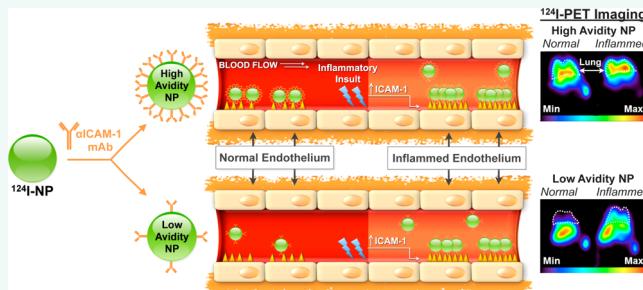


Vascular Targeting of Nanocarriers: Perplexing Aspects of the Seemingly Straightforward Paradigm

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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 bio-distribution (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.^{1,11–20} 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 μm ²⁹ 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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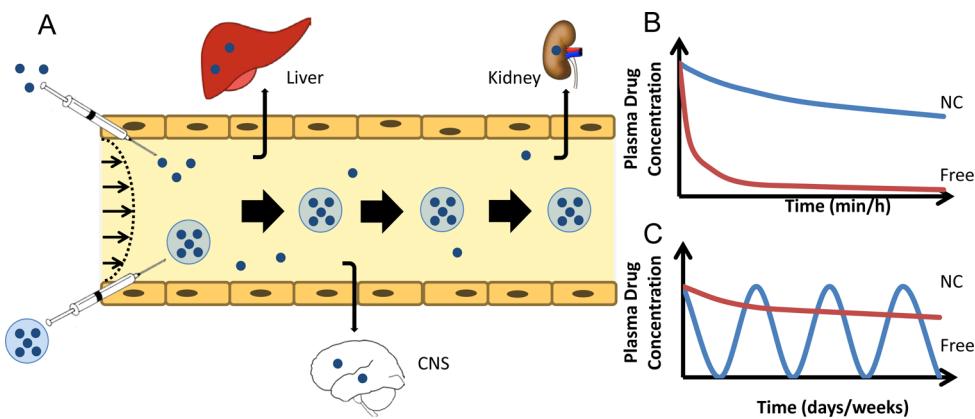


Figure 1. Drug delivery by nanocarriers: optimization of drug pharmacokinetics and biodistribution (PK and BD). (A) PK and BD of drugs (blue dots) vs drugs encapsulated in nanocarriers. Free drugs are eliminated from blood by clearing organs, including the reticuloendothelial system (RES, including liver, spleen, and lymphatic nodes) and excretory organs such as kidneys, lungs, and the bile tract (including hepatic uptake and renal filtration), and diffusion in nontarget tissues including the brain (CNS), where drugs may cause adverse effects. Long-circulating nanocarriers avoiding clearance alter PK (large arrows depict sustained drug circulation) and reduce diffusion in nontarget tissues, thereby improving BD and inhibiting adverse effects. Relative dimensions in this and other cartoons and schemas are not to scale. (B and C) Panels represent, respectively, short-term and long-term model graphs of blood level of free vs nanocarrier-bound drugs (NC). After a single injection in acute and subacute conditions, long-circulating NCs enhance area under the curve, thus reducing the effective dose (B). Hypothetically, using NCs with extended lifetime in circulation will help to maintain a stable therapeutic dose without the need for repeated injections (C).

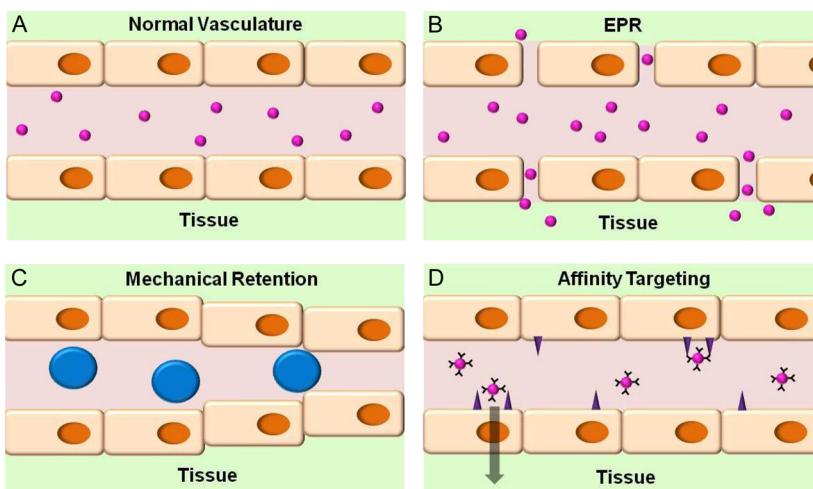


Figure 2. Passive uptake vs active targeting: differences in mechanism and potential biomedical utility. (A) Untargeted nanocarriers with size ranging from a few to a few hundred nanometers do not normally accumulate in healthy tissue with blood vessels lined by the continuous endothelial layer lacking large fenestrae typical of the reticuloendothelial system (RES). (B) Enhanced Permeation and Retention (EPR) effect. In this scenario, carriers accumulate in tissues with abnormally permeable vessels, such as in tumors fed by leaky vasculature as well as in sites of inflammation and angiogenesis (for example, wound healing). In tumors, deficient lymphatic drainage also favors the EPR effect, while high interstitial pressure opposes it (not shown). (C) Large particles, such as rigid spheres with diameter 10–50 μm (bigger than that of capillaries and precapillary arterioles), are mechanically retained downstream of the site of arterial injection in the microvasculature of an organ or tissue fed by this conduit artery. (D) Active targeting of nanocarriers coated by affinity ligands of specific determinants favors binding to endothelial cells exposing these determinants. In contrast with passive uptake (B and C), this mode guides subcellular delivery: binding to noninternalizable molecules vs those involved in cellular uptake and trafficking, respectively, favors retention on cell surface vs intracellular or transcellular delivery.

"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.^{31–35}

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

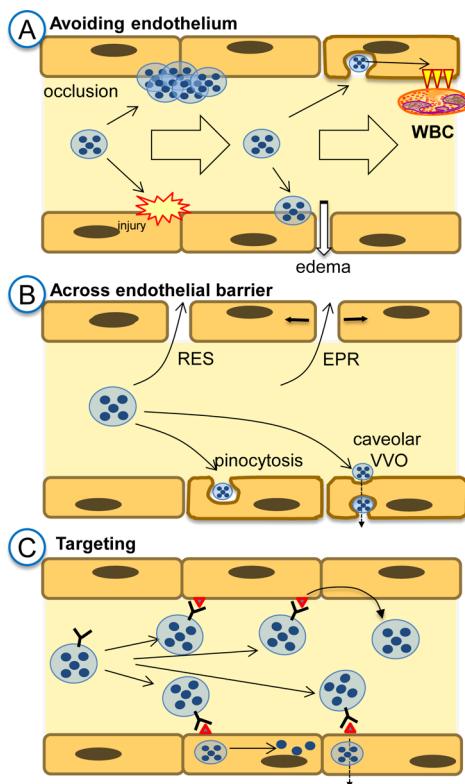


Figure 3. The vascular endothelium: a victim, barrier, and target of drug delivery. (A) In drug delivery strategies requiring cargoes to be released or act in the bloodstream, such as long-circulating reactors or slow release systems, respectively, carrier interaction with endothelium must be avoided. Otherwise, carriers adherence to endothelium may lead to vascular occlusion, endothelial damage, or pathological activation. (B) In strategies delivering drugs to the extravascular sites, endothelium is a barrier. Carriers may cross it by concentration gradient *via* large opening between endothelial cells in the RES (*e.g.*, fenestrae) and intercellular openings in abnormally leaky vessels in tumors and sites of inflammation and angiogenesis (EPR). Vesicular transendothelial pathways include fluid phase transport *via* pinocytosis and transendothelial vacuolar–vesicular organelle (VVO) that opens from the caveolae. (C) In strategies targeting drug carriers to the endothelial surface determinants, ligand-mediated anchoring may result in surface retention or internalization. Depending on the nature of anchoring molecule, choice of epitopes, and carrier design, internalization may lead to recycling to the vascular lumen, delivery to the intracellular compartments, or transfer across the endothelium.

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 ~50–500 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 venules⁵³) 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,⁹⁰ 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.^{91–94} 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.⁹⁵ 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.^{95,96}

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.^{84,97–102}

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.¹⁰³ 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.¹⁰⁴ Unless protected (see below), carriers are eliminated quickly following their injection.^{104,105} Generally, hydrophobic and charged carriers are opsonized and eliminated more rapidly than their hydrophilic and neutral counterparts.¹⁰⁶

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,¹⁰⁷ and extends circulation lifetime.¹⁰⁸ 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 length¹⁰⁹ and surface density.¹¹⁰ Ligand conjugation *via* PEG alleviates the masking effect.^{109–111} 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).¹¹² 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.¹¹³ 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.¹¹⁴

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.^{115,116} This phenomenon has been shown for a variety of carriers,

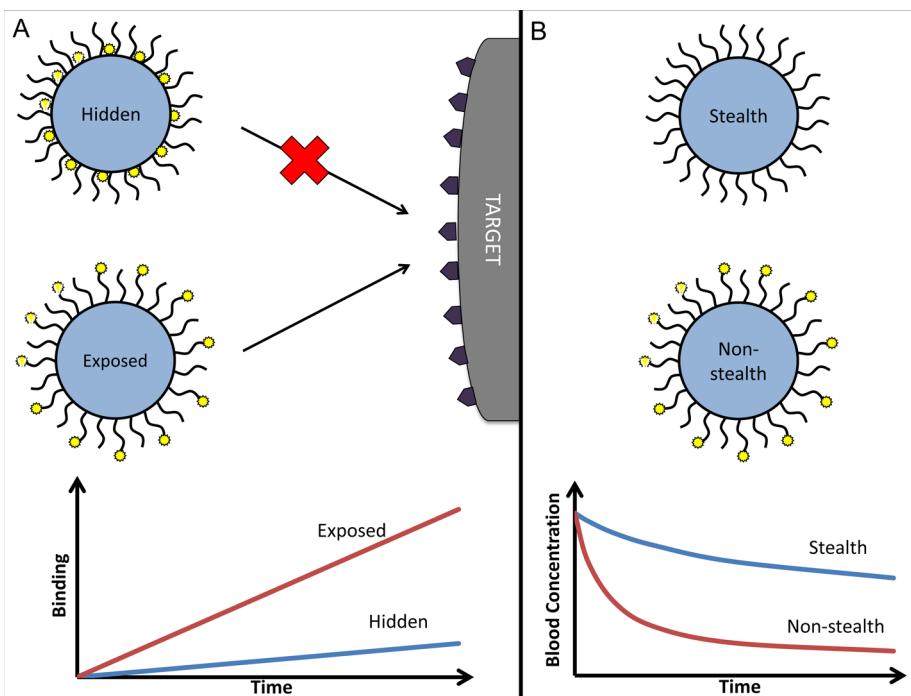


Figure 4. Balancing the stealth and avidity features of carriers: effect on targeting and PK. (A) Surface modification by hydrophilic polymeric chains (e.g., PEG) provides a carrier with stealth features but affects targeting *via* masking affinity ligands. Conjugation of ligand molecules to the end groups of polymeric chains instead of the carrier surface helps to avoid this negative interference and provides additional steric freedom for ligand–target interaction, thereby boosting binding. (B) Ligand molecules conjugated to PEG diminish its stealth effect *via* nonspecific interactions (e.g., mediated by altered charge or hydrophilic features) and ligand-mediated interactions (e.g., *via* Fc-fragment of antibodies conjugated to PEG). Ensuing acceleration of blood clearance may affect drug delivery, if a carrier's circulation time is insufficient for targeting.

ranging from proteins to liposomes.^{117–120} 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,¹¹⁵ which persist in serum and bind to reinjected PEGylated carriers, reducing the circulation time. This initial anti-PEG IgM generation occurs in the spleen¹²¹ and likely involves B-cells,¹²² as B-cell deficient mice do not exert accelerated clearance of PEG-coated carriers.¹²³

Coating carriers with molecules inhibiting activation of complement, opsonization, and phagocytosis such as sialic acid-containing glycolipids has been also pursued.^{124–126} 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.¹²⁷ Conjugation of CD47 and CD47-derived peptides to carriers helps to evade clearance *via* this natural “self-recognition” mechanism.^{127–130} 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.¹³¹ 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*.¹³²

Carrier Geometry and Plasticity. Neither too small nor too large particles circulate well. Renal filtration eliminates particles smaller than 10 nm.¹³³ In addition, they extravasate *via* transendothelial fluid phase transport pathways,^{134,135} 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.¹³⁶ 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.¹³⁷ 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.^{138–141} 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.¹⁴² 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.¹⁴³

One reason for the prolonged circulation of elongated objects is that they elude uptake by phagocytes.¹⁴⁴ Macrophages internalize opsonized disk-shaped particles contacting the cell by the rounded

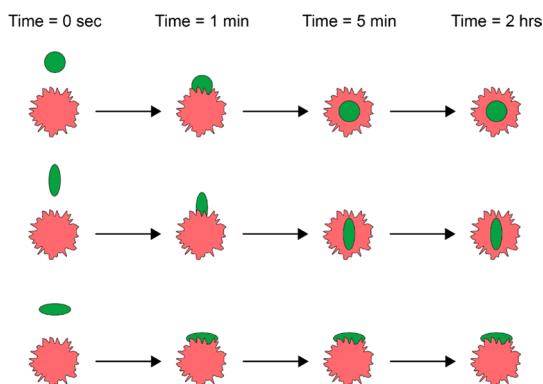


Figure 5. Carrier geometry modulates cellular uptake. Rate of intracellular uptake of elongated particles by phagocytic and other cells is markedly modulated by the axis of the particle binding to cell surface: it is faster than uptake of spherical particles of comparative maximal dimension in cases of binding *via* pointed vs flat aspects, respectively.

end of the particle as effectively as spherical particles but cannot completely internalize disk-shaped particles contacting on their flat face (Figure 5).¹⁴⁵ 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.¹⁴⁶ Indeed, red blood cell mimetic particles were shown to have extended *in vivo* circulation times.¹⁴⁷ Flexibility was tuned *via* the extent of cross-linking and resulted in plasticity similar to that of red blood cells.¹⁴⁸ Enhancing carrier's flexibility prolongs its circulation.¹⁴⁷ 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.¹⁴⁹

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.^{150,151} Filomicelles are able to persist in blood for extended periods of time by taking advantage of both their worm-like 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.¹⁵¹ Pristine filomicelles exhibit little to no cellular binding, an attractive feature for a long-circulating DDS.¹⁵²

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.¹⁵³

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)¹⁵⁴ 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,^{155–158} 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.¹⁵⁹

A direct measurement of the isotope level in drawn blood samples is arguably the best method for PK studies.^{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).^{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.¹⁶⁴ Even dense PEG-coating of polymeric carriers does not prevent eventual absorption of plasma components.¹⁶⁵ Generally, carrier interactions with body components¹⁶⁶ influence desired as well as adverse effects, including accumulation in the target and uptake by off-target cells.^{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.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.^{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^{64,173} and *in vivo* phage display⁷³ are advantageous for finding new targets, as they identify binding sites accessible from circulation in normal¹⁷⁴ and pathological vasculature.¹⁷⁵ The list of endothelial determinants theoretically useful for vascular drug targeting continues to grow.^{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,^{27,28} disappear from the endothelium in pathological conditions, which inhibits targeting.^{179,180} Constitutive molecules stably exposed in the lumen, such as PECAM, can be theoretically used for prophylactic and/or therapeutic effects.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.^{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^a

target	vascular localization	cellular localization	functions	regulation
ACE (CD143)	Ubiquitous, enriched in the lung capillaries	Cell surface single pass type I membrane protein	Constitutive Plays a key role in renin-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.	Up-regulated in inflammation
E-selectin (CD6E)	Inflamed vascular endothelium	Cell surface single pass type I membrane protein	Ligand of VLA-4	Up-regulated in inflammation
P-selectin (CD62P)	Inflamed vascular endothelium	Cytosol: Weibel-Palade bodies of endothelial cells, alpha granules of platelets	Leukocyte rolling 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	Caveolar	Metalloprotease that plays a role in inflammatory process; cleaves and inactivates circulating polypeptides such as bradykinin
PV1 (PLVAP)	Expressed in majority of tissues	Endothelial fenestrations, caveolar membrane	Formation of stomatal and fenestral diaphragms	Unknown
APN (CD13)	Vasculature of tissues that undergo angiogenesis and in multiple tumor types	Cell surface, single pass type II membrane protein; cytosol	Angiogenesis Tumor-Related	Up-regulated by VEGF <i>in vitro</i>
Integrins $\alpha_4\beta_3$, $\alpha_4\beta_5$, $\alpha_4\beta_1$	Enriched in tumor vessels and other types of angiogenesis	Cell surface	Metabolism of regulatory peptides, processing of peptide hormones (angiotensin III and IV, neuropeptides, and chemokines) angiogenesis, tumor neovascularization, and tumor metastasis	Estradiol and interleukin-8 decrease APN activity <i>in vitro</i> $\alpha_4\beta_3$ is up-regulated by bFGF, TNF
TEM-1 (CD248)	Tumor endothelial cells	Cell surface single pass type I membrane protein	Tumor angiogenesis	$\alpha_4\beta_5$ is up-regulated by VEGF, TGF- α unknown

^a EC, endothelial cells; ACE, angiotensin-converting enzyme; TM, thrombomodulin; PECAM, platelet-endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule; APN, aminopeptidase N; APP, membrane-bound aminopeptidase P; TEM-1, tumor endothelial marker-1; PV-1, plasmalemma vesicle-associated protein.

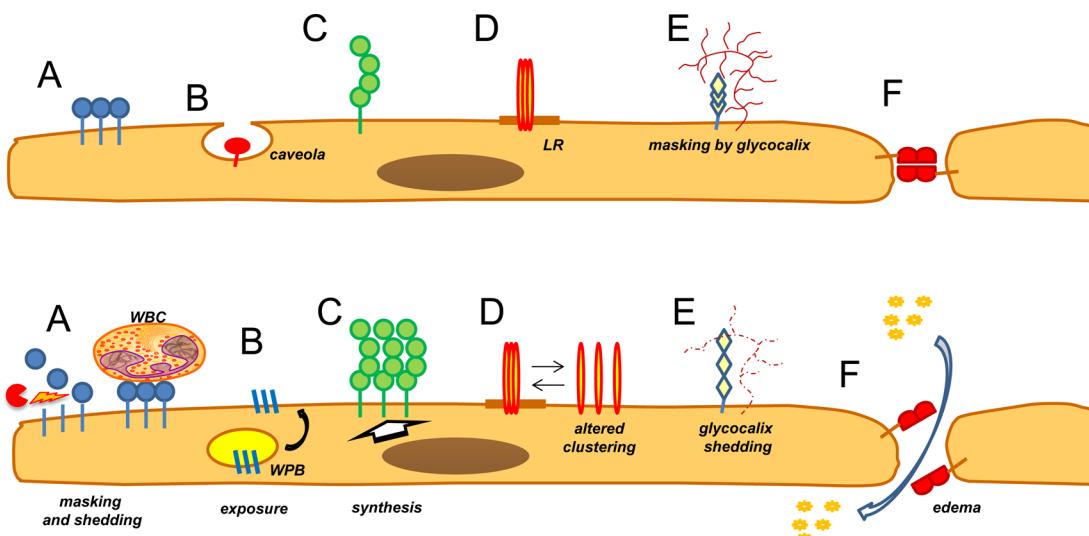


Figure 6. Endothelial determinants in normal and pathological vasculature: accessibility, regulation, and localization in specific domains of the plasmalemma. (Upper panel) Normal endothelium exposes determinants that are differently accessible to circulating carriers. They include molecules clustered in apical plasmalemma, such as ACE (A); located in the caveoli, such as APP and PV1 (B); molecules, such as ICAM-1, expressed as monomers and oligomers at basal level throughout the plasmalemma (C); molecules preferentially localized in membrane domains (e.g., lipid rafts, LR) (D); molecules partially masked by glycocalyx (E); and molecules concentrated in cellular junctions, such as PECAM and VE-cadherin (F). (Lower panel) Under pathological conditions, determinants may be masked by adherent white blood cells (WBC) and/or shed from the cells (A) (both mechanisms would inhibit targeting), whereas inducible adhesion molecules may exteriorize from intracellular stores (B), such as P-selectin from Weibel-Palade bodies (WPB), or be synthesized de novo, such as E-selectin, ICAM-1, and VCAM-1 (C). Pathological mediators also induce rearrangement of natural clusters (D); shedding of glycocalyx, thereby exposing normally masked determinants (E); and cause endothelial contraction, thereby increasing pericellular permeability and accessibility of target determinants in the junctions (F), such as VE-cadherin and PECAM.

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.^{179,182,183} 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,^{184,185} whereas adhesion molecules VCAM-1 and selectins are expressed predominantly in sites of inflammation.¹⁸⁶

Determinants differ with respect to preferential localization in domains in the endothelial plasmalemma.^{187,188} For example, PECAM and VE-cadherin are localized predominantly in cell–cell borders.^{189,190} VCAM-1 and ICAM-1 are found in tetraspanin micro-domains of the cellular apical surface,^{191–193} special types of membrane “rafts” of distinct protein and lipid composition with a diverse array of function, including adhesion, proliferation, and immune cell signaling.¹⁹⁴ 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,^{195,196} and APP and PV-1 are located within caveoli.¹³⁵ 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.⁴³ 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.¹⁹⁷ 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.¹⁹⁸ On the other hand, adherent leukocytes may mask endothelial surface from blood.¹⁶¹

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);¹⁹⁹ 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.^{67,200,201} However, thrombomodulin controls thrombin,²⁰² and its inhibition poses danger of thrombosis.^{201,203,204} This complication prohibits clinical use of this target, restricting its utility to model animal studies.^{180,201,205,206}

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.¹⁰⁷ Loading a benign agent into a carrier composed of biocompatible materials decorated by innocuous ligands may yield a combination with pro-inflammatory or adjuvant 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.^{38,90,162,207} 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 exposed on the surface of activated endothelial cells and facilitate the rolling phase of the vascular adhesion of leukocytes.²⁰⁸ Pathological factors including cytokines, oxidants, and abnormal flow cause surface exposure of P-selectin from intracellular storage within 10–30 min²⁰⁹ and induce *de novo* synthesis and surface expression of E-selectin²¹⁰ and VCAM-1.²¹¹ 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.²¹²

Carriers conjugated with antibodies to these molecules bind to activated endothelium.^{66,71,212–215} Endothelial cells internalize selectins via clathrin-coated pits,^{216–218} which favors intracellular delivery into endothelial cells of E-selectin targeted liposomes,²¹⁹ drugs,^{219,220} and genetic materials.²²¹ Inducible adhesion molecules represent excellent determinants for visualization of activated endothelium in inflammation foci by delivery of conjugated isotopes¹⁸¹ or ultrasound contrast agents.^{222,223}

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.^{224,225} 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 $(0.2–2) \times 10^6$ copies per cell,²¹¹ predominantly in the interendothelial borders.^{226,227} ICAM tends to localize in lipid rafts in the luminal membrane and may exist in either a monomer or oligomer form.¹⁹³ 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.²²⁸ In contrast to such a dramatic difference *in vitro*, ICAM is expressed in blood vessels constitutively by quiescent endothelium *in vivo* at levels of $\sim(0.2–1) \times 10^5$ 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 $(0.5–3) \times 10^5$ sites per cell in endothelium⁴⁸ and other cell types.²²⁹

CAMs are involved in adhesion and trafficking of leukocytes and in vascular signaling.²³⁰ Their clustering by multivalent ligands initiates signal transduction via their cytosolic domains.²³¹ Adhesion via these molecules supports leukocyte mobilization in sites of inflammation.^{230,232} Via its extracellular domain, endothelial PECAM engages in heterophilic binding to heparin-containing proteoglycans and integrins of leukocytes, and in homophilic PECAM–PECAM interactions.^{231,233,234} The extracellular region of ICAM binds ligands including fibrin, certain pathogens, and integrins of activated leukocytes, mediating their firm adhesion to endothelial cells.^{235,236} Therefore, PECAM and ICAM are involved in cellular recognition, adhesion, and migration of leukocytes.²³⁷ Inhibition of these functions may be beneficial in treatment of inflammation.²³⁸

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.^{78,95,160,201,205,239–241} Intravenous administration favors their uptake in the pulmonary vasculature, while local arterial infusions enrich accumulation in the downstream cardiac,²⁴² cerebral,⁹⁶ and mesentery^{243,244} vasculature.

Many groups have devised nanocarriers targeted to CAMs by conjugated antibodies.²⁴⁵ “Designer” affinity ligands for CAMs have also been devised,⁴⁰ including scFv fragments^{95,206,246} and affinity peptides selected using a phage display library.²⁴⁷ 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 paternal mouse anti-ICAM has been produced²⁴⁸ as well as multivalent Fab fragments of a monoclonal antibody to human ICAM.²⁴⁹

Some of these recombinant proteins have been clinically tested as anti-inflammatory agents and showed generally acceptable safety.^{250,251} 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.²⁵²

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.²⁵³ The carrier geometry and plasticity modulate the latter parameter and carrier ability to engage in multivalent binding.^{160,179} 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*.^{94,254–259} These models allow quantitative analysis under controlled conditions, but revealed trends need *in vivo* validation in the types of vessels²⁶⁰ and vascular areas^{261,262} of interest.

For example, several studies have indicated that binding is inversely correlated with shear stress.^{255,263} Computational analysis showed that the shear stress influence diminishes for nanocarriers coated with

antibodies at a surface density above a threshold level.²⁵³ 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.²⁶⁴ 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.²⁶⁵

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.²⁵⁴ 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.²⁵³ 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.⁹⁴

In some studies carriers were perfused in the presence of blood components.²⁵³ 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.²⁶⁶ 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.²⁶⁷ 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.^{268,269} 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,

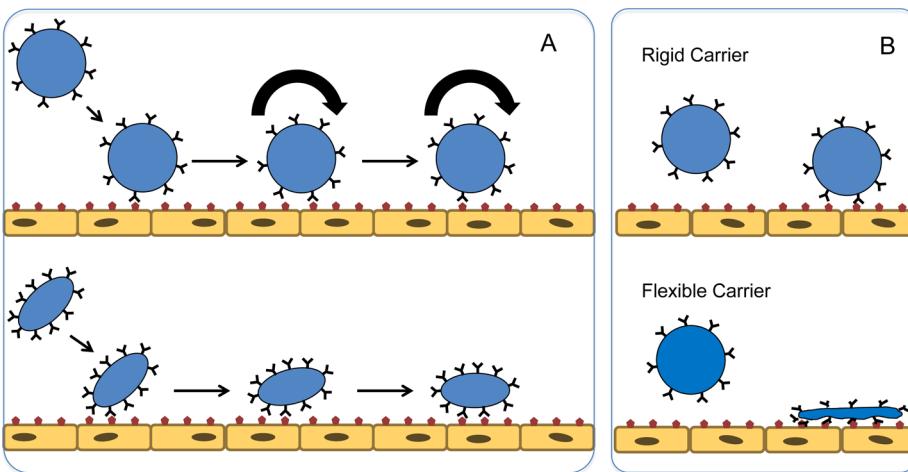


Figure 7. Carrier shape and plasticity modulate ligand-mediated anchoring on endothelium. (A) Spherical carriers bind to the endothelium in a fashion reminiscent of the phases of leukocyte adhesion (rolling, initial tethering, and firm adhesion), whereas elongated and discoid carriers “zip up” on the target molecules. (B) Endothelial binding of flexible carriers is advantageous over that of rigid carriers, because shape change and lateral diffusion of ligands afford better congruency between molecules of a ligand/receptor pair and a higher number of productive anchoring engagements.

act together to increase the carrier gradient along the cell-free-layer at the vessel walls. In smaller vessels, similar simulations show that carrier accumulation is enhanced further, likely due to the larger role volume exclusion plays since RBCs may be forced to physically contact the vessel wall.²⁶⁹

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 ~ 10 vs ~ 2 for 100 nm and 1000 nm particles, respectively), due to more pronounced mechanical retention of large untargeted carriers coated with control IgG.¹⁴³ 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.¹³⁶

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.^{197,270} 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.¹⁹⁷

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 ~ 3) functionalized with anti-ICAM showed upward of 3-fold attachment to brain endothelial cells in static cell culture.²⁷¹ Higher association of rod-shaped particles with endothelial cells was also exhibited *in vivo* as anti-ICAM coated rods exhibited ~ 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.²⁷¹

In a computational model comparing spheres and nanorods, the binding probability of a nanorod under a shear rate of 8 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).¹⁴³ 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.¹⁵² 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.⁹⁰

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.^{264,271–276} 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.²⁷⁷ 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 flow²⁷³ 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.²⁶⁴

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.²⁷⁸ 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),²⁷⁵ and anti-ICAM coated rods were shown to adhere twice as well to ICAM-expressing endothelial cells over identically coated spheres under flow.²⁷¹

Carrier plasticity is likely to contribute significantly to targeting despite the lack of studies directly confirming this. While circulating in the fluid stream, rigid and flexible carriers may retain similar morphologies. However, the latter carriers may flatten on the surface of the target (Figure 7B), thereby reducing the drag force of perfusion that leads to detachment. The same phenomenon will enhance binding via spreading over and engaging a higher number of binding sites. Additionally, lateral movement of ligand molecules in the flexible carriers favors congruency of ligand molecules interaction with multiple binding sites and their clusters.²⁷⁹ These advantages of more flexible carriers are beginning to be reported in literature where ligand presenting carriers of the same shape and identical coating are superior in terms of binding and binding strength over their rigid counterparts.²⁸⁰ Perhaps these findings will be best exploited by high-avidity multivalent binding to target sites. In addition, as noted in section Nanocarrier Behavior in the Circulation, flexible particles circulate for a longer time and have a better chance of achieving target than rigid particles. Further, rigid particles have shown to be greater than 5-fold more likely to be internalized by immune cells when compared directly to their flexible counterparts *in vitro*.²⁸¹ However, rigid particles also seem to preferentially accumulate in lung tissue much more than flexible counterparts, likely due to passive mechanical retention in lung capillaries.

The Optimal Carrier Avidity and Ligand Density. The mode of ligand conjugation modulates carrier's avidity. For example, antimyosin Fab conjugates produced with three different cross-linkers (SMCC, SPDP, and BrAc-NHS) showed different targeting, likely due to different stability of the bond and reduction of Fab affinity, among other potential factors.²⁸²

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.^{243,283} 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.^{94,253,284} 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.²⁸⁵ For example, an increase in ligand density providing higher avidity results in an increase of vascular targeting.^{253,284} 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.²⁸⁴ Yet, an excessive ligand density beyond one that saturates binding sites may be undesirable due to costs and benefit/risk ratio.²⁸⁶ Additionally, studies of nanocarrier-based vaccines imply that carriers with high ligand density are likely to cause immune reactions.^{287–289}

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 tumors²⁹⁰ or thrombi.²⁹¹ 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.^{292,293} 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.^{292,294}

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

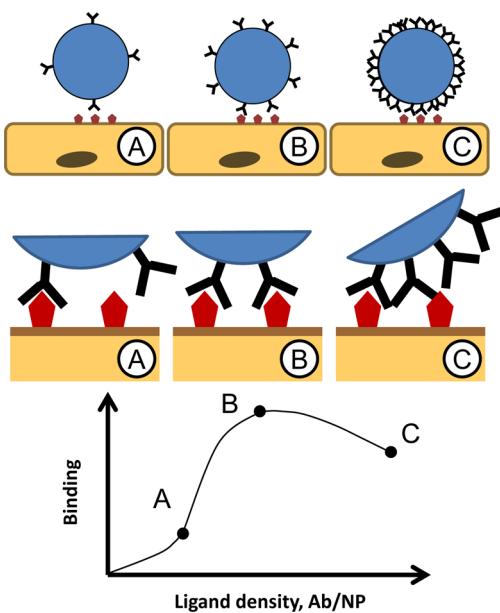


Figure 8. Maximal ligand surface density does not necessarily provide optimal carrier targeting. (A) Suboptimally low ligand density negatively impacts a carrier's ability to engage in multivalent binding, hence suboptimal targeting as depicted in the model graph. (B) Surface density of ligand copies at which they engage multiple binding sites and achieve firm binding is sufficient and optimal for targeting. (C) Excessively high surface density of ligand molecules may lead to "a ligand overcrowding" phenomenon, i.e., inhibition of multivalent engagement due to steric limitations and competition of adjacent ligand molecules for the binding sites.

parameters of ligand affinity and surface density on a carrier have to be determined empirically because surface density and clustering of target determinants *in vivo* remain to be characterized.

Varying ligand surface density also may help enhance targeting selectivity. Often, so-called "specific" markers expressed on pathologically altered target cells are also expressed at lower levels in other areas of the body. Increasing avidity of a carrier beyond a certain threshold may result in an increase in "off-target" binding to normal cells expressing target determinants at a low basal level. Specifically, in the application of imaging and detection of disease, off-target uptake should be minimized to enhance the signal-to-noise ratio of the target tissue.

This point was validated in a study on molecular imaging of pulmonary inflammation by isotope-labeled anti-ICAM nanocarriers. Admittedly, ICAM-1 is not an ideal target for this goal, since it is expressed at a relatively high basal level among the normal endothelium, while its level roughly doubles in inflamed counterpart. *In vitro* studies in static and flow-exposed cell cultures, as well as computational simulation, showed that high-avidity nanoparticles anchor effectively to both naïve and activated endothelium, whereas low-avidity (*i.e.*, low anti-ICAM density) carriers effectively engage in multivalent anchoring preferentially to

inflamed endothelium.^{94,253} Figure 9 illustrates the study that affirmed the *in vivo* effects of tissue selectivity. Specifically, 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 proteoglycans. 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

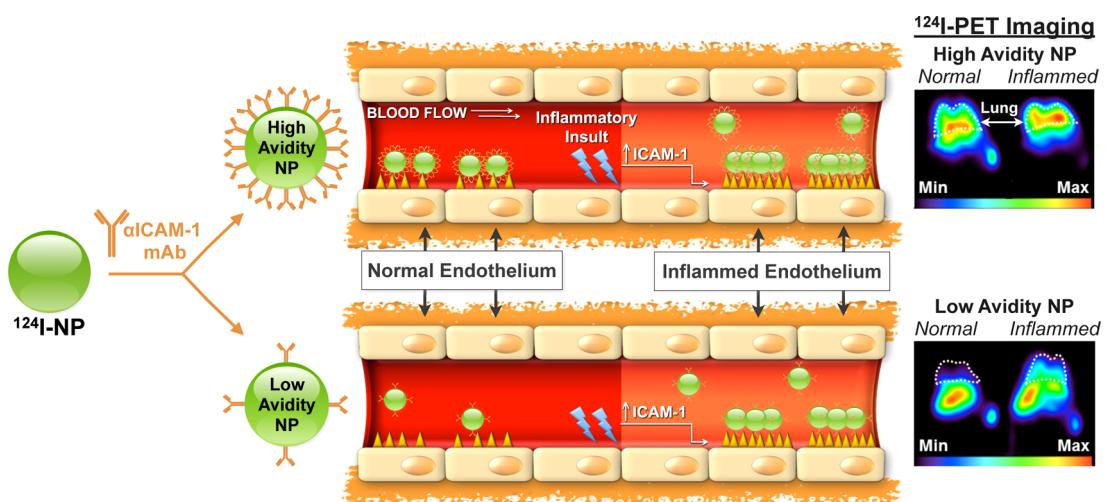


Figure 9. Controlled reduction of avidity (ligand surface density) enhances selectivity of carrier targeting to pathological endothelium. ICAM-1 is exposed on quiescent and pathological endothelium at modest basal and elevated levels, respectively. Carrier avidity to ICAM-1 is controlled by antibody surface density. High-avidity particles (approximately 150 nm diameter) carrying 200 molecules of anti-ICAM effectively bind to both quiescent and inflamed endothelial cells and show high pulmonary uptake after intravascular injection in both naïve and endotoxin-challenged mice (upper cartoons). In contrast, low-avidity particles carrying 50 anti-ICAM molecules show negligible binding to quiescent cells, whereas elevation of surface density of ICAM-1 typical of pathological endothelium allows their multivalent anchoring and binding to cytokine activated endothelium (lower cartoons). This phenomenon helps to explain the several-fold higher selectivity of targeting to pathological vs normal endothelium demonstrated by low-avidity vs high-avidity carriers in mouse model of lung inflammation (middle panels). As result, low-avidity anti-ICAM coated carriers provided PET images of the pulmonary inflammation in endotoxin-challenged mice that were more discernible from naïve animals than images provided by high-avidity carriers (far-right panels). PET imaging at 1 h after IV injection of ICAM-targeted [¹²⁴I] carriers carrying either 200 or 50 antibody molecules per particle (upper vs lower images, respectively) in naïve vs endotoxin-treated mice (left vs right). White dashed-line corresponds to lung space. (Adapted with permission from ref 156. Copyright 2013 American Chemical Society.)

2-fold more strongly than spherical counterparts. This enhanced effect was attributed to a combination of features as similar spherical particles were unable to perform as well. Indeed, it has yet to be shown whether or not these synthetic cells will be able to marginate in blood flow or perform hemostasis as well as real platelets. Yet engineering of the shape and flexibility of carriers that has been demonstrated by synthetic RBCs,¹⁴⁷ synthetic platelets,²⁸⁰ and filomicelles^{90,151} is required to perform complicated functions that spherical particles may not be able to address.

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 co-immobilized antigens²⁵⁷ or cytokine-activated cells.²⁷⁹ 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.^{257,301,302} Targeting liposomes to E-selectin and either VCAM-1 or ICAM-1 on cultured endothelial cells has also been reported.²⁷⁹ Maximal binding was observed with equimolar ratios of both ligands.^{279,303,304} 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.³⁰⁵

The dual targeting paradigm has also been used for molecular imaging: MRI-based NCs were targeted

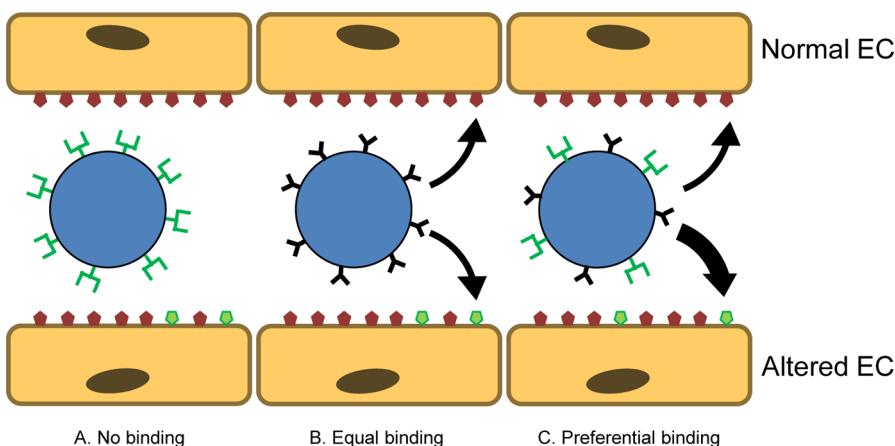


Figure 10. Carrier targeting using multiple ligands. (A) Carriers coated with ligands binding to endothelial determinants that are expressed in the area of interest selectively but scarcely (green) may have insufficient avidity for anchoring in this area. (B) Carriers coated with ligands binding to abundant endothelial determinants (red) anchor indiscriminately throughout the vasculature. (C) Carriers coated with a combination of two ligands may exert elevated basal avidity to endothelium, insufficient to provide a firm adhesion on its own but enabling binding in the case of simultaneous engagement of the scarce site-specific determinant.

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 congruity 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 vasculature is still missing. An immunochemistry-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.^{179,182}

In vitro experiments and computational studies complement expensive *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 *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.^{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 *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. Intracellular delivery and addressing to proper cellular compartments are needed for the desirable effects of many drugs, especially biotherapeutics.^{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.^{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 via vesicular micro- and macropinocytosis pathways.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 *via* caveoli^{39,319,320} and clathrin-coated³²¹ and uncoated vesicles,^{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.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 *via* its receptor into cellular vesicles.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,³²⁵ whereas endothelial cells display an opposite trend.³²⁶ Phagocytosis is viewed as a relatively minor contributor to endothelial uptake (this assumption may not necessarily reflect the phagocytic capacity of endothelium *in vivo*). Caveolar- and clathrin-mediated endocytosis are conventionally viewed as the predominant endothelial vesicular pathways.⁴⁷

Another conventional notion is that anchoring to a receptor leads to carrier uptake *via* the pathway naturally serving this molecule.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).⁶⁸ In contrast, liposomes targeted to the E-selectin, a transmembrane glycoprotein that is taken up by clathrin-mediated endocytosis,^{217,218} enter cells *via* this pathway and traffic to lysosomes.^{219,327} Similarly, antibodies to vascular cell adhesion molecule-1 (VCAM-1) are internalized by endothelial cells and addressed in the lysosomes *via* clathrin-mediated endocytosis^{327,328} and anti-VCAM conjugated compounds generally follow this fate.^{212,215,262} Antibodies to transferrin receptor (TfR) and compounds conjugated with TfR ligands also enter cells *via* this pathway, similarly to the endogenous ligand transferrin.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.^{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.²¹² Labeled VCAM-1 binding peptides undergoing enhanced endocytosis

provided improved imaging of vascular inflammation in animal models.^{212,215} 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).^{332–334}

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.^{56,335} 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.⁴⁰ 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.³³⁶ 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.^{38,337} 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.^{160,338} However, endothelial cells internalize multivalent anti-ICAM and anti-PECAM conjugates

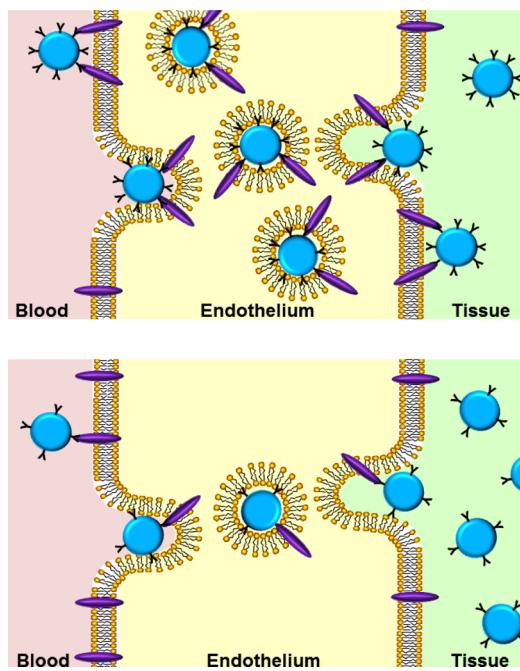


Figure 11. Optimization of transcellular transport via controlled reduction of carrier avidity. (Upper panel) Carriers with high avidity to a receptor involved in endocytosis and transcellular transport more effectively bind to and enter endothelium than carriers with low avidity (lower panel). However, high-avidity carriers less effectively dissociate from the receptor after the internalization, which may impede transfer to the tissue. In this simplified cartoon, the high and low avidity of carriers is depicted as proportional surface density of a ligand, whereas avidity may also be regulated by different affinity of the ligands coated at similar density.

and carriers coated with multiple copies of anti-CAM with a $t_{1/2}$ of ~10–20 min and uptake levels achieving 85–90% of the total amount of particles bound to the cells^{160,201,241,338} (Figure 12).

Multivalent ligands of PECAM and ICAM trigger a unique pathway, named CAM-mediated endocytosis,^{39,338} distinct from phagocytosis, pinocytosis, and the canonical endocytic pathways.²²⁸ Molecular signaling in CAM-mediated endocytosis involves sodium-proton exchanger NHE-1, release of Ca^{2+} , and a series of kinases and messengers mediating reorganization of the cytoskeleton that drives the uptake of CAM-anchored conjugates.^{228,338}

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.²²⁸ Therefore, most (not all) anti-CAM conjugates arrive in lysosomes several hours after uptake.^{39,228,239,338,339} 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.²²⁸

Co-internalized ICAM dissociates from the anti-ICAM coated carriers in the endosomes and recycles

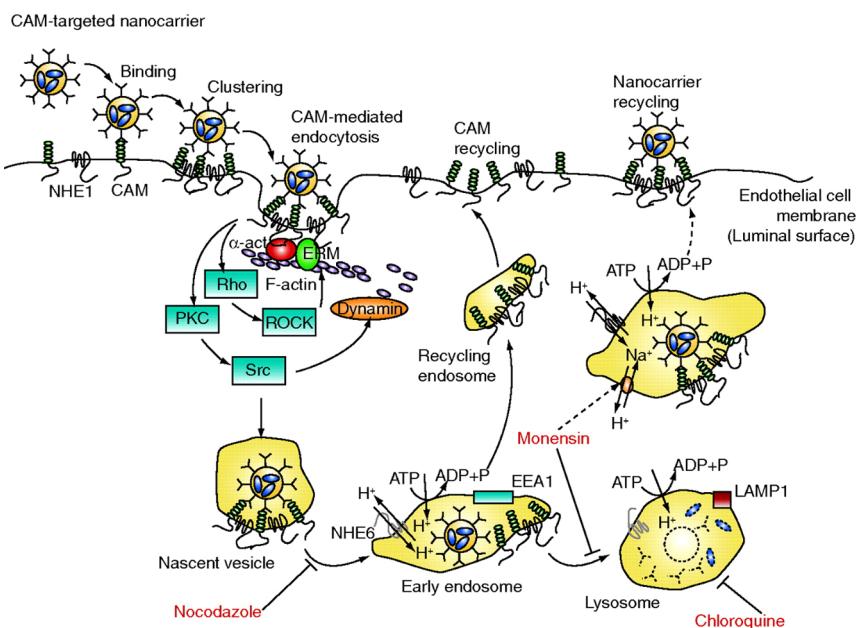


Figure 12. CAM-mediated endocytosis. Clustering cellular CAMs by multivalent anti-CAM conjugates activates a specific set of signaling kinases and the sodium/proton exchanger, NHE1, which leads to formation of actin stress fibers needed for the uptake of endocytic vesicles. Internalized carriers traffic to endosomes, where engaged CAM molecules dissociate from the carriers and recycle to the plasma membrane. Several hours after internalization, carriers arrive at lysosomes where proteolysis-sensitive carriers and cargoes are degraded. Nocodazole (which disrupts the cell microtubule network), chloroquine (which inhibits lysosomal acidification), and monensin (which enhances Na^+/H^+ exchange in endosomes and induces recycling of anti-CAM nanoconjugates to the plasma membrane) modulate intracellular traffic and effects of CAM-targeted DDS. (Reproduced with permission from B. Ding *et al.*, Advanced Drug Delivery Systems That Target the Vascular Endothelium, *Mol. Interv.* 2006, 6, 98–112.)

to the cell surface, while the carriers traffic further to lysosomes.²³⁹ This mechanism allows multiple cycles of intracellular delivery *in vitro* and *in vivo*.²³⁹ Using pharmacological agents interfering with vesicular transport and the cytoskeleton permits the deflection 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 proteins³⁴⁰ 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 caveolae³⁴¹) and exceeding of the capacity of endocytic machinery represent well-known phenomena. Endothelial cells internalize polymorphous anti-CAM conjugates with size <500 nm,³²⁶ but internalize anti-CAM coated spherical particles with diameter of up to a few micrometers,¹⁴³ 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.¹⁴³ 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.³⁴²

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.³⁴³ Nontargeted filomicelles showed 100% internalization in human derived lung endothelial cells.¹⁵¹ 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.³⁴⁴ In this study, clathrin-mediated, caveolae-mediated, and to a lesser extent macropinocytosis were involved in both

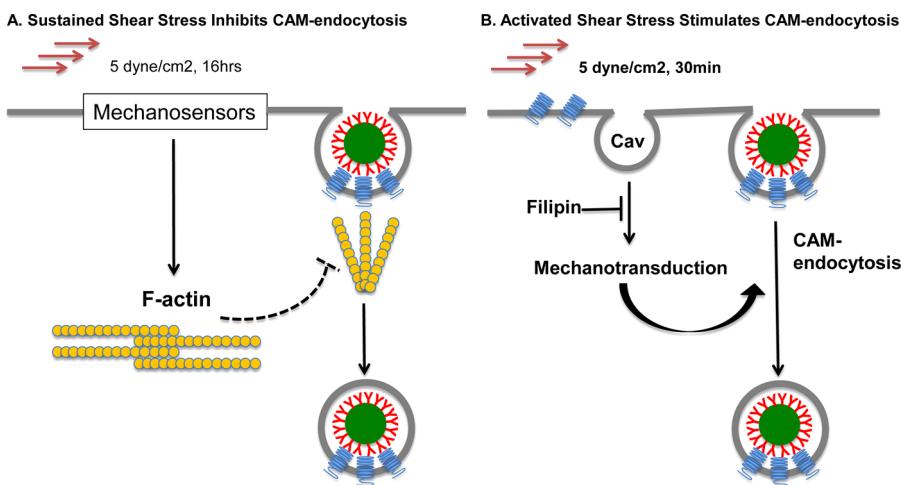


Figure 13. Flow-mediated modulation of CAM endocytosis. Endothelium *in vivo* is constantly or intermittently exposed to blood flow and chronic and acute exposure to flow differentially regulates endocytosis of CAM-targeted carriers. (A) Endothelial adaptation to chronic flow, manifested by cellular 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).

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 ~150 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 ($T_{1/2}$ was close to 20–30 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

and get internalized by cells.²⁴⁴ 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).³⁵⁰ 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,³⁵¹ and caveoli, such as APP,^{352–354} 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.¹³⁵ Some data has promoted the idea that caveoli merge into “caveolosomes” 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*.^{135,355,356} 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.^{72,314,357} In addition, many disease conditions, including inflammation, may affect this pathway.^{39,54–57,358}

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.³⁵⁹ 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.³⁶⁰ It is quite plausible that this transcellular transport pathway operates in the vascular endothelium as well and can be modulated by rational carrier design.

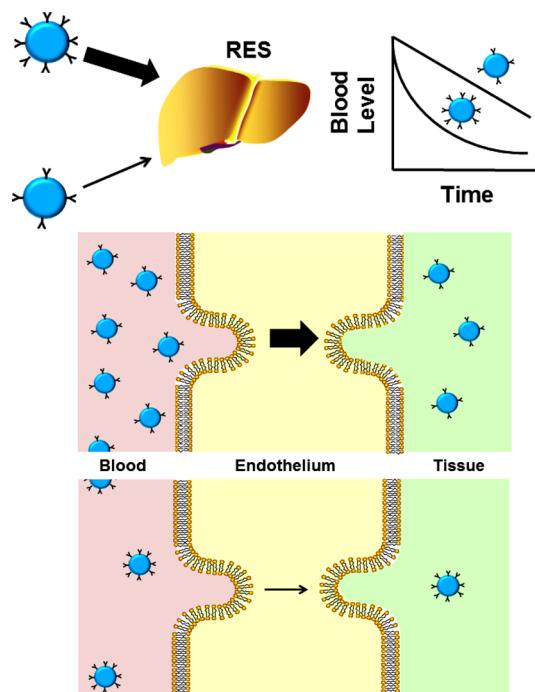


Figure 14. Enhanced blood level of low-avidity carriers as an additional factor facilitating cellular transport. PEGylated nanocarriers coated with a ligand at high density are more rapidly eliminated from blood by the clearing organs (e.g., liver) than carriers coated by a ligand at lower density (and thus retaining more preserved stealth features). This inequity in the PK may help to explain more effective cellular uptake and transport of low-avidity carriers due to elevated concentration in blood favoring binding to the target determinants.

Challenges and Perplexing Issues. All the issues discussed above in this rubric are pertinent here, including optimization of carrier labeling and tracing.³⁶¹ Data of PK/BD and targeting are vital for understanding of cellular traffic. Thus, enhanced transendothelial transport of low avidity TfR-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 (Figure 4). Second, the majority of TfR-mediated uptake occurs in liver; inhibition of hepatic uptake *via* reducing TfR avidity may also result in elevated blood level (Figure 14).

In vitro models do allow quantitative assessment of internalization^{339,361,362} and probing cellular pathways by pharmacological and genetic inhibitors.^{338,363} 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.^{356,364–369} Endothelial cells rapidly lose their phenotype in static culture.^{64,315,370–372} Use of *in vitro* flow models is more informative but is challenging and adds just one factor of vascular physiology.^{345,348,373}

Current *in vitro* models employ a limited range of flow parameters and “vessel geometries” from those observed in the body (turbulent, laminar, pulsating, etc.).^{271,374–378}

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.³⁷⁹ 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.

Conflict of Interest: The authors declare no competing financial interest.

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REFERENCES AND NOTES

- Farokhzad, O. C.; Langer, R. Impact of Nanotechnology on Drug Delivery. *ACS Nano* **2009**, *3*, 16–20.
- Langer, R. Drug Delivery and Targeting. *Nature* **1998**, *392*, 5–10.
- Wood, K. C.; Azarin, S. M.; Arap, W.; Pasqualini, R.; Langer, R.; Hammond, P. T. Tumor-Targeted Gene Delivery Using Molecularly Engineered Hybrid Polymers Functionalized with a Tumor-Homing Peptide. *Bioconjugate Chem.* **2008**, *19*, 403–405.
- Davis, M. E. The First Targeted Delivery of Sirna in Humans via a Self-Assembling, Cyclodextrin Polymer-Based Nanoparticle: From Concept to Clinic. *Mol. Pharmaceutics* **2009**, *6*, 659–668.
- Lee, J. B.; Zhang, K.; Tam, Y. Y. C.; Tam, Y. K.; Belliveau, N. M.; Sung, V. Y. C.; Lin, P. J. C.; LeBlanc, E.; Ciufolini, M. A.; Rennie, P. S.; et al. Lipid Nanoparticle Sirna Systems for Silencing the Androgen Receptor in Human Prostate Cancer *in Vivo*. *Int. J. Cancer* **2012**, *131*, E781–E790.
- Pagano, R. E.; Weinstein, J. N. Interactions of Liposomes with Mammalian Cells. *Annu. Rev. Biophys. Bioeng.* **1978**, *7*, 435–468.
- Gregoriadis, G. Liposomes in Therapeutic and Preventive Medicine: The Development of the Drug-Carrier Concept. *Ann. N.Y. Acad. Sci.* **1978**, *308*, 343–370.
- Ryman, B. E.; Tyrrell, D. A. Liposomes—Methodology and Applications. *Front. Biol.* **1979**, *48*, 549–574.
- Juliano, R. L. Drug Delivery Systems: A Brief Review. *Can. J. Physiol. Pharmacol.* **1978**, *56*, 683–690.
- Langer, R. S.; Peppas, N. A. Present and Future Applications of Biomaterials in Controlled Drug Delivery Systems. *Biomaterials* **1981**, *2*, 201–214.
- Elsabahy, M.; Wooley, K. L. Design of Polymeric Nanoparticles for Biomedical Delivery Applications. *Chem. Soc. Rev.* **2012**, *41*, 2545–2561.
- Perry, J. L.; Herlihy, K. P.; Napier, M. E.; Desimone, J. M. Print: A Novel Platform toward Shape and Size Specific Nanoparticle Theranostics. *Acc. Chem. Res.* **2011**, *44*, 990–998.
- Sawant, R. R.; Torchilin, V. P. Multifunctionality of Lipid-Core Micelles for Drug Delivery and Tumour Targeting. *Mol. Membr. Biol.* **2010**, *27*, 232–246.
- Venkataraman, S.; Hedrick, J. L.; Ong, Z. Y.; Yang, C.; Ee, P. L.; Hammond, P. T.; Yang, Y. Y. The Effects of Polymeric Nanostructure Shape on Drug Delivery. *Adv. Drug Delivery Rev.* **2011**, *63*, 1228–1246.
- van der Poll, D. G.; Kieler-Ferguson, H. M.; Floyd, W. C.; Guillaudeau, S. J.; Jerger, K.; Szoka, F. C.; Fréchet, J. M. Design, Synthesis, and Biological Evaluation of a Robust, Biodegradable Dendrimer. *Bioconjugate Chem.* **2010**, *21*, 764–773.
- Szoka, F., Jr.; Papahadjopoulos, D. Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes). *Annu. Rev. Biophys. Bioeng.* **1980**, *9*, 467–508.
- Percec, V.; Wilson, D. A.; Leowanawat, P.; Wilson, C. J.; Hughes, A. D.; Kaucher, M. S.; Hammer, D. A.; Levine, D. H.; Kim, A. J.; Bates, F. S.; et al. Self-Assembly of Janus Dendrimers into Uniform Dendrimersomes and Other Complex Architectures. *Science* **2010**, *328*, 1009–1014.

18. Eniola, A. O.; Hammer, D. A. Characterization of Biodegradable Drug Delivery Vehicles with the Adhesive Properties of Leukocytes II: Effect of Degradation on Targeting Activity. *Biomaterials* **2005**, *26*, 661–670.
19. Discher, B. M.; Won, Y. Y.; Ege, D. S.; Lee, J. C.; Bates, F. S.; Discher, D. E.; Hammer, D. A. Polymersomes: Tough Vesicles Made from Diblock Copolymers. *Science* **1999**, *284*, 1143–1146.
20. Liang, M.; Lu, J.; Kovochich, M.; Xia, T.; Ruehm, S. G.; Nel, A. E.; Tamanoi, F.; Zink, J. I. Multifunctional Inorganic Nanoparticles for Imaging, Targeting, and Drug Delivery. *ACS Nano* **2008**, *2*, 889–896.
21. Wang, Y.; Mirkin, C. A.; Park, S. J. Nanofabrication Beyond Electronics. *ACS Nano* **2009**, *3*, 1049–1056.
22. Bayer, C. L.; Peppas, N. A. Advances in Recognitive, Conductive and Responsive Delivery Systems. *J. Controlled Release* **2008**, *132*, 216–221.
23. Kieler-Ferguson, H. M.; Frechet, J. M.; Szoka, F. C., Jr. Clinical Developments of Chemotherapeutic Nanomedicines: Polymers and Liposomes for Delivery of Camptothecins and Platinum (II) Drugs. *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2013**, *5*, 130–138.
24. Batrakova, E. V.; Kabanov, A. V. Pluronic Block Copolymers: Evolution of Drug Delivery Concept from Inert Nanocarriers to Biological Response Modifiers. *J. Controlled Release* **2008**, *130*, 98–106.
25. Leucuta, S. E. Systemic and Biophase Bioavailability and Pharmacokinetics of Nanoparticulate Drug Delivery Systems. *Curr. Drug Delivery* **2013**, *10*, 208–240.
26. Fraley, R.; Papahadjopoulos, D. Liposomes: The Development of a New Carrier System for Introducing Nucleic Acid into Plant and Animal Cells. *Curr. Top. Microbiol. Immunol.* **1982**, *96*, 171–191.
27. Maeda, H. Macromolecular Therapeutics in Cancer Treatment: The Epr Effect and Beyond. *J. Controlled Release* **2012**, *164*, 138–144.
28. Uliel, L.; Royal, H. D.; Darcy, M. D.; Zuckerman, D. A.; Sharma, A.; Saad, N. E. From the Angio Suite to the Gamma-Camera: Vascular Mapping and 99mTc-MAA Hepatic Perfusion Imaging before Liver Radioembolization—A Comprehensive Pictorial Review. *J. Nucl. Med.* **2012**, *53*, 1736–1747.
29. Huo, D.; Deng, S.; Li, L.; Ji, J. Studies on the Poly(lactic-co-glycolic) Acid Microspheres of Cisplatin for Lung-Targeting. *Int. J. Pharm.* **2005**, *289*, 63–67.
30. Muller, D. W.; Gordon, D.; San, H.; Yang, Z.; Pompili, V. J.; Nabel, G. J.; Nabel, E. G. Catheter-Mediated Pulmonary Vascular Gene Transfer and Expression. *Circ. Res.* **1994**, *75*, 1039–1049.
31. Huang, R. B.; Mocherla, S.; Heslinga, M. J.; Charoenphol, P.; Eniola-Adefeso, O. Dynamic and Cellular Interactions of Nanoparticles in Vascular-Targeted Drug Delivery. *Mol. Membr. Biol.* **2010**, *27*, 312–327.
32. Corti, A.; Pastorino, F.; Curnis, F.; Arap, W.; Ponzoni, M.; Pasqualini, R. Targeted Drug Delivery and Penetration into Solid Tumors. *Med. Res. Rev.* **2012**, *32*, 1078–1091.
33. Wickline, S. A.; Neubauer, A. M.; Winter, P. M.; Caruthers, S. D.; Lanza, G. M. Molecular Imaging and Therapy of Atherosclerosis with Targeted Nanoparticles. *J. Magn. Reson. Imaging* **2007**, *25*, 667–680.
34. Noble, C. O.; Kirpotin, D. B.; Hayes, M. E.; Mamot, C.; Hong, K.; Park, J. W.; Benz, C. C.; Marks, J. D.; Drummond, D. C. Development of Ligand-Targeted Liposomes for Cancer Therapy. *Expert. Opin. Ther. Targets* **2004**, *8*, 335–353.
35. Xia, W.; Low, P. S. Folate-Targeted Therapies for Cancer. *J. Med. Chem.* **2010**, *53*, 6811–6824.
36. Saravanakumar, G.; Kim, K.; Park, J. H.; Rhee, K.; Kwon, I. C. Current Status of Nanoparticle-Based Imaging Agents for Early Diagnosis of Cancer and Atherosclerosis. *J. Biomed. Nanotechnol.* **2009**, *5*, 20–35.
37. Simone, E.; Ding, B. S.; Muzykantov, V. Targeted Delivery of Therapeutics to Endothelium. *Cell Tissue Res.* **2009**, *335*, 283–300.
38. Muro, S.; Muzykantov, V. R. Targeting of Antioxidant and Anti-Thrombotic Drugs to Endothelial Cell Adhesion Molecules. *Curr. Pharm. Des.* **2005**, *11*, 2383–2401.
39. Muro, S.; Koval, M.; Muzykantov, V. Endothelial Endocytic Pathways: Gates for Vascular Drug Delivery. *Curr. Vasc. Pharmacol.* **2004**, *2*, 281–299.
40. Muro, S. Challenges in Design and Characterization of Ligand-Targeted Drug Delivery Systems. *J. Controlled Release* **2012**, *164*, 125–137.
41. Muzykantov, V. R.; Radhakrishnan, R.; Eckmann, D. M. Dynamic Factors Controlling Targeting Nanocarriers to Vascular Endothelium. *Curr. Drug Metab.* **2012**, *13*, 70.
42. Muzykantov, V. R. Targeted Therapeutics and Nano-devices for Vascular Drug Delivery: Quo Vadis? *IUBMB Life* **2011**, *63*, 583–585.
43. Muzykantov, V. R. Biomedical Aspects of Targeted Delivery of Drugs to Pulmonary Endothelium. *Expert Opin. Drug Delivery* **2005**, *2*, 909–926.
44. Igarashi, E. Factors Affecting Toxicity and Efficacy of Polymeric Nanomedicines. *Toxicol. Appl. Pharmacol.* **2008**, *229*, 121–134.
45. Wischke, C.; Kruger, A.; Roch, T.; Pierce, B. F.; Li, W.; Jung, F.; Lendlein, A. Endothelial Cell Response to (Co)Polymer Nanoparticles Depending on the Inflammatory Environment and Comonomer Ratio. *Eur. J. Pharm. Biopharm.* **2013**, *84*, 288–296.
46. Garcia, A. N.; Vogel, S. M.; Komarova, Y. A.; Malik, A. B. Permeability of Endothelial Barrier: Cell Culture and *in Vivo* Models. *Methods Mol. Biol. (N.Y., NY, U.S.)* **2011**, *763*, 333–354.
47. Aird, W. C. Endothelium in Health and Disease. *Pharmacol. Rep.* **2008**, *60*, 139–143.
48. Thomas, S. R.; Witting, P. K.; Drummond, G. R. Redox Control of Endothelial Function and Dysfunction: Molecular Mechanisms and Therapeutic Opportunities. *Antioxid. Redox Signaling* **2008**, *10*, 1713–1765.
49. Stan, R. V.; Tse, D.; Deharvengt, S. J.; Smits, N. C.; Xu, Y.; Luciano, M. R.; McGarry, C. L.; Buitendijk, M.; Nemani, K. V.; Elgueta, R.; et al. The Diaphragms of Fenestrated Endothelia: Gatekeepers of Vascular Permeability and Blood Composition. *Dev. Cell* **2011**, *23*, 1203–1218.
50. Stan, R. V. Endothelial Stomat and Fenestral Diaphragms in Normal Vessels and Angiogenesis. *J. Cell. Mol. Med.* **2007**, *11*, 621–643.
51. Feng, D.; Nagy, J. A.; Pyne, K.; Hammel, I.; Dvorak, H. F.; Dvorak, A. M. Pathways of Macromolecular Extravasation across Microvascular Endothelium in Response to Vpf/Vegf and Other Vasoactive Mediators. *Microcirculation* **1999**, *6*, 23–44.
52. Thomsen, L. B.; Lichota, J.; Eskehave, T. N.; Linemann, T.; Mortensen, J. H.; du Jardin, K. G.; Moos, T. Brain Delivery Systems via Mechanism Independent of Receptor-Mediated Endocytosis and Adsorptive-Mediated Endocytosis. *Curr. Pharm. Biotechnol.* **2012**, *13*, 2349–2354.
53. Feng, D.; Nagy, J. A.; Dvorak, H. F.; Dvorak, A. M. Ultrastructural Studies Define Soluble Macromolecular, Particulate, and Cellular Transendothelial Cell Pathways in Venules, Lymphatic Vessels, and Tumor-Associated Microvessels in Man and Animals. *Microsc. Res. Technol.* **2002**, *57*, 289–326.
54. Carver, L. A.; Schnitzer, J. E. Caveolae: Mining Little Caves for New Cancer Targets. *Nat. Rev. Cancer* **2003**, *3*, 571–581.
55. Dejana, E. Endothelial Adherens Junctions: Implications in the Control of Vascular Permeability and Angiogenesis. *J. Clin. Invest.* **1996**, *98*, 1949–1953.
56. Pardridge, W. M.; Buciak, J.; Yang, J.; Wu, D. Enhanced Endocytosis in Cultured Human Breast Carcinoma Cells and *in Vivo* Biodistribution in Rats of a Humanized Monoclonal Antibody after Cationization of the Protein. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 548–554.
57. Predescu, D.; Vogel, S. M.; Malik, A. B. Functional and Morphological Studies of Protein Transcytosis in Continuous Endothelia. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2004**, *287*, L895–901.
58. Simionescu, N. Cellular Aspects of Transcapillary Exchange. *Physiol. Rev.* **1983**, *63*, 1536–1579.

59. Wang, Z.; Tiruppathi, C.; Cho, J.; Minshall, R. D.; Malik, A. B. Delivery of Nanoparticle: Complexed Drugs across the Vascular Endothelial Barrier via Caveolae. *IUBMB Life* **2011**, *63*, 659–667.
60. Davies, P. F. Endothelial Mechanisms of Flow-Mediated Athero-Protection and Susceptibility. *Circ. Res.* **2007**, *101*, 10–12.
61. Kowalski, P. S.; Leus, N. G.; Scherphof, G. L.; Ruiters, M. H.; Kamps, J. A.; Molema, G. Targeted Sirna Delivery to Diseased Microvascular Endothelial Cells: Cellular and Molecular Concepts. *IUBMB Life* **2011**, *63*, 648–658.
62. Hood, E.; Simone, E.; Wattamwar, P.; Dziubla, T.; Muzykantov, V. Nanocarriers for Vascular Delivery of Antioxidants. *Nanomedicine (London, U.K.)* **2011**, *6*, 1257–1272.
63. Muzykantov, V. R. Immunotargeting of Drugs to the Pulmonary Vascular Endothelium as a Therapeutic Strategy. *Pathophysiology* **1998**, *5*, 15–33.
64. Oh, P.; Li, Y.; Yu, J.; Durr, E.; Krasinska, K. M.; Carver, L. A.; Testa, J. E.; Schnitzer, J. E. Subtractive Proteomic Mapping of the Endothelial Surface in Lung and Solid Tumours for Tissue-Specific Therapy. *Nature* **2004**, *429*, 629–635.
65. Schnitzer, J. E. Vascular Targeting as a Strategy for Cancer Therapy. *N. Engl. J. Med.* **1998**, *339*, 472–474.
66. Spragg, D. D.; Alford, D. R.; Greferath, R.; Larsen, C. E.; Lee, K. D.; Gurtner, G. C.; Cybulsky, M. I.; Tosi, P. F.; Nicolau, C.; Gimbrone, M. A., Jr. Immunotargeting of Liposomes to Activated Vascular Endothelial Cells: A Strategy for Site-Selective Delivery in the Cardiovascular System. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8795–8800.
67. Kennel, S. J.; Lee, R.; Bultman, S.; Kabalka, G. Rat Monoclonal Antibody Distribution in Mice: An Epitope inside the Lung Vascular Space Mediates Very Efficient Localization. *Int. J. Radiat. Appl. Instrum., Part B* **1990**, *17*, 193–200.
68. McIntosh, D. P.; Tan, X. Y.; Oh, P.; Schnitzer, J. E. Targeting Endothelium and Its Dynamic Caveolae for Tissue-Specific Transcytosis *In Vivo*: A Pathway to Overcome Cell Barriers to Drug and Gene Delivery. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1996–2001.
69. Muzykantov, V. R.; Danilov, S. M. Targeting of Radiolabeled Monoclonal Antibody against Ace to the Pulmonary Endothelium. In *Targeted Delivery of Imaging Agents*; Torchilin, V., Ed. CRC Press: Boca Raton, FL, 1995; pp 465–485.
70. Goetz, D. J.; el-Sabban, M. E.; Hammer, D. A.; Pauli, B. U. Lu-Ecam-1-Mediated Adhesion of Melanoma Cells to Endothelium under Conditions of Flow. *Int. J. Cancer* **1996**, *65*, 192–199.
71. Huang, X.; Molema, G.; King, S.; Watkins, L.; Edgington, T. S.; Thorpe, P. E. Tumor Infarction in Mice by Antibody-Directed Targeting of Tissue Factor to Tumor Vasculature. *Science* **1997**, *275*, 547–550.
72. Stan, R. V.; Ghitescu, L.; Jacobson, B. S.; Palade, G. E. Isolation, Cloning, and Localization of Rat Pv-1, a Novel Endothelial Caveolar Protein. *J. Cell Biol.* **1999**, *145*, 1189–1198.
73. Rajotte, D.; Arap, W.; Hagedorn, M.; Koivunen, E.; Pasqualini, R.; Ruoslahti, E. Molecular Heterogeneity of the Vascular Endothelium Revealed by *In Vivo* Phage Display. *J. Clin. Invest.* **1998**, *102*, 430–437.
74. Danilov, S. M.; Muzykantov, V. R.; Martynov, A. V.; Atochina, E. N.; Sakharov, I.; Trakht, I. N.; Smirnov, V. N. Lung Is the Target Organ for a Monoclonal Antibody to Angiotensin-Converting Enzyme. *Lab. Invest.* **1991**, *64*, 118–124.
75. Pasqualini, R.; McDonald, D. M.; Arap, W. Vascular Targeting and Antigen Presentation. *Nat. Immunol.* **2001**, *2*, 567–568.
76. Muzykantov, V. R.; Atochina, E. N.; Ischiropoulos, H.; Danilov, S. M.; Fisher, A. B. Immunotargeting of Antioxidant Enzyme to the Pulmonary Endothelium. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5213–5218.
77. Reynolds, P. N.; Nicklin, S. A.; Kaliberova, L.; Boatman, B. G.; Grizzle, W. E.; Balyasnikova, I. V.; Baker, A. H.; Danilov, S. M.; Curiel, D. T. Combined Transductional and Transcriptional Targeting Improves the Specificity of Transgene Expression *In Vivo*. *Nat. Biotechnol.* **2001**, *19*, 838–842.
78. Hamilton, A. J.; Huang, S. L.; Warnick, D.; Rabbat, M.; Kane, B.; Nagaraj, A.; Klegerman, M.; McPherson, D. D. Intravascular Ultrasound Molecular Imaging of Atherosoma Components *In Vivo*. *J. Am. Coll. Cardiol.* **2004**, *43*, 453–460.
79. Kastrup, C. J.; Nahrendorf, M.; Figueiredo, J. L.; Lee, H.; Kambhampati, S.; Lee, T.; Cho, S.-W.; Gorbatov, R.; Iwamoto, Y.; Dang, T. T. Painting Blood Vessels and Atherosclerotic Plaques with an Adhesive Drug Depot. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 21444–21449.
80. Majmudar, M. D.; Yoo, J.; Keliher, E. J.; Truelove, J. J.; Iwamoto, Y.; Sena, B.; Dutta, P.; Borodovsky, A.; Fitzgerald, K.; Di Carli, M. F. Polymeric Nanoparticle Pet/Mr Imaging Allows Macrophage Detection in Atherosclerotic Plaques. *Circ. Res.* **2013**, *112*, 755–761.
81. Schmieder, A. H.; Wang, K.; Zhang, H.; Senpan, A.; Pan, D.; Keupp, J.; Caruthers, S. D.; Wickline, S. A.; Shen, B.; Wagner, E. M. Characterization of Early Neovascular Response to Acute Lung Ischemia Using Simultaneous 19f/1h Mr Molecular Imaging. *Angiogenesis* **2014**, *17*, 51–60.
82. Pan, D.; Pramanik, M.; Senpan, A.; Allen, J. S.; Zhang, H.; Wickline, S. A.; Wang, L. V.; Lanza, G. M. Molecular Photoacoustic Imaging of Angiogenesis with Integrin-Targeted Gold Nanobeacons. *FASEB J.* **2011**, *25*, 875–882.
83. Lanza, G.; Winter, P.; Caruthers, S.; Hughes, M.; Hu, G.; Schmieder, A.; Wickline, S. Theragnostics for Tumor and Plaque Angiogenesis with Perfluorocarbon Nanoemulsions. *Angiogenesis* **2010**, *13*, 189–202.
84. Sadeghi, M. M.; Glover, D. K.; Lanza, G. M.; Fayad, Z. A.; Johnson, L. L. Imaging Atherosclerosis and Vulnerable Plaque. *J. Nucl. Med.* **2010**, *51*, 51S–65S.
85. Kim, Y.; Lobatto, M. E.; Kawahara, T.; Chung, B. L.; Mieszawska, A. J.; Sanchez-Gaytan, B. L.; Fay, F.; Senders, M. L.; Calcagno, C.; Bechart, J.; et al. Probing Nanoparticle Translocation across the Permeable Endothelium in Experimental Atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 1078–1083.
86. Tahara, N.; Mukherjee, J.; de Haas, H. J.; Petrov, A. D.; Tawakol, A.; Haider, N.; Tahara, A.; Constantinescu, C. C.; Zhou, J.; Boersma, H. H. 2-Deoxy-2-[¹⁸F] Fluoro-D-Mannose Positron Emission Tomography Imaging in Atherosclerosis. *Nat. Med.* **2014**, *20*, 215–219.
87. Kim, Y.; Fay, F.; Cormode, D. P.; Sanchez-Gaytan, B. L.; Tang, J.; Hennessy, E. J.; Ma, M.; Moore, K.; Farokhzad, O. C.; Fisher, E. A. Single Step Reconstitution of Multi-functional High-Density Lipoprotein-Derived Nanomaterials Using Microfluidics. *ACS Nano* **2013**, *7*, 9975–9983.
88. Mieszawska, A. J.; Kim, Y.; Gianella, A.; van Rooy, I.; Priem, B.; Labarre, M. P.; Ozcan, C.; Cormode, D. P.; Petrov, A.; Langer, R. Synthesis of Polymer–Lipid Nanoparticles for Image-Guided Delivery of Dual Modality Therapy. *Bioconjugate Chem.* **2013**, *24*, 1429–1434.
89. Hara, T.; Bhayana, B.; Thompson, B.; Kessinger, C. W.; Khatri, A.; McCarthy, J. R.; Weissleder, R.; Lin, C. P.; Tearney, G. J.; Jaffer, F. A. Molecular Imaging of Fibrin Deposition in Deep Vein Thrombosis Using Fibrin-Targeted near-Infrared Fluorescence. *JACC* **2012**, *5*, 607–615.
90. Shuveev, V. V.; Ilies, M. A.; Simone, E.; Zaitsev, S.; Kim, Y.; Cai, S.; Mahmud, A.; Dziubla, T.; Muro, S.; Discher, D. E.; et al. Endothelial Targeting of Antibody-Decorated Polymeric Filomicelles. *ACS Nano* **2011**, *5*, 6991–6999.
91. Haun, J. B.; Pepper, L. R.; Boder, E. T.; Hammer, D. A. Using Engineered Single-Chain Antibodies to Correlate Molecular Binding Properties and Nanoparticle Adhesion Dynamics. *Langmuir* **2011**, *27*, 13701–13712.
92. Haun, J. B.; Hammer, D. A. Quantifying Nanoparticle Adhesion Mediated by Specific Molecular Interactions. *Langmuir* **2008**, *24*, 8821–8832.

93. Eniola, A. O.; Krasik, E. F.; Smith, L. A.; Song, G.; Hammer, D. A. I-Domain of Lymphocyte Function-Associated Antigen-1 Mediates Rolling of Polystyrene Particles on ICAM-1 under Flow. *Biophys. J.* **2005**, *89*, 3577–3588.
94. Calderon, A. J.; Muzykantov, V.; Muro, S.; Eckmann, D. M. Flow Dynamics, Binding and Detachment of Spherical Carriers Targeted to ICAM-1 on Endothelial Cells. *Biorheology* **2009**, *46*, 323–341.
95. Scherpereel, A.; Rome, J. J.; Wiewrodt, R.; Watkins, S. C.; Harshaw, D. W.; Alder, S.; Christofidou-Solomidou, M.; Haut, E.; Murciano, J. C.; Nakada, M.; et al. Platelet-Endothelial Cell Adhesion Molecule-1-Directed Immunotargeting to Cardiopulmonary Vasculature. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 777–786.
96. Danielyan, K.; Ding, B.-S.; Gottstein, C.; Cines, D. B.; Muzykantov, V. R. Delivery of Anti-Platelet-Endothelial Cell Adhesion Molecule Single-Chain Variable Fragment-Urokinase Fusion Protein to the Cerebral Vasculature Lyses Arterial Clots and Attenuates Postischemic Brain Edema. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 947–952.
97. Matter, C. M.; Schuler, P. K.; Alessi, P.; Meier, P.; Ricci, R.; Zhang, D.; Halin, C.; Castellani, P.; Zardi, L.; Hofer, C. K. Molecular Imaging of Atherosclerotic Plaques Using a Human Antibody against the Extra-Domain B of Fibronectin. *Circ. Res.* **2004**, *95*, 1225–1233.
98. Virmani, R.; Kolodgie, F. D.; Burke, A. P.; Finn, A. V.; Gold, H. K.; Tuleenko, T. N.; Wrenn, S. P.; Narula, J. Atherosclerotic Plaque Progression and Vulnerability to Rupture Angiogenesis as a Source of Intraplaque Hemorrhage. *Arterioscler., Thromb., Vasc. Biol.* **2005**, *25*, 2054–2061.
99. Winter, P. M.; Neubauer, A. M.; Caruthers, S. D.; Harris, T. D.; Robertson, J. D.; Williams, T. A.; Schmieder, A. H.; Hu, G.; Allen, J. S.; Lacy, E. K. Endothelial $\text{Av}\beta 3$ Integrin—Targeted Fumagillin Nanoparticles Inhibit Angiogenesis in Atherosclerosis. *Arterioscler., Thromb., Vasc. Biol.* **2006**, *26*, 2103–2109.
100. Moreno, P. R.; Purushothaman, K.-R.; Sirol, M.; Levy, A. P.; Fuster, V. Neovascularization in Human Atherosclerosis. *Circulation* **2006**, *113*, 2245–2252.
101. Gerrit, L.; Sijbrands, E. J.; Valkema, R.; Folkert, J.; Feinstein, S. B.; Van der Steen, A. F.; Daemen, M. J.; Schinkel, A. F. Molecular Imaging of Inflammation and Intraplaque Vasa Vasorum: A Step Forward to Identification of Vulnerable Plaques? *J. Nucl. Cardiol* **2010**, *17*, 897–912.
102. Schinkel, A. F.; Krueger, C. G.; Tellez, A.; Granada, J. F.; Reed, J. D.; Hall, A.; Zang, W.; Owens, C.; Kaluza, G. L.; Staub, D. Contrast-Enhanced Ultrasound for Imaging Vasa Vasorum: Comparison with Histopathology in a Swine Model of Atherosclerosis. *Eur. J. Echocardiogr.* **2010**, *11*, 659–664.
103. Korin, N.; Kanapathipillai, M.; Matthews, B. D.; Crescente, M.; Brill, A.; Mammoto, T.; Ghosh, K.; Jurek, S.; Bencherif, S. A.; Bhatta, D.; et al. Shear-Activated Nanotherapeutics for Drug Targeting to Obstructed Blood Vessels. *Science* **2012**, *337*, 738–742.
104. Owens, D. E., III; Peppas, N. A. Opsonization, Biodistribution, and Pharmacokinetics of Polymeric Nanoparticles. *Int. J. Pharm.* **2006**, *307*, 93–102.
105. Roser, M.; Fischer, D.; Kissel, T. Surface-Modified Biodegradable Albumin Nano- and Microspheres. II: Effect of Surface Charges on *in Vitro* Phagocytosis and Biodistribution in Rats. *Eur. J. Pharm. Biopharm.* **1998**, *46*, 255–63.
106. Carrstensen, H.; Muller, R. H.; Muller, B. W. Particle Size, Surface Hydrophobicity and Interaction with Serum of Parenteral Fat Emulsions and Model Drug Carriers as Parameters Related to Res Uptake. *Clin. Nutr.* **1992**, *11*, 289–97.
107. Howard, M. D.; Jay, M.; Dziubla, T. D.; Lu, X. Pegylation of Nanocarrier Drug Delivery Systems: State of the Art. *J. Biomed. Nanotechnol.* **2008**, *4*, 133–148.
108. Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R. Biodegradable Long-Circulating Polymeric Nanospheres. *Science* **1994**, *263*, 1600–1603.
109. Mori, A.; Klibanov, A. L.; Torchilin, V. P.; Huang, L. Influence of the Steric Barrier Activity of Amphiphatic Poly(Ethyleneglycol) and Ganglioside Gm1 on the Circulation Time of Liposomes and on the Target Binding of Immunoliposomes *in Vivo*. *FEBS Lett.* **1991**, *284*, 263–266.
110. Torchilin, V. P.; Klibanov, A. L.; Huang, L.; O'Donnell, S.; Nossiff, N. D.; Khaw, B. A. Targeted Accumulation of Polyethylene Glycol-Coated Immunoliposomes in Infarcted Rabbit Myocardium. *FASEB J.* **1992**, *6*, 2716–2719.
111. Klibanov, A. L.; Maruyama, K.; Beckerleg, A. M.; Torchilin, V. P.; Huang, L. Activity of Amphiphatic Poly(Ethylene Glycol) 5000 to Prolong the Circulation Time of Liposomes Depends on the Liposome Size and Is Unfavorable for Immunoliposome Binding to Target. *Biochim. Biophys. Acta* **1991**, *1062*, 142–148.
112. Sawant, R. R.; Sawant, R. M.; Kale, A. A.; Torchilin, V. P. The Architecture of Ligand Attachment to Nanocarriers Controls Their Specific Interaction with Target Cells. *J. Drug Targeting* **2008**, *16*, 596–600.
113. Biswas, S.; Dodwadkar, N. S.; Sawant, R. R.; Torchilin, V. P. Development of the Novel Peg-Pe-Based Polymer for the Reversible Attachment of Specific Ligands to Liposomes: Synthesis and *in Vitro* Characterization. *Bioconjugate Chem.* **2011**, *22*, 2005–2013.
114. Liu, J.; Huang, Y.; Kumar, A.; Tan, A.; Jin, S.; Mozhi, A.; Liang, X. J. Ph-Sensitive Nano-Systems for Drug Delivery in Cancer Therapy. *Biotechnol. Adv.* **2013**, *10.1016/j.biotechadv.2013.11.009*.
115. Ishida, T.; Maeda, R.; Ichihara, M.; Mukai, Y.; Motoki, Y.; Manabe, Y.; Irimura, K.; Kiwada, H. The Accelerated Clearance on Repeated Injection of Pegylated Liposomes in Rats: Laboratory and Histopathological Study. *Cell. Mol. Biol. Lett.* **2002**, *7*, 286.
116. Ishida, T.; Maeda, R.; Ichihara, M.; Irimura, K.; Kiwada, H. Accelerated Clearance of Pegylated Liposomes in Rats after Repeated Injections. *J. Controlled Release* **2003**, *88*, 35–42.
117. Lu, W.; Wan, J.; She, Z.; Jiang, X. Brain Delivery Property and Accelerated Blood Clearance of Cationic Albumin Conjugated Pegylated Nanoparticle. *J. Controlled Release* **2007**, *118*, 38–53.
118. Ishida, T.; Ichihara, M.; Wang, X.; Yamamoto, K.; Kimura, J.; Majima, E.; Kiwada, H. Injection of Pegylated Liposomes in Rats Elicits Peg-Specific IgM, Which Is Responsible for Rapid Elimination of a Second Dose of Pegylated Liposomes. *J. Controlled Release* **2006**, *112*, 15–25.
119. Dams, E. T.; Laverman, P.; Oyen, W. J.; Storm, G.; Scherphof, G. L.; van Der Meer, J. W.; Corstens, F. H.; Boerman, O. C. Accelerated Blood Clearance and Altered Biodistribution of Repeated Injections of Sterically Stabilized Liposomes. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 1071–1079.
120. Laverman, P.; Carstens, M. G.; Boerman, O. C.; Dams, E. T.; Oyen, W. J.; van Rooijen, N.; Corstens, F. H.; Storm, G. Factors Affecting the Accelerated Blood Clearance of Polyethylene Glycol-Liposomes Upon Repeated Injection. *J. Pharmacol. Exp. Ther.* **2001**, *298*, 607–612.
121. Ishida, T.; Ichihara, M.; Wang, X.; Kiwada, H. Spleen Plays an Important Role in the Induction of Accelerated Blood Clearance of Pegylated Liposomes. *J. Controlled Release* **2006**, *115*, 243–250.
122. Zandvoort, A.; Timens, W. The Dual Function of the Splenic Marginal Zone: Essential for Initiation of Anti-Ti-2 Responses but Also Vital in the General First-Line Defense against Blood-Borne Antigens. *Clin. Exp. Immunol.* **2002**, *130*, 4–11.
123. Ichihara, M.; Shimizu, T.; Imoto, A.; Hashiguchi, Y.; Uehara, Y.; Ishida, T.; Kiwada, H. Anti-Peg IgM Response against Pegylated Liposomes in Mice and Rats. *Pharmaceutics* **2010**, *3*, 1–11.
124. Park, Y. S.; Huang, L. Effect of Chemically Modified Gm1 and Neoglycolipid Analogs of Gm1 on Liposome Circulation Time: Evidence Supporting the Dysopsonin Hypothesis. *Biochim. Biophys. Acta* **1993**, *1166*, 105–114.
125. Michalek, M. T.; Mold, C.; Bremer, E. G. Inhibition of the Alternative Pathway of Human Complement by

- Structural Analogues of Sialic Acid. *J. Immunol.* **1988**, *140*, 1588–1594.
126. Shichijo, S.; Alving, C. R. Inhibitory Effects of Gangliosides on Immune Reactions of Antibodies to Neutral Glycolipids in Liposomes. *Biochim. Biophys. Acta* **1986**, *858*, 118–124.
 127. Rodriguez, P. L.; Harada, T.; Christian, D. A.; Pantano, D. A.; Tsai, R. K.; Discher, D. E. Minimal “Self” Peptides That Inhibit Phagocytic Clearance and Enhance Delivery of Nanoparticles. *Science* **2013**, *339*, 971–975.
 128. Stachelek, S. J.; Finley, M. J.; Alferiev, I. S.; Wang, F.; Tsai, R. K.; Eckells, E. C.; Tomczyk, N.; Connolly, J. M.; Discher, D. E.; Eckmann, D. M.; et al. The Effect of Cd47 Modified Polymer Surfaces on Inflammatory Cell Attachment and Activation. *Biomaterials* **2011**, *32*, 4317–4326.
 129. Tsai, R. K.; Rodriguez, P. L.; Discher, D. E. Self Inhibition of Phagocytosis: The Affinity of ‘Marker of Self’ Cd47 for Sirpalpha Dictates Potency of Inhibition but Only at Low Expression Levels. *Blood Cells Mol. Dis.* **2010**, *45*, 67–74.
 130. Subramanian, S.; Parthasarathy, R.; Sen, S.; Boder, E. T.; Discher, D. E. Species- and Cell Type-Specific Interactions between Cd47 and Human Sirpalpha. *Blood* **2006**, *107*, 2548–2556.
 131. Hu, C. M.; Zhang, L.; Aryal, S.; Cheung, C.; Fang, R. H.; Zhang, L. Erythrocyte Membrane-Camouflaged Polymeric Nanoparticles as a Biomimetic Delivery Platform. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 10980–10985.
 132. Parodi, A.; Quattrocchi, N.; van de Ven, A. L.; Chiappini, C.; Evangelopoulos, M.; Martinez, J. O.; Brown, B. S.; Khaled, S. Z.; Yazdi, I. K.; Enzo, M. V.; et al. Synthetic Nanoparticles Functionalized with Biomimetic Leukocyte Membranes Possess Cell-Like Functions. *Nat. Nanotechnol.* **2013**, *8*, 61–68.
 133. Choi, H. S.; Liu, W.; Misra, P.; Tanaka, E.; Zimmer, J. P.; Ipe, B. I.; Bawendi, M. G.; Frangioni, J. V. Renal Clearance of Quantum Dots. *Nat. Biotechnol.* **2007**, *25*, 1165–1170.
 134. Predescu, S. A.; Predescu, D. N.; Malik, A. B. Molecular Determinants of Endothelial Transcytosis and Their Role in Endothelial Permeability. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2007**, *293*, L823–L842.
 135. Oh, P.; Borgstrom, P.; Witkiewicz, H.; Li, Y.; Borgstrom, B. J.; Chrustina, A.; Iwata, K.; Zinn, K. R.; Baldwin, R.; Testa, J. E.; et al. Live Dynamic Imaging of Caveolae Pumping Targeted Antibody Rapidly and Specifically across Endothelium in the Lung. *Nat. Biotechnol.* **2007**, *25*, 327–337.
 136. Shuvaev, V. V.; Tliba, S.; Pick, J.; Arguiri, E.; Christofidou-Solomidou, M.; Albelda, S. M.; Muzykantov, V. R. Modulation of Endothelial Targeting by Size of Antibody-Antioxidant Enzyme Conjugates. *J. Controlled Release* **2011**, *149*, 236–241.
 137. Daum, N.; Tscheke, C.; Neumeyer, A.; Schneider, M. Novel Approaches for Drug Delivery Systems in Nanomedicine: Effects of Particle Design and Shape. *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2012**, *4*, 52–65.
 138. Simone, E. A.; Dziubla, T. D.; Muzykantov, V. R. Polymeric Carriers: Role of Geometry in Drug Delivery. *Expert Opin. Drug Delivery* **2008**, *5*, 1283–1300.
 139. Liu, Y.; Tan, J.; Thomas, A.; Ou-Yang, D.; Muzykantov, V. R. The Shape of Things to Come: Importance of Design in Nanotechnology for Drug Delivery. *Ther. Delivery* **2012**, *3*, 181–194.
 140. Champion, J. A.; Katare, Y. K.; Mitragotri, S. Particle Shape: A New Design Parameter for Micro- and Nanoscale Drug Delivery Carriers. *J. Controlled Release* **2007**, *121*, 3–9.
 141. Mitragotri, S. In Drug Delivery, Shape Does Matter. *Pharm. Res.* **2009**, *26*, 232–234.
 142. Herd, H.; Daum, N.; Jones, A. T.; Huwer, H.; Ghandehari, H.; Lehr, C. M. Nanoparticle Geometry and Surface Orientation Influence Mode of Cellular Uptake. *ACS Nano* **2013**, *7*, 1961–1973.
 143. Muro, S.; Garnacho, C.; Champion, J. A.; Leferovich, J.; Gajewski, C.; Schuchman, E. H.; Mitragotri, S.; Muzykantov, V. R. Control of Endothelial Targeting and Intracellular Delivery of Therapeutic Enzymes by Modulating the Size and Shape of ICAM-1-Targeted Carriers. *Mol. Ther.* **2008**, *16*, 1450–1458.
 144. Champion, J. A.; Mitragotri, S. Shape Induced Inhibition of Phagocytosis of Polymer Particles. *Pharm. Res.* **2009**, *26*, 244–249.
 145. Champion, J.; Mitragotri, S. Role of Target Geometry in Phagocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 4930–4934.
 146. Muzykantov, V. R. Drug Delivery by Red Blood Cells: Vascular Carriers Designed by Mother Nature. *Expert Opin. Drug Delivery* **2010**, *7*, 403–427.
 147. Merkel, T. J.; Jones, S. W.; Herlihy, K. P.; Kersey, F. R.; Shields, A. R.; Napier, M.; Luft, J. C.; Wu, H.; Zamboni, W. C.; Wang, A. Z.; et al. Using Mechanobiological Mimicry of Red Blood Cells to Extend Circulation Times of Hydrogel Microparticles. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 586–591.
 148. Fung, Y.-c. *Biomechanics: Mechanical Properties of Living Tissues*; Springer: New York, 1993.
 149. Merkel, T. J.; Chen, K.; Jones, S. W.; Pandya, A. A.; Tian, S.; Napier, M. E.; Zamboni, W. E.; DeDimone, J. M. The Effect of Particle Size on the Biodistribution of Low-Modulus Hydrogel Print Particles. *J. Controlled Release* **2012**, *162*, 37–44.
 150. Simone, E. A.; Dziubla, T. D.; Colon-Gonzalez, F.; Discher, D. E.; Muzykantov, V. R. Effect of Polymer Amphiphilicity on Loading of a Therapeutic Enzyme into Protective Filamentous and Spherical Polymer Nanocarriers. *Biomacromolecules* **2007**, *8*, 3914–3921.
 151. Geng, Y.; Dalhaimer, P.; Cai, S.; Tsai, R.; Tewari, M.; Minko, T.; Discher, D. E. Shape Effects of Filaments versus Spherical Particles in Flow and Drug Delivery. *Nat. Nanotechnol.* **2007**, *2*, 249–255.
 152. Dalhaimer, P.; Engler, A. J.; Parthasarathy, R.; Discher, D. E. Targeted Worm Micelles. *Biomacromolecules* **2004**, *5*, 1714–1719.
 153. Venditto, V. J.; Szoka, F. C., Jr. Cancer Nanomedicines: So Many Papers and So Few Drugs!. *Adv. Drug Delivery Rev.* **2013**, *65*, 80–88.
 154. Pollinger, K.; Hennig, R.; Ohlmann, A.; Fuchshofer, R.; Wenzel, R.; Breunig, M.; Tessmar, J.; Tamm, E. R.; Goepfertich, A. Ligand-Functionalized Nanoparticles Target Endothelial Cells in Retinal Capillaries after Systemic Application. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 6115–6120.
 155. Brinkhuis, R. P.; Stojanov, K.; Laverman, P.; Eilander, J.; Zuhorn, I. S.; Rutjes, F. P.; van Hest, J. C. Size Dependent Biodistribution and Spect Imaging of (111) in-Labeled Polymersomes. *Bioconjugate Chem.* **2012**, *26*, 958–965.
 156. Zern, B. J.; Chacko, A.-M.; Liu, J.; Greineder, C. F.; Blankemeyer, E. R.; Radhakrishnan, R.; Muzykantov, V. Reduction of Nanoparticle Avidity Enhances the Selectivity of Vascular Targeting and Pet Detection of Pulmonary Inflammation. *ACS Nano* **2013**, *7*, 2461–2469.
 157. Rossin, R.; Muro, S.; Welch, M. J.; Muzykantov, V. R.; Schuster, D. P. In Vivo Imaging of 64cu-Labeled Polymer Nanoparticles Targeted to the Lung Endothelium. *J. Nucl. Med.* **2008**, *49*, 103–111.
 158. Danilov, S. M.; Martynov, A. V.; Klibanov, A. L.; Slinkin, M. A.; Sakharov, I.; Malov, A. G.; Sergienko, V. B.; Vedernikov, A.; Muzykantov, V. R.; Torchilin, V. P. Radioimmunoimaging of Lung Vessels: An Approach Using Indium-111-Labeled Monoclonal Antibody to Angiotensin-Converting Enzyme. *J. Nucl. Med.* **1989**, *30*, 1686–1692.
 159. Simone, E. A.; Zern, B. J.; Chacko, A. M.; Mikitsh, J. L.; Blankemeyer, E. R.; Muro, S.; Stan, R. V.; Muzykantov, V. R. Endothelial Targeting of Polymeric Nanoparticles Stably Labeled with the Pet Imaging Radioisotope Iodine-124. *Biomaterials* **2012**, *33*, 5406–5413.
 160. Muzykantov, V. R.; Christofidou-Solomidou, M.; Balyasnikova, I.; Harshaw, D. W.; Schultz, L.; Fisher, A. B.; Albelda, S. M. Streptavidin Facilitates Internalization and Pulmonary Targeting of an Anti-Endothelial Cell Antibody (Platelet-Endothelial Cell Adhesion Molecule 1): A Strategy for Vascular Immunotargeting of Drugs. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 2379–2384.

161. Muzykantov, V. R.; Puchnina, E. A.; Atochina, E. N.; Hiemish, H.; Slinkin, M. A.; Meertsuk, F. E.; Danilov, S. M. Endotoxin Reduces Specific Pulmonary Uptake of Radiolabeled Monoclonal Antibody to Angiotensin-Converting Enzyme. *J. Nucl. Med.* **1991**, *32*, 453–460.
162. Pan, H.; Myerson, J. W.; Hu, L.; Marsh, J. N.; Hou, K.; Scott, M. J.; Allen, J. S.; Hu, G.; San Roman, S.; Lanza, G. M.; et al. A Programmable Nanoparticle Functionalization for *in Vivo* Targeting. *FASEB J.* **2013**, *27*, 255–264.
163. Muzykantov, V. R.; Gavriluk, V. D.; Reinecke, A.; Atochina, E. N.; Kuo, A.; Barnathan, E. S.; Fisher, A. B. The Functional Effects of Biotinylation of Anti-Angiotensin-Converting Enzyme Monoclonal Antibody in Terms of Targeting *in Vivo*. *Anal. Biochem.* **1995**, *226*, 279–287.
164. Tenzer, S.; Docter, D.; Rosfa, S.; Włodarski, A.; Kuharev, J.; Rekik, A.; Knauer, S. K.; Bantz, C.; Nawroth, T.; Bier, C.; et al. Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis. *ACS Nano* **2011**, *5*, 7155–7167.
165. Monopoli, M. P.; Aberg, C.; Salvati, A.; Dawson, K. A. Biomolecular Coronas Provide the Biological Identity of Nanosized Materials. *Nat. Nanotechnol.* **2012**, *7*, 779–786.
166. Andersen, A. J.; Hashemi, S. H.; Andresen, T. L.; Hunter, A. C.; Moghimi, S. M. Complement: Alive and Kicking Nanomedicines. *J. Biomed. Nanotechnol.* **2009**, *5*, 364–372.
167. Salvati, A.; Pitek, A. S.; Monopoli, M. P.; Prapainop, K.; Bombelli, F. B.; Hristov, D. R.; Kelly, P. M.; Aberg, C.; Mahon, E.; Dawson, K. A. Transferrin-Functionalized Nanoparticles Lose Their Targeting Capabilities When a Biomolecule Corona Adsorbs on the Surface. *Nat. Nanotechnol.* **2013**, *8*, 137–143.
168. Lesniak, A.; Fenaroli, F.; Monopoli, M. P.; Aberg, C.; Dawson, K. A.; Salvati, A. Effects of the Presence or Absence of a Protein Corona on Silica Nanoparticle Uptake and Impact on Cells. *ACS Nano* **2012**, *6*, 5845–5857.
169. Tenzer, S.; Docter, D.; Kuharev, J.; Musyanovich, A.; Fetz, V.; Hecht, R.; Schlenk, F.; Fischer, D.; Kiouoptsi, K.; Reinhardt, C.; et al. Rapid Formation of Plasma Protein Corona Critically Affects Nanoparticle Pathophysiology. *Nat. Nanotechnol.* **2013**, *8*, 772–781.
170. Muzykantov, V. R.; Smirnov, M. D.; Samokhin, G. P. Avidin Attachment to Biotinylated Erythrocytes Induces Homologous Lysis *via* the Alternative Pathway of Complement. *Blood* **1991**, *78*, 2611–2618.
171. Lesniak, A.; Campbell, A.; Monopoli, M. P.; Lynch, I.; Salvati, A.; Dawson, K. A. Serum Heat Inactivation Affects Protein Corona Composition and Nanoparticle Uptake. *Biomaterials* **2010**, *31*, 9511–9518.
172. Muzykantov, V. R.; Smirnov, M. D.; Samokhin, G. P. Streptavidin-Induced Lysis of Homologous Biotinylated Erythrocytes. Evidence against the Key Role of the Avidin Charge in Complement Activation *via* the Alternative Pathway. *FEBS Lett.* **1991**, *280*, 112–114.
173. Durr, E.; Yu, J.; Krasinska, K. M.; Carver, L. A.; Yates, J. R.; Testa, J. E.; Oh, P.; Schnitzer, J. E. Direct Proteomic Mapping of the Lung Microvascular Endothelial Cell Surface *in Vivo* and in Cell Culture. *Nat. Biotechnol.* **2004**, *22*, 985–992.
174. Hajitou, A.; Trepel, M.; Lilley, C. E.; Soghomonyan, S.; Alauddin, M. M.; Marini, F. C., III; Restel, B. H.; Ozawa, M. G.; Moya, C. A.; Rangel, R.; et al. A Hybrid Vector for Ligand-Directed Tumor Targeting and Molecular Imaging. *Cell* **2006**, *125*, 385–398.
175. Nicklin, S. A.; White, S. J.; Watkins, S. J.; Hawkins, R. E.; Baker, A. H. Selective Targeting of Gene Transfer to Vascular Endothelial Cells by Use of Peptides Isolated by Phage Display. *Circulation* **2000**, *102*, 231–237.
176. Christofidou-Solomidou, M.; Muzykantov, V. R. Antioxidant Strategies in Respiratory Medicine. *Treat. Respir. Med.* **2006**, *5*, 47–78.
177. Muzykantov, V. R. Targeting of Superoxide Dismutase and Catalase to Vascular Endothelium. *J. Controlled Release* **2001**, *71*, 1–21.
178. Pasqualini, R.; Arap, W.; McDonald, D. M. Probing the Structural and Molecular Diversity of Tumor Vasculature. *Trends Mol. Med.* **2002**, *8*, 563–571.
179. Danilov, S. M.; Gavriluk, V. D.; Franke, F. E.; Pauls, K.; Harshaw, D. W.; McDonald, T. D.; Miletich, D. J.; Muzykantov, V. R. Lung Uptake of Antibodies to Endothelial Antigens: Key Determinants of Vascular Immunotargeting. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2001**, *280*, L1335–L1347.
180. Shuvaev, V. V.; Christofidou-Solomidou, M.; Scherpereel, A.; Simone, E.; Arguiri, E.; Tliba, S.; Pick, J.; Kennel, S.; Albelda, S. M.; Muzykantov, V. R. Factors Modulating the Delivery and Effect of Enzymatic Cargo Conjugated with Antibodies Targeted to the Pulmonary Endothelium. *J. Controlled Release* **2007**, *118*, 235–244.
181. Keelan, E. T.; Harrison, A. A.; Chapman, P. T.; Binns, R. M.; Peters, A. M.; Haskard, D. O. Imaging Vascular Endothelial Activation: An Approach Using Radiolabeled Monoclonal Antibodies against the Endothelial Cell Adhesion Molecule E-Selectin. *J. Nucl. Med.* **1994**, *35*, 276–281.
182. Metzger, R.; Franke, F. E.; Bohle, R. M.; Alhenc-Gelas, F.; Danilov, S. M. Heterogeneous Distribution of Angiotensin I-Converting Enzyme (Cd143) in the Human and Rat Vascular Systems: Vessel, Organ and Species Specificity. *Microvasc. Res.* **2011**, *81*, 206–215.
183. Ford, V. A.; Stringer, C.; Kennel, S. J. Thrombomodulin Is Preferentially Expressed in Balb/C Lung Microvessels. *J. Biol. Chem.* **1992**, *267*, 5446–5450.
184. Carson-Walter, E. B.; Winans, B. N.; Whiteman, M. C.; Liu, Y.; Jarvela, S.; Haapasalo, H.; Tyler, B. M.; Huso, D. L.; Johnson, M. D.; Walter, K. A. Characterization of Tem1/Endosialin in Human and Murine Brain Tumors. *BMC Cancer* **2009**, *9*, 417.
185. Pasqualini, R.; Koivunen, E.; Kain, R.; Lahdenranta, J.; Sakamoto, M.; Stryhn, A.; Ashmun, R. A.; Shapiro, L. H.; Arap, W.; Ruoslahti, E. Aminopeptidase N Is a Receptor for Tumor-Homing Peptides and a Target for Inhibiting Angiogenesis. *Cancer Res.* **2000**, *60*, 722–727.
186. Pober, J. S.; Sessa, W. C. Evolving Functions of Endothelial Cells in Inflammation. *Nat. Rev. Immunol.* **2007**, *7*, 803–815.
187. Aird, W. C.; Edelberg, J. M.; Weiler-Guettler, H.; Simmons, W. W.; Smith, T. W.; Rosenberg, R. D. Vascular Bed-Specific Expression of an Endothelial Cell Gene Is Programmed by the Tissue Microenvironment. *J. Cell Bio.* **1997**, *138*, 1117–1124.
188. Aird, W. C. Phenotypic Heterogeneity of the Endothelium: I. Structure, Function, and Mechanisms. *Circ. Res.* **2007**, *100*, 158–173.
189. Newman, P. J.; Albelda, S. M. Cellular and Molecular Aspects of PECAM-1. *Nouv. Rev. Fr. Hematol.* **1992**, *34* (Suppl.), S9–13.
190. Giannotta, M.; Trani, M.; Dejana, E. Ve-Cadherin and Endothelial Adherens Junctions: Active Guardians of Vascular Integrity. *Dev. Cell* **2013**, *26*, 441–454.
191. Barreiro, O.; Yanez-Mo, M.; Sala-Valdes, M.; Gutierrez-Lopez, M. D.; Ovalle, S.; Higginbottom, A.; Monk, P. N.; Cabanas, C.; Sanchez-Madrid, F. Endothelial Tetraspanin Microdomains Regulate Leukocyte Firm Adhesion During Extravasation. *Blood* **2005**, *105*, 2852–2861.
192. Cook-Mills, J. M.; Marchese, M. E.; Abdala-Valencia, H. Vascular Cell Adhesion Molecule-1 Expression and Signaling During Disease: Regulation by Reactive Oxygen Species and Antioxidants. *Antioxid. Redox Signaling* **2011**, *15*, 1607–1638.
193. Almenar-Queralt, A.; Duperray, A.; Miles, L. A.; Felez, J.; Altieri, D. C. Apical Topography and Modulation of ICAM-1 Expression on Activated Endothelium. *Am. J. Pathol.* **1995**, *147*, 1278–1288.
194. Wright, M. D.; Moseley, G. W.; van Spriel, A. B. Tetraspanin Microdomains in Immune Cell Signalling and Malignant Disease. *Tissue Antigens* **2004**, *64*, 533–542.
195. Ghitescu, L.; Jacobson, B. S.; Crine, P. A Novel, 85 kDa Endothelial Antigen Differentiates Plasma Membrane Microdomains in Lung Alveolar Capillaries. *Endothelium* **1999**, *6*, 241–250.

196. Murciano, J. C.; Harshaw, D. W.; Ghitescu, L.; Danilov, S. M.; Muzykantov, V. R. Vascular Immunotargeting to Endothelial Surface in a Specific Macrodomain in Alveolar Capillaries. *Am. J. Respir. Crit. Care Med.* **2001**, *164*, 1295–1302.
197. Garnacho, C.; Albelda, S. M.; Muzykantov, V. R.; Muro, S. Differential Intra-Endothelial Delivery of Polymer Nanocarriers Targeted to Distinct PECAM-1 Epitopes. *J. Controlled Release* **2008**, *130*, 226–233.
198. Mulivor, A. W.; Lipowsky, H. H. Role of Glycocalyx in Leukocyte-Endothelial Cell Adhesion. *Am. J. Physiol.: Heart Circ. Physiol.* **2002**, *283*, H1282–1291.
199. Skidgel, R. A. Bradykinin-Degrading Enzymes: Structure, Function, Distribution, and Potential Roles in Cardiovascular Pharmacology. *J. Cardiovasc. Pharmacol.* **1992**, *20* (Suppl 9), S4–9.
200. Maruyama, K.; Kennel, S. J.; Huang, L. Lipid Composition Is Important for Highly Efficient Target Binding and Retention of Immunoliposomes. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5744–5748.
201. Christofidou-Solomidou, M.; Kennel, S.; Scherpereel, A.; Wiewrodt, R.; Solomides, C. C.; Pietra, G. G.; Murciano, J. C.; Shah, S. A.; Ischiropoulos, H.; Albelda, S. M.; et al. Vascular Immunotargeting of Glucose Oxidase to the Endothelial Antigens Induces Distinct Forms of Oxidant Acute Lung Injury: Targeting to Thrombomodulin, but Not to PECAM-1, Causes Pulmonary Thrombosis and Neutrophil Transmigration. *Am. J. Pathol.* **2002**, *160*, 1155–1169.
202. Esmon, C. T. Thrombomodulin as a Model of Molecular Mechanisms That Modulate Protease Specificity and Function at the Vessel Surface. *FASEB J.* **1995**, *9*, 946–955.
203. Brisson, C.; Archipoff, G.; Hartmann, M. L.; Hanau, D.; Beretz, A.; Freyssinet, J. M.; Cazenave, J. P. Antibodies to Thrombomodulin Induce Receptor-Mediated Endocytosis in Human Saphenous Vein Endothelial Cells. *Thromb. Haemost.* **1992**, *68*, 737–743.
204. Guermazi, S.; Mellouli, F.; Trabelsi, S.; Bejaoui, M.; Dellagi, K. Anti-Thrombomodulin Antibodies and Venous Thrombosis. *Blood Coagulation Fibrinolysis* **2004**, *15*, 553–558.
205. Christofidou-Solomidou, M.; Scherpereel, A.; Wiewrodt, R.; Ng, K.; Sweitzer, T.; Arguiri, E.; Shuvaev, V.; Solomides, C. C.; Albelda, S. M.; Muzykantov, V. R. PECAM-Directed Delivery of Catalase to Endothelium Protects against Pulmonary Vascular Oxidative Stress. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2003**, *285*, L283–292.
206. Gow, A. J.; Branco, F.; Christofidou-Solomidou, M.; Black-Schultz, L.; Albelda, S. M.; Muzykantov, V. R. Immunotargeting of Glucose Oxidase: Intracellular Production of H₂O₂ and Endothelial Oxidative Stress. *Am. J. Physiol.* **1999**, *277*, L271–281.
207. Chacko, A. M.; Hood, E. D.; Zern, B. J.; Muzykantov, V. R. Targeted Nanocarriers for Imaging and Therapy of Vascular Inflammation. *Curr. Opin. Colloid Interface Sci.* **2011**, *16*, 215–227.
208. Springer, T. A. Adhesion Receptors of the Immune System. *Nature* **1990**, *346*, 425–434.
209. Kumasaka, T.; Quinlan, W. M.; Doyle, N. A.; Condon, T. P.; Sligh, J.; Takei, F.; Beaudet, A.; Bennett, C. F.; Doerschuk, C. M. Role of the Intercellular Adhesion Molecule-1(ICAM-1) in Endotoxin-Induced Pneumonia Evaluated Using ICAM-1 Antisense Oligonucleotides, Anti-ICAM-1 Monoclonal Antibodies, and ICAM-1 Mutant Mice. *J. Clin. Invest.* **1996**, *97*, 2362–2369.
210. Kishimoto, T. K.; Rothlein, R. Integrins, ICAMs, and Selectins: Role and Regulation of Adhesion Molecules in Neutrophil Recruitment to Inflammatory Sites. *Adv. Pharmacol.* **1994**, *25*, 117–169.
211. Albelda, S. M. Endothelial and Epithelial Cell Adhesion Molecules. *Am. J. Respir. Cell Mol. Biol.* **1991**, *4*, 195–203.
212. Kelly, K. A.; Allport, J. R.; Tsourkas, A.; Shinde-Patil, V. R.; Josephson, L.; Weissleder, R. Detection of Vascular Adhesion Molecule-1 Expression Using a Novel Multimodal Nanoparticle. *Circ. Res.* **2005**, *96*, 327–336.
213. Cybulsky, M. I.; Gimbrone, M. A., Jr. Endothelial Expression of a Mononuclear Leukocyte Adhesion Molecule during Atherogenesis. *Science* **1991**, *251*, 788–791.
214. Taichman, D. B.; Cybulsky, M. I.; Djaffar, I.; Longenecker, B. M.; Teixido, J.; Rice, G. E.; Aruffo, A.; Bevilacqua, M. P. Tumor Cell Surface Alpha 4 Beta 1 Integrin Mediates Adhesion to Vascular Endothelium: Demonstration of an Interaction with the N-Terminal Domains of Incam-110/Vcam-1. *Cell Regul.* **1991**, *2*, 347–355.
215. Tsourkas, A.; Shinde-Patil, V. R.; Kelly, K. A.; Patel, P.; Wolley, A.; Allport, J. R.; Weissleder, R. *In Vivo* Imaging of Activated Endothelium Using an Anti-Vcam-1 Magneto-optical Probe. *Bioconjugate Chem.* **2005**, *16*, 576–581.
216. Kuijpers, T. W.; Raleigh, M.; Kavanagh, T.; Janssen, H.; Calafat, J.; Roos, D.; Harlan, J. M. Cytokine-Activated Endothelial Cells Internalize E-Selectin into a Lysosomal Compartment of Vesiculotubular Shape. A Tubulin-Driven Process. *J. Immunol.* **1994**, *152*, 5060–5069.
217. Straley, K. S.; Green, S. A. Rapid Transport of Internalized P-Selectin to Late Endosomes and the Tgn: Roles in Regulating Cell Surface Expression and Recycling to Secretory Granules. *J. Cell Biol.* **2000**, *151*, 107–116.
218. von Asmuth, E. J.; Smeets, E. F.; Ginsel, L. A.; Onderwater, J. J.; Leeuwenberg, J. F.; Buurman, W. A. Evidence for Endocytosis of E-Selectin in Human Endothelial Cells. *Eur. J. Immunol.* **1992**, *22*, 2519–2526.
219. Kessner, S.; Krause, A.; Rothe, U.; Bendas, G. Investigation of the Cellular Uptake of E-Selectin-Targeted Immunoliposomes by Activated Human Endothelial Cells. *Biochim. Biophys. Acta* **2001**, *1514*, 177–190.
220. Kok, R. J.; Everts, M.; Asgeirsdottir, S. A.; Meijer, D. K.; Moelma, G. Cellular Handling of a Dexamethasone-Anti-E-Selectin Immunoconjugate by Activated Endothelial Cells: Comparison with Free Dexamethasone. *Pharm. Res.* **2002**, *19*, 1730–1735.
221. Harari, O. A.; Wickham, T. J.; Stocker, C. J.; Kovacs, I.; Segal, D. M.; Huehns, T. Y.; Sarrat, C.; Haskard, D. O. Targeting an Adenoviral Gene Vector to Cytokine-Activated Vascular Endothelium via E-Selectin. *Gene Ther.* **1999**, *6*, 801–807.
222. Lindner, J. R.; Song, J.; Christiansen, J.; Klibanov, A. L.; Xu, F.; Ley, K. Ultrasound Assessment of Inflammation and Renal Tissue Injury with Microbubbles Targeted to P-Selectin. *Circulation* **2001**, *104*, 2107–2112.
223. Lindner, J. R.; Klibanov, A. L.; Ley, K. Targeting Inflammation. In *Biomedical Aspects of Drug Targeting*; Muzykantov, V. R., Torchilin, V. P., Eds.; Kluwer Academic Publishers: Boston, MA, 2003; pp 149–172.
224. Carpen, O.; Pallai, P.; Staunton, D. E.; Springer, T. A. Association of Intercellular Adhesion Molecule-1 (ICAM-1) with Actin-Containing Cytoskeleton and Alpha-Actinin. *J. Cell Biol.* **1992**, *118*, 1223–1234.
225. Treutiger, C. J.; Heddini, A.; Fernandez, V.; Muller, W. A.; Wahlgren, M. PECAM-1/Cd31, an Endothelial Receptor for Binding Plasmodium Falciparum-Infected Erythrocytes. *Nat. Med.* **1997**, *3*, 1405–1408.
226. Romer, L. H.; McLean, N. V.; Yan, H. C.; Daise, M.; Sun, J.; DeLisser, H. M. Ifn-Gamma and Tnf-Alpha Induce Redistribution of PECAM-1 (Cd31) on Human Endothelial Cells. *J. Immunol.* **1995**, *154*, 6582–6592.
227. Scalia, R.; Lefer, A. M. *In Vivo* Regulation of PECAM-1 Activity During Acute Endothelial Dysfunction in the Rat Mesenteric Microvasculature. *J. Leukocyte Biol.* **1998**, *64*, 163–169.
228. Muro, S.; Cui, X.; Gajewski, C.; Murciano, J. C.; Muzykantov, V. R.; Koval, M. Slow Intracellular Trafficking of Catalase Nanoparticles Targeted to ICAM-1 Protects Endothelial Cells from Oxidative Stress. *Am. J. Physiol.: Cell Physiol.* **2003**, *285*, C1339–1347.
229. Rothlein, R.; Wegner, C. Role of Intercellular Adhesion Molecule-1 in the Inflammatory Response. *Kidney Int.* **1992**, *41*, 617–619.
230. Hubbard, A. K.; Rothlein, R. Intercellular Adhesion Molecule-1 (ICAM-1) Expression and Cell Signaling Cascades. *Free Radical Biol. Med.* **2000**, *28*, 1379–1386.
231. Cao, M. Y.; Huber, M.; Beauchemin, N.; Famiglietti, J.; Albelda, S. M.; Veillette, A. Regulation of Mouse PECAM-1 Tyrosine Phosphorylation by the Src and Csk Families of

- Protein-Tyrosine Kinases. *J. Biol. Chem.* **1998**, *273*, 15765–15772.
232. Cao, G.; O'Brien, C. D.; Zhou, Z.; Sanders, S. M.; Greenbaum, J. N.; Makrigiannakis, A.; DeLisser, H. M. Involvement of Human PECAM-1 in Angiogenesis and *in Vitro* Endothelial Cell Migration. *Am. J. Physiol.: Cell Physiol.* **2002**, *282*, C1181–1190.
233. DeLisser, H. M.; Christofidou-Solomidou, M.; Strieter, R. M.; Burdick, M. D.; Robinson, C. S.; Wexler, R. S.; Kerr, J. S.; Garlanda, C.; Merwin, J. R.; Madri, J. A.; et al. M. Involvement of Endothelial PECAM-1/Cd31 in Angiogenesis. *Am. J. Pathol.* **1997**, *151*, 671–677.
234. DeLisser, H. M.; Yan, H. C.; Newman, P. J.; Muller, W. A.; Buck, C. A.; Albelda, S. M. Platelet/Endothelial Cell Adhesion Molecule-1 (Cd31)-Mediated Cellular Aggregation Involves Cell Surface Glycosaminoglycans. *J. Biol. Chem.* **1993**, *268*, 16037–16046.
235. Diamond, M. S.; Staunton, D. E.; de Fougerolles, A. R.; Stacker, S. A.; Garcia-Aguilar, J.; Hibbs, M. L.; Springer, T. A. ICAM-1 (Cd54): A Counter-Receptor for Mac-1 (Cd11b/Cd18). *J. Cell Biol.* **1990**, *111*, 3129–3139.
236. Jun, C. D.; Shimaoka, M.; Carman, C. V.; Takagi, J.; Springer, T. A. Dimerization and the Effectiveness of ICAM-1 in Mediating Lfa-1-Dependent Adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6830–6835.
237. Nakada, M. T.; Amin, K.; Christofidou-Solomidou, M.; O'Brien, C. D.; Sun, J.; Gurubhagavatula, I.; Heavner, G. A.; Taylor, A. H.; Paddock, C.; Sun, Q. H.; et al. Antibodies against the First Ig-Like Domain of Human Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) That Inhibit PECAM-1-Dependent Homophilic Adhesion Block *in Vivo* Neutrophil Recruitment. *J. Immunol.* **2000**, *164*, 452–462.
238. Murohara, T.; Delyani, J. A.; Albelda, S. M.; Lefer, A. M. Blockade of Platelet Endothelial Cell Adhesion Molecule-1 Protects against Myocardial Ischemia and Reperfusion Injury in Cats. *J. Immunol.* **1996**, *156*, 3550–3557.
239. Muro, S.; Gajewski, C.; Koval, M.; Muzykantov, V. R. ICAM-1 Recycling in Endothelial Cells: A Novel Pathway for Sustained Intracellular Delivery and Prolonged Effects of Drugs. *Blood* **2005**, *105*, 650–658.
240. Panes, J.; Perry, M. A.; Anderson, D. C.; Muzykantov, V. R.; Carden, D. L.; Miyasaka, M.; Granger, D. N. Portal Hypertension Enhances Endotoxin-Induced Intercellular Adhesion Molecule 1 Up-Regulation in the Rat. *Gastroenterology* **1996**, *110*, 866–874.
241. Murciano, J. C.; Muro, S.; Koniaris, L.; Christofidou-Solomidou, M.; Harshaw, D. W.; Albelda, S. M.; Granger, D. N.; Cines, D. B.; Muzykantov, V. R. ICAM-Directed Vascular Immunotargeting of Antithrombotic Agents to the Endothelial Luminal Surface. *Blood* **2003**, *101*, 3977–3984.
242. Scherpereel, A.; Wiewrodt, R.; Christofidou-Solomidou, M.; Gervais, R.; Murciano, J. C.; Albelda, S. M.; Muzykantov, V. R. Cell-Selective Intracellular Delivery of a Foreign Enzyme to Endothelium *In Vivo* Using Vascular Immunotargeting. *FASEB J.* **2001**, *15*, 416–426.
243. Muro, S.; Dziubla, T.; Qiu, W.; Leferovich, J.; Cui, X.; Berk, E.; Muzykantov, V. R. Endothelial Targeting of High-Affinity Multivalent Polymer Nanocarriers Directed to Intercellular Adhesion Molecule 1. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 1161–1169.
244. Garnacho, C.; Dhami, R.; Simone, E.; Dziubla, T.; Leferovich, J.; Schuchman, E. H.; Muzykantov, V.; Muro, S. Delivery of Acid Sphingomyelinase in Normal and Niemann-Pick Disease Mice Using Intercellular Adhesion Molecule-1-Targeted Polymer Nanocarriers. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 400–408.
245. Hua, S.; Chang, H. I.; Davies, N. M.; Cabot, P. J. Targeting of ICAM-1-Directed Immunoliposomes Specifically to Activated Endothelial Cells with Low Cellular Uptake: Use of an Optimized Procedure for the Coupling of Low Concentrations of Antibody to Liposomes. *J. Liposome Res.* **2011**, *21*, 95–105.
246. Ding, B. S.; Gottstein, C.; Grunow, A.; Kuo, A.; Ganguly, K.; Albelda, S. M.; Cines, D. B.; Muzykantov, V. R. Endothelial Targeting of a Recombinant Construct Fusing a PECAM-1 Single-Chain Variable Antibody Fragment (Scfv) with Prourokinase Facilitates Prophylactic Thrombolysis in the Pulmonary Vasculature. *Blood* **2005**, *106*, 4191–4198.
247. Belizaire, A. K.; Tchistiakova, L.; St-Pierre, Y.; Alakhov, V. Identification of a Murine ICAM-1-Specific Peptide by Subtractive Phage Library Selection on Cells. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 625–630.
248. Luo, G. X.; Kohlstaedt, L. A.; Charles, C. H.; Gorfain, E.; Morantte, I.; Williams, J. H.; Fang, F. Humanization of an Anti-ICAM-1 Antibody with over 50-fold Affinity and Functional Improvement. *J. Immunol. Methods* **2003**, *275*, 31–40.
249. Charles, C. H.; Luo, G. X.; Kohlstaedt, L. A.; Morantte, I. G.; Gorfain, E.; Cao, L.; Williams, J. H.; Fang, F. Prevention of Human Rhinovirus Infection by Multivalent Fab Molecules Directed against ICAM-1. *Antimicrob. Agents Chemother.* **2003**, *47*, 1503–1508.
250. Furuya, K.; Takeda, H.; Azhar, S.; McCarron, R. M.; Chen, Y.; Ruetzler, C. A.; Wolcott, K. M.; DeGraba, T. J.; Rothlein, R.; Hugli, T. E.; et al. Examination of Several Potential Mechanisms for the Negative Outcome in a Clinical Stroke Trial of Enlimomab, a Murine Anti-Human Intercellular Adhesion Molecule-1 Antibody: A Bedside-to-Bench Study. *Stroke* **2001**, *32*, 2665–2674.
251. Rothlein, R.; Mainolfi, E. A.; Kishimoto, T. K. Treatment of Inflammation with Anti-ICAM-1. *Res. Immunol.* **1993**, *144*, 735–739. discussion 754–762.
252. Garnacho, C.; Serrano, D.; Muro, S. A Fibrinogen-Derived Peptide Provides Intercellular Adhesion Molecule-1-Specific Targeting and Intraendothelial Transport of Polymer Nanocarriers in Human Cell Cultures and Mice. *J. Pharmacol. Exp. Ther.* **2012**, *340*, 638–647.
253. Liu, J.; Weller, G. E.; Zern, B.; Ayyaswamy, P. S.; Eckmann, D. M.; Muzykantov, V. R.; Radhakrishnan, R. Computational Model for Nanocarrier Binding to Endothelium Validated Using *In Vivo*, *In Vitro*, and Atomic Force Microscopy Experiments. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 16530–16535.
254. Charoenphol, P.; Huang, R. B.; Eniola-Adefeso, O. Potential Role of Size and Hemodynamics in the Efficacy of Vascular-Targeted Spherical Drug Carriers. *Biomaterials* **2010**, *31*, 1392–1402.
255. Blackwell, J. E.; Dapia, N. M.; Dickerson, J. B.; Berg, E. L.; Goetz, D. J. Ligand Coated Nanosphere Adhesion to E- and P-Selectin under Static and Flow Conditions. *Ann. Biomed. Eng.* **2001**, *29*, 523–533.
256. Swaminathan, T. N.; Liu, J.; Balakrishnan, U.; Ayyaswamy, P. S.; Radhakrishnan, R.; Eckmann, D. M. Dynamic Factors Controlling Carrier Anchoring on Vascular Cells. *IUBMB Life* **2011**, *63*, 640–647.
257. Omolola Eniola, A.; Hammer, D. A. *In Vitro* Characterization of Leukocyte Mimetic for Targeting Therapeutics to the Endothelium Using Two Receptors. *Biomaterials* **2005**, *26*, 7136–7144.
258. Charoenphol, P.; Onyskiw, P. J.; Carrasco-Teja, M.; Eniola-Adefeso, O. Particle-Cell Dynamics in Human Blood Flow: Implications for Vascular-Targeted Drug Delivery. *J. Biomechanics* **2012**, *45*, 2822–2828.
259. Namdee, K.; Thompson, A. J.; Charoenphol, P.; Eniola-Adefeso, O. Margination Propensity of Vascular-Targeted Spheres from Blood Flow in a Microfluidic Model of Human Microvessels. *Langmuir* **2013**, *29*, 2530–2535.
260. Kiani, M. F.; Yuan, H.; Chen, X.; Smith, L.; Gaber, M. W.; Goetz, D. J. Targeting Microparticles to Select Tissue via Radiation-Induced Upregulation of Endothelial Cell Adhesion Molecules. *Pharm. Res.* **2002**, *19*, 1317–1322.
261. Sakhalkar, H. S.; Hanes, J.; Fu, J.; Benavides, U.; Malgor, R.; Borruso, C. L.; Kohn, L. D.; Kurjiaka, D. T.; Goetz, D. J. Enhanced Adhesion of Ligand-Conjugated Biodegradable Particles to Colitic Venules. *FASEB J.* **2005**, *19*, 792–794.
262. Sakhalkar, H. S.; Dalal, M. K.; Salem, A. K.; Ansari, R.; Fu, J.; Kiani, M. F.; Kurjiaka, D. T.; Hanes, J.; Shakesheff, K. M.; Goetz, D. J. Leukocyte-Inspired Biodegradable Particles

- That Selectively and Avidly Adhere to Inflamed Endothelium *in Vitro* and *in Vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15895–15900.
263. Lin, A.; Sabnis, A.; Kona, S.; Nattama, S.; Patel, H.; Dong, J.-F.; Nguyen, K. T. Shear-Regulated Uptake of Nanoparticles by Endothelial Cells and Development of Endothelial-Targeting Nanoparticles. *J. Biomed. Mater. Res., Part A* **2010**, *93A*, 833–842.
264. Decuzzi, P.; Lee, S.; Bhushan, B.; Ferrari, M. A Theoretical Model for the Margination of Particles within Blood Vessels. *Ann. Biomed. Eng.* **2005**, *33*, 179–190.
265. Gentile, F.; Curcio, A.; Indolfi, C.; Ferrari, M.; Decuzzi, P. The Margination Propensity of Spherical Particles for Vascular Targeting in the Microcirculation. *J. Nanobiotechnol.* **2008**, *6*, 9.
266. Desjardins, C.; Duling, B. R. Heparinase Treatment Suggests a Role for the Endothelial Cell Glycocalyx in Regulation of Capillary Hematocrit. *Am. J. Physiol.* **1990**, *258*, H647–654.
267. Samokhin, G. P.; Smirnov, M. D.; Muzykantov, V. R.; Domogatsky, S. P.; Smirnov, V. N. Red Blood Cell Targeting to Collagen-Coated Surfaces. *FEBS Lett.* **1983**, *154*, 257–261.
268. Zhang, J.; Johnson, P. C.; Popel, A. S. Effects of Erythrocyte Deformability and Aggregation on the Cell Free Layer and Apparent Viscosity of Microscopic Blood Flows. *Microvasc. Res.* **2009**, *77*, 265–272.
269. Tan, J.; Thomas, A.; Liu, Y. Influence of Red Blood Cells on Nanoparticle Targeted Delivery in Microcirculation. *Soft Matter* **2011**, *8*, 1934–1946.
270. Chrastina, A.; Valadon, P.; Massey, K. A.; Schnitzer, J. E. Lung Vascular Targeting Using Antibody to Aminopeptidase P: Ct-Spect Imaging, Biodistribution, and Pharmacokinetic Analysis. *J. Vasc. Res.* **2010**, *47*, 531–543.
271. Kolhar, P.; Anselmo, A. C.; Gupta, V.; Pant, K.; Prabhakarpandian, B.; Ruoslahti, E.; Mitragotri, S. Using Shape Effects to Target Antibody-Coated Nanoparticles to Lung and Brain Endothelium. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 10753–10758.
272. Prabhakarpandian, B.; Pant, K.; Scott, R. C.; Patillo, C. B.; Irimia, D.; Kiani, M. F.; Sundaram, S. Synthetic Microvascular Networks for Quantitative Analysis of Particle Adhesion. *Biomed. Microdevices* **2008**, *10*, 585–595.
273. Decuzzi, P.; Ferrari, M. The Adhesive Strength of Non-Spherical Particles Mediated by Specific Interactions. *Biomaterials* **2006**, *27*, 5307–5314.
274. Decuzzi, P.; Ferrari, M. The Role of Specific and Non-Specific Interactions in Receptor-Mediated Endocytosis of Nanoparticles. *Biomaterials* **2007**, *28*, 2915–2922.
275. Doshi, N.; Prabhakarpandian, B.; Rea-Ramsey, A.; Pant, K.; Sundaram, S.; Mitragotri, S. Flow and Adhesion of Drug Carriers in Blood Vessels Depend on Their Shape: A Study Using Model Synthetic Microvascular Networks. *J. Controlled Release* **2010**, *146*, 196–200.
276. Rosano, J. M.; Tousi, N.; Scott, R. C.; Krynska, B.; Rizzo, V.; Prabhakarpandian, B.; Pant, K.; Sundaram, S.; Kiani, M. F. A Physiologically Realistic *In Vitro* Model of Microvascular Networks. *Biomed. Microdevices* **2009**, *11*, 1051–1057.
277. Shah, S.; Liu, Y.; Hu, W.; Gao, J. Modeling Particle Shape-Dependent Dynamics in Nanomedicine. *J. Nanosci. Nanotechnol.* **2011**, *11*, 919–928.
278. Gentile, F.; Chiappini, C.; Fine, D.; Bhavane, R. C.; Peluccio, M. S.; Cheng, M. M.; Liu, X.; Ferrari, M.; Decuzzi, P. The Effect of Shape on the Margination Dynamics of Non-Neutral Buoyant Particles in Two-Dimensional Shear Flows. *J. Biomechanics* **2008**, *41*, 2312–2318.
279. Gunawan, R. C.; Auguste, D. T. The Role of Antibody Synergy and Membrane Fluidity in the Vascular Targeting of Immunoliposomes. *Biomaterials* **2010**, *31*, 900–907.
280. Doshi, N.; Orje, J. N.; Molins, B.; Smith, J. W.; Mitragotri, S.; Ruggeri, Z. M. Platelet Mimetic Particles for Targeting Thrombi in Flowing Blood. *Adv. Mater.* **2012**, *24*, 3864–3869.
281. Beningo, K. A.; Wang, Y.-I. Fc-Receptor-Mediated Phagocytosis Is Regulated by Mechanical Properties of the Target. *J. Cell Sci.* **2002**, *115*, 849–856.
282. Trubetskoy, V. S.; Narula, J.; Khaw, B. A.; Torchilin, V. P. Chemically Optimized Antimyosin Fab Conjugates with Chelating Polymers: Importance of the Nature of the Protein-Polymer Single Site Covalent Bond for Bio distribution and Infarction Localization. *Bioconjugate Chem.* **1993**, *4*, 251–255.
283. Muro, S. New Biotechnological and Nanomedicine Strategies for Treatment of Lysosomal Storage Disorders. *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2010**, *2*, 189–204.
284. Calderon, A. J.; Bhowmick, T.; Leferovich, J.; Burman, B.; Pichette, B.; Muzykantov, V.; Eckmann, D. M.; Muro, S. Optimizing Endothelial Targeting by Modulating the Antibody Density and Particle Concentration of Anti-ICAM Coated Carriers. *J. Controlled Release* **2011**, *150*, 37–44.
285. Adams, G. P.; Tai, M. S.; McCartney, J. E.; Marks, J. D.; Stafford, W. F., III; Houston, L. L.; Huston, J. S.; Weiner, L. M. Avidity-Mediated Enhancement of *In Vivo* Tumor Targeting by Single-Chain Fv Dimers. *Clin. Cancer Res.* **2006**, *12*, 1599–1605.
286. Cheng, Z.; Al Zaki, A.; Hui, J. Z.; Muzykantov, V. R.; Tsourkas, A. Multifunctional Nanoparticles: Cost versus Benefit of Adding Targeting and Imaging Capabilities. *Science* **2012**, *338*, 903–910.
287. Irvine, D. J.; Swartz, M. A.; Szeto, G. L. Engineering Synthetic Vaccines Using Cues from Natural Immunity. *Nat. Mater.* **2013**, *12*, 978–990.
288. Liu, H.; Moynihan, K. D.; Zheng, Y.; Szeto, G. L.; Li, A. V.; Huang, B.; Van Egeren, D. S.; Park, C.; Irvine, D. J. Structure-Based Programming of Lymph-Node Targeting in Molecular Vaccines. *Nature* **2014**, *507*, 519–522.
289. Metcalfe, S. M.; Fahmy, T. M. Targeted Nanotherapy for Induction of Therapeutic Immune Responses. *Trends Mol. Med.* **2012**, *18*, 72–80.
290. Adams, G. P.; Schier, R.; McCall, A. M.; Simmons, H. H.; Horak, E. M.; Alpaugh, R. K.; Marks, J. D.; Weiner, L. M. High Affinity Restricts the Localization and Tumor Penetration of Single-Chain Fv Antibody Molecules. *Cancer Res.* **2001**, *61*, 4750–4755.
291. Sakharov, D. V.; Rijken, D. C. Superficial Accumulation of Plasminogen During Plasma Clot Lysis. *Circulation* **1995**, *92*, 1883–1890.
292. Elias, D. R.; Poloukhtine, A.; Popik, V.; Tsourkas, A. Effect of Ligand Density, Receptor Density, and Nanoparticle Size on Cell Targeting. *Nanomedicine* **2013**, *9*, 194–201.
293. Fakhari, A.; Baoum, A.; Siahaan, T. J.; Le, K. B.; Berkland, C. Controlling Ligand Surface Density Optimizes Nanoparticle Binding to ICAM-1. *J. Pharm. Sci.* **2011**, *100*, 1045–1056.
294. Gu, F.; Zhang, L.; Teply, B. A.; Mann, N.; Wang, A.; Radovic-Moreno, A. F.; Langer, R.; Farokhzad, O. C. Precise Engineering of Targeted Nanoparticles by Using Self-Assembled Biointegrated Block Copolymers. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2586–2591.
295. Levi, M.; Friederich, P. W.; Middleton, S.; de Groot, P. G.; Wu, Y. P.; Harris, R.; Biemond, B. J.; Heijnen, H. F.; Levin, J.; ten Cate, J. W. Fibrinogen-Coated Albumin Microcapsules Reduce Bleeding in Severely Thrombocytopenic Rabbits. *Nat. Med.* **1999**, *5*, 107–111.
296. Bertram, J. P.; Williams, C. A.; Robinson, R.; Segal, S. S.; Flynn, N. T.; Lavik, E. B. Intravenous Hemostat: Nanotechnology to Halt Bleeding. *Sci. Transl. Med.* **2009**, *1*, 11ra22.
297. Modery-Pawlowski, C. L.; Tian, L. L.; Ravikumar, M.; Wong, T. L.; Sen Gupta, A. *In Vitro* and *In Vivo* Hemostatic Capabilities of a Functionally Integrated Platelet-Mimetic Liposomal Nanoconstruct. *Biomaterials* **2013**, *34*, 3031–3041.
298. Modery-Pawlowski, C. L.; Tian, L. L.; Pan, V.; McCrae, K. R.; Mitragotri, S.; Sen Gupta, A. Approaches to Synthetic Platelet Analogs. *Biomaterials* **2012**, *34*, 526–541.
299. Lashof-Sullivan, M.; Shoffstall, A.; Lavik, E. Intravenous Hemostats: Challenges in Translation to Patients. *Nanoscale* **2013**, *5*, 10719–10728.

300. Shoffstall, A. J.; Everhart, L. M.; Varley, M. E.; Soehnlen, E. S.; Shick, A. M.; Ustin, J. S.; Lavik, E. B. Tuning Ligand Density on Intravenous Hemostatic Nanoparticles Dramatically Increases Survival Following Blunt Trauma. *Biomacromolecules* **2013**, *14*, 2790–2797.
301. Sun, D.; Nakao, S.; Xie, F.; Zandi, S.; Schering, A.; Hafezi-Moghadam, A. Superior Sensitivity of Novel Molecular Imaging Probe: Simultaneously Targeting Two Types of Endothelial Injury Markers. *FASEB J.* **2010**, *24*, 1532–1540.
302. Eniola, A. O.; Willcox, P. J.; Hammer, D. A. Interplay between Rolling and Firm Adhesion Elucidated with a Cell-Free System Engineered with Two Distinct Receptor-Ligand Pairs. *Biophys. J.* **2003**, *85*, 2720–2731.
303. Gunawan, R. C.; Auguste, D. T. Immunoliposomes That Target Endothelium *In Vitro* Are Dependent on Lipid Raft Formation. *Mol. Pharmaceutics* **2010**, *7*, 1569–1575.
304. Gunawan, R. C.; Almeda, D.; Auguste, D. T. Complementary Targeting of Liposomes to IL-1 α and TNF- α Activated Endothelial Cells via the Transient Expression of Vcam1 and E-Selectin. *Biomaterials* **2011**, *32*, 9848–9853.
305. Papademetriou, I. T.; Garnacho, C.; Schuchman, E. H.; Muro, S. In Vivo Performance of Polymer Nanocarriers Dually-Targeted to Epitopes of the Same or Different Receptors. *Biomaterials* **2013**, *34*, 3459–3466.
306. McAtee, M. A.; Schneider, J. E.; Ali, Z. A.; Warrick, N.; Bursill, C. A.; von zur Muhlen, C.; Greaves, D. R.; Neubauer, S.; Channon, K. M.; Choudhury, R. P. Magnetic Resonance Imaging of Endothelial Adhesion Molecules in Mouse Atherosclerosis Using Dual-Targeted Microparticles of Iron Oxide. *Arterioscler., Thromb., Vasc. Biol.* **2008**, *28*, 77–83.
307. Weller, G. E.; Villanueva, F. S.; Tom, E. M.; Wagner, W. R. Targeted Ultrasound Contrast Agents: *In Vitro* Assessment of Endothelial Dysfunction and Multi-Targeting to ICAM-1 and Sialyl Lewisx. *Biotechnol. Bioeng.* **2005**, *92*, 780–788.
308. Danilov, S.; Jaspard, E.; Churakova, T.; Towbin, H.; Savoie, F.; Wei, L.; Alhenc-Gelas, F. Structure-Function Analysis of Angiotensin I-Converting Enzyme Using Monoclonal Antibodies: Selective Inhibition of the Amino-Terminal Active Site. *J. Biol. Chem.* **1994**, *269*, 26806–26814.
309. Balyasnikova, I. V.; Karran, E. H.; Albrecht, R. F., II; Danilov, S. M. Epitope-Specific Antibody-Induced Cleavage of Angiotensin-Converting Enzyme from the Cell Surface. *Biochem. J.* **2002**, *362*, 585–595.
310. Gordon, K.; Balyasnikova, I. V.; Nesterovitch, A. B.; Schwartz, D. E.; Sturrock, E. D.; Danilov, S. M. Fine Epitope Mapping of Monoclonal Antibodies 9b9 and 3g8 to the N Domain of Angiotensin-Converting Enzyme (Cd143) Defines a Region Involved in Regulating Angiotensin-Converting Enzyme Dimerization and Shedding. *Tissue Antigens* **2010**, *75*, 136–150.
311. Chacko, A. M.; Nayak, M.; Greineder, C. F.; Delisser, H. M.; Muzykantov, V. R. Collaborative Enhancement of Antibody Binding to Distinct PECAM-1 Epitopes Modulates Endothelial Targeting. *PLoS One* **2012**, *7*, No. e34958.
312. Chen, X. Y.; Wang, S. M.; Li, N.; Hu, Y.; Zhang, Y.; Xu, J. F.; Li, X.; Ren, J.; Su, B.; Yuan, W. Z.; Teng, X. R.; Zhang, R. X.; Jiang, D. H.; Mulet, X.; Li, H. P. Creation of Lung-Targeted Dexamethasone Immunoliposome and Its Therapeutic Effect on Bleomycin-Induced Lung Injury in Rats. *PLoS One* **2013**, *8*, No. e58275.
313. Aird, W. C.; Kwaan, H. C. Under-Recognized Significance of Endothelial Heterogeneity: Hemostasis, Thrombosis, and Beyond. *Semin. Thromb. Hemostasis* **2010**, *36*, 225–226.
314. Schnitzer, J. E. Caveolae: From Basic Trafficking Mechanisms to Targeting Transcytosis for Tissue-Specific Drug and Gene Delivery *In Vivo*. *Adv. Drug Delivery Rev.* **2001**, *49*, 265–280.
315. Balyasnikova, I. V.; Danilov, S. M.; Muzykantov, V. R.; Fisher, A. B. Modulation of Angiotensin-Converting Enzyme in Cultured Human Vascular Endothelial Cells. *In Vitro Cell. Dev. Biol.: Anim.* **1998**, *34*, 545–554.
316. Biswas, S.; Torchilin, V. P. Nanopreparations for Organelle-Specific Delivery in Cancer. *Adv. Drug Delivery Rev.* **2013**, *66*, 26–41.
317. Agarwal, R.; Roy, K. Intracellular Delivery of Polymeric Nanocarriers: A Matter of Size, Shape, Charge, Elasticity and Surface Composition. *Ther. Delivery* **2013**, *4*, 705–723.
318. Predescu, S. A.; Predescu, D. N.; Malik, A. B. Molecular Determinants of Endothelial Transcytosis and Their Role in Endothelial Permeability. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2007**, *293*, L823–842.
319. Mukherjee, S.; Ghosh, R. N.; Maxfield, F. R. Endocytosis. *Physiol. Rev.* **1997**, *77*, 759–803.
320. Caron, E.; Hall, A. *Phagocytosis*; Oxford University Press: Oxford, U.K., 2001; p 58–77.
321. Popova, N. V.; Deyev, I. E.; Petrenko, A. G. Clathrin-Mediated Endocytosis and Adaptor Proteins. *Acta Nat.* **2013**, *5*, 62–73.
322. Mellman, I. Endocytosis and Molecular Sorting. *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 575–625.
323. Minshall, R. D.; Tiruppathi, C.; Vogel, S. M.; Niles, W. D.; Gilchrist, A.; Hamm, H. E.; Malik, A. B. Endothelial Cell-Surface Gp60 Activates Vesicle Formation and Trafficking via G(I)-Coupled Src Kinase Signaling Pathway. *J. Cell Biol.* **2000**, *150*, 1057–1070.
324. Predescu, D.; Predescu, S.; Malik, A. B. Transport of Nitrated Albumin across Continuous Vascular Endothelium. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13932–13937.
325. Koval, M.; Preiter, K.; Adles, C.; Stahl, P. D.; Steinberg, T. H. Size of IgG-Opsonized Particles Determines Macrophage Response During Internalization. *Exp. Cell Res.* **1998**, *242*, 265–273.
326. Wiewrodt, R.; Thomas, A. P.; Cipelletti, L.; Christofidou-Solomidou, M.; Weitz, D. A.; Feinstein, S. I.; Schaffer, D.; Albelda, S. M.; Koval, M.; Muzykantov, V. R. Size-Dependent Intracellular Immunotargeting of Therapeutic Cargoes into Endothelial Cells. *Blood* **2002**, *99*, 912–922.
327. Everts, M.; Kok, R. J.; Asgeirsdottir, S. A.; Melgert, B. N.; Moolenaar, T. J.; Koning, G. A.; van Luyn, M. J.; Meijer, D. K.; Moelma, G. Selective Intracellular Delivery of Dexamethasone into Activated Endothelial Cells Using an E-Selectin-Directed Immunoconjugate. *J. Immunol.* **2002**, *168*, 883–889.
328. Ricard, I.; Payet, M. D.; Dupuis, G. Vcam-1 Is Internalized by a Clathrin-Related Pathway in Human Endothelial Cells but Its Alpha 4 Beta 1 Integrin Counter-Receptor Remains Associated with the Plasma Membrane in Human T Lymphocytes. *Eur. J. Immunol.* **1998**, *28*, 1708–1718.
329. Pardridge, W. M. Blood-Brain Barrier Drug Targeting: The Future of Brain Drug Development. *Mol. Interventions* **2003**, *3*, 90–105.
330. Tagliabue, E.; Centis, F.; Campiglio, M.; Mastrianni, A.; Martignone, S.; Pellegrini, R.; Casalini, P.; Lanzi, C.; Menard, S.; Colnaghi, M. I. Selection of Monoclonal Antibodies Which Induce Internalization and Phosphorylation of P185her2 and Growth Inhibition of Cells with Her2/Neu Gene Amplification. *Int. J. Cancer* **1991**, *47*, 933–937.
331. Feero, W. G.; Li, S.; Rosenblatt, J. D.; Sirianni, N.; Morgan, J. E.; Partridge, T. A.; Huang, L.; Hoffman, E. P. Selection and Use of Ligands for Receptor-Mediated Gene Delivery to Myogenic Cells. *Gene Ther.* **1997**, *4*, 664–674.
332. Zhou, Y.; Zhao, L.; Marks, J. D. Selection and Characterization of Cell Binding and Internalizing Phage Antibodies. *Arch. Biochem. Biophys.* **2012**, *526*, 107–113.
333. Zhou, Y.; Zou, H.; Zhang, S.; Marks, J. D. Internalizing Cancer Antibodies from Phage Libraries Selected on Tumor Cells and Yeast-Displayed Tumor Antigens. *J. Mol. Biol.* **2010**, *404*, 88–99.
334. Heitner, T.; Moor, A.; Garrison, J. L.; Marks, C.; Hasan, T.; Marks, J. D. Selection of Cell Binding and Internalizing Epidermal Growth Factor Receptor Antibodies from a Phage Display Library. *J. Immunol. Methods* **2001**, *248*, 17–30.

335. Anderson, D. C.; Manger, R.; Schroeder, J.; Woodle, D.; Barry, M.; Morgan, A. C.; Fritzberg, A. R. Enhanced *in Vitro* Tumor Cell Retention and Internalization of Antibody Derivatized with Synthetic Peptides. *Bioconjugate Chem.* **1993**, *4*, 10–18.
336. Couch, J. A.; Yu, Y. J.; Zhang, Y.; Tarrant, J. M.; Fuji, R. N.; Meilandt, W. J.; Solanoy, H.; Tong, R. K.; Hoyte, K.; Luk, W.; et al. Addressing Safety Liabilities of TfR Bispecific Antibodies That Cross the Blood-Brain Barrier. *Sci. Transl. Med.* **2013**, *5*, 183ra57.
337. Ilan, N.; Mohsenin, A.; Cheung, L.; Madri, J. A. PECAM-1 Shedding During Apoptosis Generates a Membrane-Anchored Truncated Molecule with Unique Signaling Characteristics. *FASEB J.* **2001**, *15*, 362–372.
338. Muro, S.; Wiewrodt, R.; Thomas, A.; Koniaris, L.; Albelda, S. M.; Muzykantov, V. R.; Koval, M. A Novel Endocytic Pathway Induced by Clustering Endothelial ICAM-1 or PECAM-1. *J. Cell Sci.* **2003**, *116*, 1599–1609.
339. Muro, S.; Muzykantov, V. R.; Murciano, J. C. Characterization of Endothelial Internalization and Targeting of Antibody-Enzyme Conjugates in Cell Cultures and in Laboratory Animals. *Methods Mol. Biol.* **2004**, *283*, 21–36.
340. Ding, B. S.; Hong, N.; Christofidou-Solomidou, M.; Gottstein, C.; Albelda, S. M.; Cines, D. B.; Fisher, A. B.; Muzykantov, V. R. Anchoring Fusion Thrombomodulin to the Endothelial Lumen Protects against Injury-Induced Lung Thrombosis and Inflammation. *Am. J. Respir. Crit. Care Med.* **2009**, *180*, 247–256.
341. Pelkmans, L.; Helenius, A. Endocytosis via Caveolae. *Traffic* **2002**, *3*, 311–320.
342. Kolhar, P.; Mitragotri, S. Polymer Microparticles Exhibit Size and Shape Dependent Accumulation around the Nucleus after Endocytosis. *Adv. Funct. Mater.* **2012**, *22*, 3759–3764.
343. Kolhar, P.; Doshi, N.; Mitragotri, S. Polymer Nanoneedle-Mediated Intracellular Drug Delivery. *Small* **2011**, *7*, 2094–2100.
344. Gratton, S. E.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. The Effect of Particle Design on Cellular Internalization Pathways. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 11613–11618.
345. Bhowmick, T.; Berk, E.; Cui, X.; Muzykantov, V. R.; Muro, S. Effect of Flow on Endothelial Endocytosis of Nanocarriers Targeted to ICAM-1. *J. Controlled Release* **2012**, *157*, 485–492.
346. Samuel, S. P.; Jain, N.; O'Dowd, F.; Paul, T.; Kashanin, D.; Gerard, V. A.; Gun'ko, Y. K.; Prina-Mello, A.; Volkov, Y. Multifactorial Determinants That Govern Nanoparticle Uptake by Human Endothelial Cells under Flow. *Int. J. Nanomed.* **2012**, *7*, 2943–2956.
347. Terrisse, A. D.; Puech, N.; Allart, S.; Gourdy, P.; Xuereb, J. M.; Payrastre, B.; Sie, P. Internalization of Microparticles by Endothelial Cells Promotes Platelet/Endothelial Cell Interaction under Flow. *J. Thromb. Haemost.* **2010**, *8*, 2810–2819.
348. Han, J.; Zern, B. J.; Shuvaev, V. V.; Davies, P. F.; Muro, S.; Muzykantov, V. Acute and Chronic Shear Stress Differently Regulate Endothelial Internalization of Nanocarriers Targeted to Platelet-Endothelial Cell Adhesion Molecule-1. *ACS Nano* **2012**, *6*, 8824–8836.
349. Serrano, D.; Bhowmick, T.; Chadha, R.; Garnacho, C.; Muro, S. Intercellular Adhesion Molecule 1 Engagement Modulates Sphingomyelinase and Ceramide, Supporting Uptake of Drug Carriers by the Vascular Endothelium. *Arterioscler., Thromb., Vasc. Biol.* **2012**, *32*, 1178–1185.
350. Ansar, M.; Serrano, D.; Papademetriou, I.; Bhowmick, T. K.; Muro, S. Biological Functionalization of Drug Delivery Carriers to Bypass Size Restrictions of Receptor-Mediated Endocytosis Independently from Receptor Targeting. *ACS Nano* **2013**, *7*, 10597–10611.
351. Zhang, Y.; Schlachetzki, F.; Pardridge, W. M. Global Non-Viral Gene Transfer to the Primate Brain Following Intravenous Administration. *Mol. Ther.* **2003**, *7*, 11–18.
352. Schnitzer, J. E.; Oh, P.; McIntosh, D. P. Role of Gtp Hydrolysis in Fission of Caveolae Directly from Plasma Membranes. *Science* **1996**, *274*, 239–242.
353. Vogel, S. M.; Easington, C. R.; Minshall, R. D.; Niles, W. D.; Tiruppathi, C.; Hollenberg, S. M.; Parrillo, J. E.; Malik, A. B. Evidence of Transcellular Permeability Pathway in Microvessels. *Microvasc. Res.* **2001**, *61*, 87–101.
354. Dvorak, A. M.; Feng, D. The Vesiculo-Vacuolar Organelle (Vvo). A New Endothelial Cell Permeability Organelle. *J. Histochem. Cytochem.* **2001**, *49*, 419–432.
355. Iversen, T. G.; Frerker, N.; Sandvig, K. Uptake of Ricin-Quantum Dot Nanoparticles by a Macropinocytosis-Like Mechanism. *J. Nanobiotechnol.* **2012**, *10*, 33.
356. Wang, Z.; Tiruppathi, C.; Minshall, R. D.; Malik, A. B. Size and Dynamics of Caveolae Studied Using Nanoparticles in Living Endothelial Cells. *ACS Nano* **2009**, *3*, 4110–4116.
357. Schnitzer, J. E.; Liu, J.; Oh, P. Endothelial Caveolae Have the Molecular Transport Machinery for Vesicle Budding, Docking, and Fusion Including Vamp, Nsf, Snap, Annexins, and Gtpases. *J. Biol. Chem.* **1995**, *270*, 14399–14404.
358. Mehta, D.; Bhattacharya, J.; Matthay, M. A.; Malik, A. B. Integrated Control of Lung Fluid Balance. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2004**, *287*, L1081–1090.
359. Ghaffarian, R.; Bhowmick, T.; Muro, S. Transport of Nanocarriers across Gastrointestinal Epithelial Cells by a New Transcellular Route Induced by Targeting ICAM-1. *J. Controlled Release* **2012**, *163*, 25–33.
360. Mane, V.; Muro, S. Biodistribution and Endocytosis of ICAM-1-Targeting Antibodies versus Nanocarriers in the Gastrointestinal Tract in Mice. *Int. J. Nanomed.* **2012**, *7*, 4223–4237.
361. Neumeyer, A.; Bukowski, M.; Veith, M.; Lehr, C. M.; Daum, N. Propidium Iodide Labeling of Nanoparticles as a Novel Tool for the Quantification of Cellular Binding and Uptake. *Nanomedicine* **2011**, *7*, 410–419.
362. Shuvaev, V. V.; Dziubla, T.; Wiewrodt, R.; Muzykantov, V. R. Streptavidin-Biotin Crosslinking of Therapeutic Enzymes with Carrier Antibodies: Nanoconjugates for Protection against Endothelial Oxidative Stress. *Methods Mol. Biol.* **2004**, *283*, 3–19.
363. Garnacho, C.; Shuvaev, V.; Thomas, A.; McKenna, L.; Sun, J.; Koval, M.; Albelda, S.; Muzykantov, V.; Muro, S. Rhoa Activation and Actin Reorganization Involved in Endothelial Cam-Mediated Endocytosis of Anti-PECAM Carriers: Critical Role for Tyrosine 686 in the Cytoplasmic Tail of PECAM-1. *Blood* **2008**, *111*, 3024–3033.
364. Kim, J. A.; Aberg, C.; Salvati, A.; Dawson, K. A. Role of Cell Cycle on the Cellular Uptake and Dilution of Nanoparticles in a Cell Population. *Nat. Nanotechnol.* **2012**, *7*, 62–68.
365. Georgieva, J. V.; Kalicharan, D.; Couraud, P. O.; Romero, I. A.; Weksler, B.; Hoekstra, D.; Zuhorn, I. S. Surface Characteristics of Nanoparticles Determine Their Intracellular Fate in and Processing by Human Blood-Brain Barrier Endothelial Cells *in Vitro*. *Mol. Ther.* **2011**, *19*, 318–325.
366. Kastl, L.; Sasse, D.; Wulf, V.; Hartmann, R.; Mircheski, J.; Ranke, C.; Carregal-Romero, S.; Martinez-Lopez, J. A.; Fernandez-Chacon, R.; Parak, W. J.; et al. Multiple Internalization Pathways of Polyelectrolyte Multilayer Capsules into Mammalian Cells. *ACS Nano* **2013**, *7*, 6605–6618.
367. Vasdekis, A. E.; Scott, E. A.; O'Neil, C. P.; Psaltis, D.; Hubbell, J. A. Precision Intracellular Delivery Based on Optofluidic Polymersome Rupture. *ACS Nano* **2012**, *6*, 7850–7857.
368. Kardara, M.; Hatziantoniou, S.; Sfika, A.; Vassiliou, A. G.; Mourelatos, E.; Muagkou, C.; Armaganidis, A.; Roussos, C.; Orfanos, S. E.; Kotanidou, A.; et al. Caveolar Uptake and Endothelial-Protective Effects of Nanostructured Lipid Carriers in Acid Aspiration Murine Acute Lung Injury. *Pharm. Res.* **2013**, *30*, 1836–1847.
369. Rejman, J.; Oberle, V.; Zuhorn, I. S.; Hoekstra, D. Size-Dependent Internalization of Particles via the Pathways of Clathrin- and Caveolae-Mediated Endocytosis. *Biochem. J.* **2004**, *377*, 159–169.
370. Minami, T.; Sugiyama, A.; Wu, S. Q.; Abid, R.; Kodama, T.; Aird, W. C. Thrombin and Phenotypic Modulation of the Endothelium. *Arterioscler., Thromb., Vasc. Biol.* **2004**, *24*, 41–53.

371. Comhair, S. A.; Xu, W.; Mavrakis, L.; Aldred, M. A.; Asosingh, K.; Erzurum, S. C. Human Primary Lung Endothelial Cells in Culture. *Am. J. Respir. Cell Mol. Biol.* **2012**, *46*, 723–730.
372. Wagner, S.; Zensi, A.; Wien, S. L.; Tschickardt, S. E.; Maier, W.; Vogel, T.; Worek, F.; Pietrzik, C. U.; Kreuter, J.; von Briesen, H. Uptake Mechanism of Apoe-Modified Nanoparticles on Brain Capillary Endothelial Cells as a Blood-Brain Barrier Model. *PLoS One* **2012**, *7*, e32568.
373. Yang, H.; Zhao, F.; Li, Y.; Xu, M.; Li, L.; Wu, C.; Miyoshi, H.; Liu, Y. Vcam-1-Targeted Core/Shell Nanoparticles for Selective Adhesion and Delivery to Endothelial Cells with Lipopolysaccharide-Induced Inflammation under Shear Flow and Cellular Magnetic Resonance Imaging *in Vitro*. *Int. J. Nanomed.* **2013**, *8*, 1897–1906.
374. Davies, P. F. Hemodynamic Shear Stress and the Endothelium in Cardiovascular Pathophysiology. *Nat. Clin. Pract. Cardiovasc. Med.* **2009**, *6*, 16–26.
375. Baker, B. M.; Trappmann, B.; Stapleton, S. C.; Toro, E.; Chen, C. S. Microfluidics Embedded within Extracellular Matrix to Define Vascular Architectures and Pattern Diffusive Gradients. *Lab Chip* **2013**, *13*, 3246–3252.
376. Kusunose, J.; Zhang, H.; Gagnon, M. K.; Pan, T.; Simon, S. I.; Ferrara, K. W. Microfluidic System for Facilitated Quantification of Nanoparticle Accumulation to Cells under Laminar Flow. *Ann. Biomed. Eng.* **2013**, *41*, 89–99.
377. Khan, O. F.; Chamberlain, M. D.; Sefton, M. V. Toward an *in Vitro* Vasculature: Differentiation of Mesenchymal Stromal Cells within an Endothelial Cell-Seeded Modular Construct in a Microfluidic Flow Chamber. *Tissue Eng., Part A* **2012**, *18*, 744–756.
378. Young, E. W.; Simmons, C. A. Macro- and Microscale Fluid Flow Systems for Endothelial Cell Biology. *Lab Chip* **2010**, *10*, 143–160.
379. Wadia, J. S.; Stan, R. V.; Dowdy, S. F. Transducible Tat-Ha Fusogenic Peptide Enhances Escape of Tat-Fusion Proteins after Lipid Raft Macropinocytosis. *Nat. Med.* **2004**, *10*, 310–315.
380. Elsaesser, A.; Barnes, C. A.; McKerr, G.; Salvati, A.; Lynch, I.; Dawson, K. A.; Howard, C. V. Quantification of Nanoparticle Uptake by Cells Using an Unbiased Sampling Method and Electron Microscopy. *Nanomedicine (London, U.K.)* **2011**, *6*, 1189–1198.
381. ur Rehman, Z.; Hoekstra, D.; Zuhorn, I. S. Mechanism of Polyplex- and Lipoplex-Mediated Delivery of Nucleic Acids: Real-Time Visualization of Transient Membrane Destabilization without Endosomal Lysis. *ACS Nano* **2013**, *7*, 3767–3777.
382. Fiandra, L.; Mazzucchelli, S.; De Palma, C.; Colombo, M.; Allevi, R.; Sommaruga, S.; Clementi, E.; Bellini, M.; Prosperi, D.; Corsi, F. Assessing the *in Vivo* Targeting Efficiency of Multifunctional Nanoconstructs Bearing Antibody-Derived Ligands. *ACS Nano* **2013**, *7*, 6092–6102.