injected with an unlabeled paired mAb.

This highly unusual empirical finding can be interpreted as an increase in accessibility of an epitope to its ligand due to conformational changes in the target determinant molecule induced by binding of a paired “stimulatory” ligand to its adjacent epitope. In theory, it may find utility in targeting strategies. The premise of this strategy, “collaborative enhancement”, is that the initial binding event can lead to an increased binding event of the secondary targeting agent. With this strategy, a therapeutic effect was realized with increased activity of a scFv targeted to PECAM on endothelial cells.311 This phenomenon offers a new paradigm for optimizing the endothelial-targeted delivery of diagnostic agents and therapeutics. It will be interesting to see if this strategy of enhanced targeting can be applied to NC platforms for drug delivery and imaging of disease.

**Challenges and Perplexing Issues.** The assessment of carrier fate within the target tissue of live animals is such a challenging endeavor that PK-related issues pale in comparison. No method affords accurate data of binding per cell at a given location in vivo. The best data sets are provided by combining isotope tracing demonstrating uptake in an organ and microscopy imaging cellular distribution. Such a level of rigor is needed to characterize new, targeted DDS. Otherwise, studies may look enigmatic, such as vascular pulmonary targeting by an antibody to surfactant protein SP-A,312 the alveolar component inaccessible for liposomes in blood (which is difficult to comprehend except as an artifact of passive retention in the pulmonary capillaries).

Even at the current limited level of understanding of target biology and processes to intervene, many targeting paradigms are incredibly simplistic. For example, tens of thousands of publications characterize ACE, PECAM and ICAM-1: structure, functions, tissue distribution, and role in a plethora of human maladies. Yet, data on their distribution in the vascular lumen.
throughout blood vessels and in the endothelial cell plasmalemma are fragmentary, at best. Immunostaining, in situ hybridization, Western-blotting, and PCR do not distinguish surface and intracellular proteins. Isotope-labeled antibodies detect surface target, but offer no insight into the protein organization in oligomers or/and clusters and their distribution in the plasmalemma. Systematic information on normal and pathological distribution of any of the endothelial target molecules in the in the vasculature is still missing. An immunohistochemistry-based atlas of the vascular distribution of ACE, the target of a widely used inhibitor therapy, is arguably the best attempt so far, but ACE organization in the endothelial plasmalemma in the vessels is still enigmatic.\textsuperscript{179,182}

\textit{In vitro} experiments and computational studies complement expensive \textit{in vivo} studies but must be interpreted carefully. In the context of targeting, issues of cell culture include (i) degeneration of the cell phenotype; (ii) conditions irrelevant to \textit{in vivo} (high dose, incubation without washing, prolonged exposure); and (iii) lack of a proper cell environment (for example, tissue components, flow, blood). In particular, endothelial cells grown in cell culture are known to lose phenotypic features—caveolae, glycocalyx, ACE and other marker proteins, basal level of ICAM-1, etc.\textsuperscript{64,313–315} Studies using immobilized molecules seem even less relevant. For example, studies of dual ligand targeting using densely co-immobilized target molecules hardly represent the \textit{in vivo} situation accurately, especially in the case that one of the targets is scarce in the vasculature. Most likely, this strategy may only be useful for a few determinants as both must coexist in close proximity to each other in order to permit simultaneous anchoring of both ligands.

**Cellular Uptake and Trafficking of Targeted Carriers.** Intra-cellular delivery and addressing to proper cellular compartments are needed for the desirable effects of many drugs, especially biotherapeutics.\textsuperscript{114,316,317} Endothelial cells, like other cell types in the body, except professional phagocytes, exert fairly selective mechanisms for the uptake and transport of macromolecules and nanoparticles.\textsuperscript{59,318} Without targeting, such compounds poorly enter cells unless exposed at high concentration for a prolonged time.

Anchoring on selected surface determinants offers mechanisms to boost the uptake and control the rate and pathway of internalization, subsequent trafficking, and fate of nanocarriers. The conventional wisdom is that anchoring of a drug or carrier to a molecule involved in uptake of its natural ligand(s) provides the intracellular delivery. Targeting nanocarriers to endothelial cells provides ample examples illustrating this principle but also examples of less intuitive and somewhat paradoxical pathways for intracellular drug delivery.

**Entering through Open Doors: Targeting Carriers to Determinants Involved in Natural Endocytic Pathways.** The endothelium exerts active fluid phase uptake \textit{via} vesicular micro- and macropinocytosis pathways.\textsuperscript{39} They transport compounds present in the blood at high concentrations, but efficacy and specificity of these pathways seem unlikely to meet the requirements for drug targeting.

Uptake of ligands that bind to internalizing receptors is more effective, specific, and controlled than passive fluid phase uptake. Like other cells, endothelial cells internalize ligands by phagocytosis and receptor-mediated endocytosis \textit{via} caveolae\textsuperscript{39,319,320} and clathrin-coated\textsuperscript{221} and uncoated vesicles,\textsuperscript{322–324} as well as noncanonical pathways (see below). Endocytic, phagocytic, and pinocytic pathways have selectivity to certain ligands and pharmacological or genetic methods of inhibition (to a degree) and deliver cargoes to distinct destinations.\textsuperscript{38}

Diverse cell types and phenotypes internalize and traffic carriers differently. For example, phagocytes internalize ligands of Fc-receptors, whereas non-phagocytic cells internalize transferrin \textit{via} its receptor into cellular vesicles.\textsuperscript{322} Further, the lysosomal traffic of IgG-opsonized particles in phagocytes increases proportionally to their size in the range of 100–10 000 nm in diameter,\textsuperscript{325} whereas endothelial cells display an opposite trend.\textsuperscript{326} Phagocytosis is viewed as a relatively minor contributor to endothelial uptake (this assumption may not necessarily reflect the phagocytic capacity of endothelium \textit{in vivo}). Caveolar- and clathrin-mediated endocytosis are conventionally viewed as the predominant endothelial vesicular pathways.\textsuperscript{47}

Another conventional notion is that anchoring to a receptor leads to carrier uptake \textit{via} the pathway naturally serving this molecule.\textsuperscript{39} For example, antibodies against gp90, a 90 kDa glycoprotein located in the caveoli, and compounds conjugated to these antibodies enter vesicular organelles from caveoli (see below).\textsuperscript{68} In contrast, liposomes targeted to the E-selectin, a transmembrane glycoprotein that is taken up by clathrin-mediated endocytosis,\textsuperscript{217,218} enter cells \textit{via} this pathway and traffic to lysosomes.\textsuperscript{219,327} Similarly, antibodies to vascular cell adhesion molecule-1 (VCAM-1) are internalized by endothelial cells and addressed in the lysosomes \textit{via} clathrin-mediated endocytosis\textsuperscript{327,328} and anti-VCAM conjugated compounds generally follow this fate.\textsuperscript{212,215,262} Antibodies to transferrin receptor (TfR) and compounds conjugated with TfR ligands also enter cells \textit{via} this pathway, similarly to the endogenous ligand transferrin.\textsuperscript{329}

Ligands binding to distinct epitopes of anchoring molecules may enter cells differently. Selection of ligands facilitating cellular uptake is an important and so far mostly empirical task.\textsuperscript{330,331} With the use of phage-display library, the series of peptides binding to diverse VCAM-1 epitopes was identified, some of which have shown enhanced uptake.\textsuperscript{212} Labeled VCAM-1 binding peptides undergoing enhanced endocytosis...
provided improved imaging of vascular inflammation in animal models. Phage display and other high-throughput methods facilitate selection of internalizable antibodies and their fragments (keeping in mind that multivalent interaction of the ligand-exposing phages is different from that of ligand).123–125

Ligand modification affects internalization; obviously, loss of affinity cancels specific uptake mechanism. Modifications of antibodies that change their charge or enable hydrophobic interactions with phospholipids in cellular membranes stimulate their internalization. Yet, such modifications reduce targeting specificity. This concern is relevant to use of relatively promiscuous secondary interactions, such as those mediated by charge or hydrophobic interactions.

It has been recognized for a long time that conversion of monovalent or bivalent ligands into multivalent carriers that can engage numerous copies of the receptors enhances internalization. An extensive clustering of receptors eliciting strong endocytic signaling, as well as dissociation of pre-existing clusters and “unnatural” signaling, among other factors, may be involved in the enhanced uptake of multivalent carriers. Generally, this high carrier avidity is viewed as favorable for intracellular delivery. However, excessive avidity may be detrimental for the subsequent dissociation of the carrier from the receptors during intracellular trafficking. This intriguing scenario has been illustrated in a study of transferrin-mediated transcytosis across the blood–brain barrier. High avidity particles got stuck in the endothelium, whereas particles coated with a lower affinity antibody were able to dissociate from the receptor on the basal surface of endothelium, thereby enhancing delivery to the brain (Figure 11).

However, coating a nanocarrier with internalizable molecules does not necessarily result in internalization. For example, the uptake of a large, micrometer-size carrier may exceed cellular endocytic capacity; formation of such a large vacuole would require prohibitively extensive mobilization of cell membrane and cytoskeleton. Further, coupling ligands to carriers impedes their interaction with receptors and epitopes localized in invaginations and domains of the plasmalemma inaccessible to the particles of such size. For example, conjugation of ligands of caveolar epitopes to carriers larger than the diameter of the neck of caveoli (70 nm) abolishes endothelial targeting and transfer.54

Walking through the Walls: Intracellular Uptake via “Noninternalizable” Cellular Determinants, PECAM and ICAM. PECAM and ICAM exert low level of uptake of their antibodies. For example, the rate of internalization of labeled anti-PECAM and anti-ICAM by endothelial cells in culture rather marginally (by 5–10%) exceeds the background uptake level on ice. However, endothelial cells internalize multivalent anti-ICAM and anti-PECAM conjugates and carriers coated with multiple copies of anti-CAM with a t1/2 of ∼10–20 min and uptake levels achieving 85–90% of the total amount of particles bound to the cells (Figure 12).

Multivalent ligands of PECAM and ICAM trigger a unique pathway, named CAM-mediated endocytosis, distinct from phagocytosis, pinocytosis, and the canonical endocytic pathways. Molecular signaling in CAM-mediated endocytosis involves sodium-proton exchanger NHE-1, release of Ca2+, and a series of kinases and messengers mediating reorganization of the cytoskeleton that drives the uptake of CAM-anchored conjugates.

Internalized anti-CAM conjugates initially reside in the nascent vesicles negative for endosomal markers (for <60 min), subsequently traffic to EEA-1 positive sorting endosomes (1 to 2 h post internalization), and reach lysosomes about 3 h after uptake in endothelial cells. Therefore, most (not all) anti-CAM conjugates arrive in lysosomes several hours after uptake. This pace of vesicular traffic is fairly slow compared with the classical endocytic pathways that deliver their ligands to lysosomes 10–60 min after internalization in endothelial cells.

Co-internalized ICAM dissociates from the anti-ICAM coated carriers in the endosomes and recycles...
to the cell surface, while the carriers traffic further to lysosomes. This mechanism allows multiple cycles of intracellular delivery in vitro and in vivo. Using pharmacological agents interfering with vesicular transport and the cytoskeleton permits the de-flection of vesicular transport from the lysosomal destination and facilitates recycling of the internalized carriers to the cell surface. A body of evidence accumulated in vitro and in animal models indicates that PECAM and ICAM represent highly unusual endothelial targets providing either surface anchoring or internalization with the choice controlled by the parameters of the DDS design, i.e., valence of binding. This feature permits targeting of drugs that either need to be retained on the cell surface such as monovalent antithrombotic fusion proteins or delivered inside the cell.

**Intracellular Delivery of Targeted Carriers: Geometry Does Matter.** Carrier size, shape, and plasticity modulate cellular uptake and trafficking. Depending on cell type, their functional states, and selection of anchoring molecules, the effects and mechanisms of the modulation vary. Size-mediated restriction of accessibility to the binding site or the endocytic entry (e.g., in caveolae) and exceeding of the capacity of endocytic machinery represent well-known phenomena. Endothelial cells internalize polymorphous anti-CAM conjugates with size <500 nm, but internalize anti-CAM coated spherical particles with diameter of up to a few micrometers, revealing the endocytic potency typical of phagocytes.

Our current understanding of the geometrical control of targeted DDS uptake is empirical and fragmentary. For example, in one prototype study, ICAM-targeted disks entered endothelial cells more slowly than spherical carriers of a similar size, whereas the pace of traffic through the vesicular compartments was controlled by size: smaller particles arrived at the lysosomes faster, regardless of their shape. In a follow-up study, co-delivery of differently shaped, or sized, carriers was investigated for preferential perinuclear distribution. Larger particles (either spheres or rods) were more likely to localize in this region as compared to smaller particles. In the same study, rods were far less likely to accumulate in perinuclear regions as compared to spheres of the same volume.

In extreme cases, high aspect ratio carriers (aspect ratio close to 10, i.e., needle-shaped particles) can breach the membrane of cells delivering cargo into the cytoplasm. Nontargeted filomicelles showed 100% internalization in human derived lung endothelial cells. While internalization studies with endothelial cells have been limited, internalization of different shaped carriers by cancer cells has been widely explored and in some cases contrast what is reported for endothelial cells in terms of internalization. For instance, in the HeLa cancer cell line, higher aspect ratio particles exhibited higher internalization rates than those exhibited by spherical carriers. In this study, clathrin-mediated, caveolae-mediated, and to a lesser extent macropinocytosis were involved in both...
the nanocarrier and microcarrier uptake; however, it is believed that they play a more important role for the internalization of nanocarriers.

**Biological Modulation of Carrier Intracellular Delivery.**

Binding to distinct epitopes on the same anchoring molecule may lead to different outcomes. For example, spherical nanocarriers (diameter \(\sim 150 \text{ nm}\)) directed to adjacent PECAM epitopes are internalized and trafficked by endothelial cells differently. Testing of a series of monoclonal antibodies directed to distinct epitopes of PECAM revealed marked differences in the rate of internalization of nanoparticles coated by different clones of anti-PECAM: one anti-PECAM coated nanocarrier failed to enter the endothelium, whereas three other types of anti-PECAM nanocarriers exhibited markedly different kinetics of trafficking from early endosomes to lysosomes despite the fact that they all entered cells with a similar rate \((T_{1/2} \text{ was close to } 20-30 \text{ min and max uptake reached } 80-90\%))^{197}

The functional status of target cells and their microenvironment modulate endocytosis. Thus, activated endothelium internalizes ICAM-targeted nanocarriers faster than the quiescent endothelium does both in vitro and in vivo.\(^{345}\) The effects of hydrodynamic factors are more enigmatic and less well-studied. The majority of publications on cellular uptake and trafficking of nanocarriers have employed static cell lines. A few studies have attempted to define the role of flow in endothelial uptake of nanoparticles using flow chambers and microfluidics; most dealt with non-targeted particles.\(^{346,347}\) However, recent studies revealed interesting effects of chronic and acute flow on endothelial uptake of CAM-targeted particles (Figure 13). Experiments in flow chambers revealed that prolonged exposure to flow leads to partial, yet significant, inhibition of endocytosis of nanocarriers targeted to ICAM and PECAM.\(^{159,160}\) These results correlated with in vivo data showing less effective internalization of anti-ICAM nanocarriers in arterioles relative to capillaries, i.e., vascular areas in which endothelial cells do and do not adapt to flow, respectively.\(^{345}\) This effect is attributed to organization of the actin cytoskeleton into stress fibers in the course of endothelial adaptation to flow, which impedes actin involvement in the endocytosis.\(^{345,348}\) In contrast, exposure to acute shear stress (which happens in reperfusion and in physiological hyperperfusion in exertion) accelerated endocytosis of PECAM-targeted nanocarriers.\(^{348}\) Results of pharmacological studies suggest that this effect is due to mechanical stimulation of the signaling mechanisms including caveoli (Figure 13). The rheological regulation of intracellular delivery represents an intriguing area of bioengineering and biomedicine.

Very recently, a novel mode of biological modulation of internalization of targeted nanocarriers has been described. It is mediated by activation of the enzyme(s) metabolizing sphingomyelin in the plasma-lemma and induced by binding of nanocarriers to ICAM-1 in endothelial and other cell types. Studies in cell culture and in genetically modified mice revealed that clustering of ICAM-1 leads to activation of sphingomyelinase and subsequent cleavage of its substrate, stimulating endocytosis of anti-ICAM coated carriers ranging in size from <100 to >1000 nm in diameter.\(^{349}\) This mechanism does not operate in sphingomyelinase-deficient cells and animals; however, carriers coated with both anti-ICAM and the enzyme, devised to deliver enzyme replacement therapy in Neumann-Pick syndrome, do compensate for the genetic defect.

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**Figure 13. Flow-mediated modulation of CAM endocytosis.** Endothelium in vivo is constantly or intermittently exposed to blood flow and chronic and acute exposure to flow differentially regulates endocytosis of CAM-targeted carriers. (A) Endothelial adaptation to chronic flow, manifested by cellular alignment with flow direction and formation of actin stress fibers, inhibits anti-CAM/carrier endocytosis, likely via impaired recruitment of actin in the stress fibers needed for endocytosis. (B) In contrast, acute exposure of endothelium to flow (imitating reperfusion of exertion perfusion) stimulates endocytosis of carriers, likely through mechanisms involving signaling via cholesterol-rich domains of plasmalemma such as caveoli (cav).
and get internalized by cells. Furthermore, co-immobilization of sphingomyelinase with ligands anchoring carriers to transferrin receptor permitted internalization of carriers larger than those normally permitted by clathrin vesicles (<200 nm). This very unusual, serendipitously discovered mechanism further extends the versatility of targeted nanocarriers for intracellular delivery.

**Transendothelial Delivery.** Adhesion of carriers to endothelium may favor their transport across the cells via diverse mechanisms. For example, elevation of the local concentration of a carrier in close proximity to the vessel wall may facilitate its passive entrance into transcellular and pericellular pathways, on the condition that binding is reversible and the gradient direction supports extravasation.

However, anchoring to some endothelial molecules provides more specific and effective mechanisms. Some ligands of receptors involved in endocytosis via clathrin-coated pits, such as transferrin receptor, and caveolae, such as APP, are capable of crossing the endothelial barrier. These pathways provide an opportunity for transendothelial transport of DDS with size suitable for these endocytic vesicles (<100 nm). For example, antibodies to caveolar APP undergo fast transport across the endothelium, but particles >100 nm do not enter this pathway. Some data has supported the idea that caveolae merge into “cavesthesomes” supporting uptake of large particles, but it is unclear to what extent data obtained in static cell cultures reflect this aspect of endothelial physiology in vivo. However, the potential side effects of engaging caveolar determinants must be more fully understood in order to define the biomedical utility of this transcellular pathway. In addition, many disease conditions, including inflammation, may affect this pathway.

In this context, it is intriguing to explore endothelial transport opportunities offered by CAM-mediated endocytosis, which can allow entrance of objects up to several micrometers. It has recently been reported that gastrointestinal epithelial cells, which normally express ICAM, take up anti-ICAM coated nanocarriers (~100 nm diameter spheres) via CAM-mediated endocytosis and transport the carriers across the cellular monolayer without cell damage or disruption of intercellular junctions. Further, this team reported that orally administered anti-ICAM coated nanocarriers enter endocytic pathway(s) in the epithelial cells in the gastroenteral tract and that this process may be differentially modulated by auxiliary drugs that regulate intestinal digestion and peristalsis, opening an opportunity for oral delivery of nanocarriers into the vascular compartment. It is quite plausible that this transcellular transport pathway operates in the vascular endothelium as well and can be modulated by rational carrier design.

**Challenges and Perplexing Issues.** All the issues discussed above in this rubric are pertinent here, including optimization of carrier labeling and tracing. Data of PK/BD and targeting are vital for understanding of cellular traffic. Thus, enhanced transendothelial transport of low avidity TR-targeted carriers (attributed to facilitated intracellular dissociation from the receptor, Figure 12) can be also explained by elevation of the blood/tissue gradient via two mechanisms. First, carriers with lower ligand density retain better stealth features (attributed to facilitated intracellular dissociation from the receptor). Second, the majority of TR-mediated uptake occurs in liver; inhibition of hepatic uptake via reducing TR avidity may also result in elevated blood level (Figure 14).

**In vitro models do allow quantitative assessment of internalization** and probing cellular pathways by pharmacological and genetic inhibitors. Yet, keeping in mind limited specificity and efficacy and considerable toxicity of these agents, their effects need validation using classical ligands of the pathway of interest. Lack of systemic and microenvironmental factors is a common shortcoming of cell culture studies. Endothelial cells rapidly lose their phenotype in static culture. Use of in vitro flow models is more informative but is challenging and adds just one factor of vascular physiology.
Current in vitro models employ a limited range of flow parameters and "vessel geometries" from those observed in the body (turbulent, laminar, pulsating, etc.).[27,1374–378]

Prolonged incubations of serum-starved static cells with high doses of particles and use of acidic or proteolytic elution are prone to false-positive results. "Metabolism-independent" internalization of TAT-conjugates that turned out to be endocytosis serves as an example.[378] Background "uptake" level on ice should not exceed 5–10%; higher levels are indicative of an artifact. Even meticulously performed, common microscopy-based methods are not truly quantitative, while more precise methods are low-throughput.[380,381]

Electron microscopy remains the gold standard for evaluating cellular localization of carriers and cargoes.[382] No doubt, much needed new approaches to inquire into a carrier's fate in the body at the subcellular level are evolving.

Vascular Targeting: Perspectives. In our opinion, successful endothelial nanomedicine requires both adequate understanding of biomedical factors pertinent the medical objectives and optimal nanocarrier design. The latter includes quantitative real-time tracing of both carrier and cargo, high-resolution localization in tissues, balanced combination of stealth and targeting features (e.g., attained by responsiveness to local microenvironment), and adequate (not necessarily the highest) avidity. Carrier elongated geometry and plasticity allowing flow alignment and lateral diffusivity of ligands are likely to improve circulation, targeting and subcellular delivery. Targeting these new DDS to the determinants optimally serving given biomedical context will yield the winning combination.

Endothelial targeting is developing at a vigorous pace. Since first reports two decades ago, hundreds of studies have elaborated the strategy, which is on the verge of translation into the clinical domain. This objective represents a set of paramount challenges. Animal studies only partially reflect therapy of patients with unique individual profiles. Mice provide high-throughput models including mutants lacking or expressing genes of interest, but interspecies differences are difficult to overestimate. Clinical relevance is inversely proportional to species availability. Many industrial and healthcare counterparts hesitate to embrace complex and costly DDS. Undoubtedly, well-defined targeted DDS will find additional utility as tools for probing of physiological pathways in animals. And, of course, the targeted endothelial nanomedicine formulations enabling novel therapeutic mechanisms and qualitatively improving the outcome of dangerous human maladies will eventually be translated into the clinical domain.

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