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Vascular Accessibility of Endothelial Targeted Ferritin Nanoparticles

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Abstract

Targeting nanocarriers to the endothelium, using affinity ligands to cell adhesion molecules such as ICAM-1 and PECAM-1, holds promise to improve the pharmacotherapy of many disease conditions. This approach capitalizes on the observation that antibody-targeted carriers of 100 nm and above accumulate in the pulmonary vasculature more effectively than free antibodies.

Targeting of prospective nanocarriers in the 10–50 nm range, however, has not been studied. To address this intriguing issue, we conjugated monoclonal antibodies (Ab) to ICAM-1 and PECAM-1 or their single chain antigen-binding fragments (scFv) to ferritin nanoparticles (FNPs, size 12 nm), thereby producing Ab/FNPs and scFv/FNPs. Targeted FNPs retained their typical symmetric core-shell structure with sizes of 20–25 nm and ~4–5 Ab (or ~7–9 scFv) per particle. Ab/FNPs and scFv/FNPs, but not control IgG/FNPs, bound specifically to cells expressing target molecules and accumulated in the lungs after intravenous injection, with pulmonary targeting an order of magnitude higher than free Ab. Most intriguing, the targeting of Ab/FNPs to ICAM-1, but not PECAM-1, surpassed that of larger Ab/carriers targeted by the same ligand. These results indicate that: i) FNPs may provide a platform for targeting endothelial adhesion molecules with carriers in the 20 nm size range, which has not been previously reported; and, ii) ICAM-1 and PECAM-1 (known to localize in different domains of endothelial plasmalemma) differ in their accessibility to circulating objects of this size, common for blood components and nanocarriers.

TOC Graphic

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Supporting Information. Additional FNP characterization and biodistribution data; This material is available free of charge via the Internet at <http://pubs.acs.org>.

Differences in pulmonary targeting of small and large nanocarriers to vascular cell adhesion molecules ICAM-1 and PECAM-1.

Keywords

Ferritin; immunotargeting; vascular endothelium; cell adhesion molecules; nanoparticles

Introduction

Since the 2005 FDA-approval of nanoparticle albumin-bound paclitaxel (Abraxane), protein-based carriers have garnered significant interest as translatable drug delivery platforms.^{1–3} Both naturally occurring (e.g., albumin, lipoproteins, ferritin, gelatin, elastin, etc.), and genetically engineered proteins (e.g., silk-like polypeptides, virus-like particles, etc.) have been utilized as nanoparticle-based delivery systems,^{4–8} with the ultimate aim of producing biocompatible, stable, and versatile diagnostic and therapeutic agents. Self-assembly of proteins into multimeric nanoparticles can occur naturally, as with ferritin and virus-like particles, or may be induced via desolvation, crosslinking, or through thermally triggered self-assembly.^{9–12}

Ferritin, the body's major iron storage protein, is perhaps the most studied protein-based carrier. Mammalian ferritin self-assembles into 12 nm, highly symmetric, spherical nanoparticles, comprising 24 subunits of two distinct types: a heavy chain (21 kDa) and a light chain (19 kDa).^{13–16} The exact composition of FNPs – i.e., the proportion of heavy and light chain subunits – varies from organ to organ.¹⁷ In all cases, the assembled subunits surround a central cavity, with an internal diameter of ~8 nm. This interior compartment, capable of carrying up to 4500 atoms of iron,^{15, 16} is a primary reason that ferritin has attracted interest as a drug delivery platform. Indeed, investigators have loaded not only metals into FNPs, but a variety of imaging contrast agents^{18–22} and small-molecule therapeutics^{23–30}

Ferritin exerts its natural transporting function via binding to endogenous receptors on a number of different cell types – most prominently the human transferrin receptor 1 (TfR1)³¹ From the standpoint of drug delivery this means that FNPs must be “re-directed” to a target

organ and/or cell, while simultaneously decreasing receptor-mediated uptake by off-target cells. Some success has been achieved by attaching affinity ligands to the FNP surface, either via chemical conjugation, or in the case of recombinant ferritin, by direct fusion to the heavy or light chain gene. Using these techniques, several groups have reported targeting of FNPs to cancer cells,^{23–27} extracellular matrix proteins,³² leukocytes,³³ and abnormal vasculature via RGD-targeting to $\alpha_v\beta_3$.³⁴

The pulmonary endothelium represents an important therapeutic target in a plethora of prevalent human diseases with high morbidity and mortality. Preferential delivery to this region of the vasculature can be achieved using “vascular immunotargeting” – i.e., coupling of drugs, imaging agents, or their carriers to antibodies and other ligands specific for endothelial determinants. Surface molecules used for immunotargeting include intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, angiotensin-converting enzyme (ACE), aminopeptidase P (APP), thrombomodulin (TM), and plasmalemma vesicle protein-1 (PV1).^{35–40} “Complementary targeting”, in which carriers bind multiple endothelial determinants has also been explored to improve binding.^{41–43}

The foundational work in this area has focused on ligands and nanoparticles with size of ~100 nm and above bearing these ligands, whereas few studies quantitatively compared vascular immunotargeting of ligands with ligand-bearing particles smaller than 50 nm. FNPs, in theory, provide this opportunity, but FNPs targeting to the pulmonary endothelium has not been studied. Here, we describe endothelial targeting of FNP directed to ICAM-1 or PECAM-1 using Ab or Ab fragments (scFv) conjugated to ferritin. Our results indicate: (i) targeted FNPs bind specifically to endothelial cells *in vitro* and *in vivo*, (ii) conjugation of ligands reduces natural receptor-mediated binding of FNPs and (iii) FNPs pulmonary targeting directed to endothelial determinant ICAM-1 markedly surpasses targeting of bigger carriers to ICAM-1, likely due to a favorable combination of multivalent avidity and size.

Results

Synthesis and characterization of targeted FNPs

We conjugated the targeting ligands to FNPs using bi-functional conjugation chemistry. Maleimide groups were added to FNP using SMCC, whereas protected sulfhydryl groups were added to Ab or scFv using SATA. The stoichiometry of the latter reaction was adjusted to ensure that no more than one sulfhydryl was added per Ab or scFv, in order to prevent crosslinking of FNPs in the subsequent thiol/maleimide reaction.

The stoichiometric molar ratio of FNP-maleimide to Ab- or scFv-sulfhydryl reaction was varied to determine its effect on the number of Ab or scFv moieties ultimately conjugated to each FNP. The latter was determined via Western blotting and radioisotope tracing, in which ¹²⁵I-labeled Ab or scFv was used to quantify the amount of targeting ligand conjugated per particle.

Supplemental Figure 1 shows densitometric analysis of Western blots of FNP conjugates. By this method, conjugation using a stoichiometric molar ratio of 10:1 Ab:FNP resulted in conjugates with a ratio of approximately 5 Abs/conjugate. The two upper bands, near the 82 kDa ladder marker, represent ferritin heavy and light chain respectively, conjugated to the antibody heavy chain. As expected, decreasing the starting molar ratio of Ab:FNP resulted in a smaller percentage of conjugated subunits, corresponding to a lower number of Ab per FNP. In contrast, increasing the stoichiometric ratio of scFv:FNP from 10:1 to 30:1 did not significantly affect the percentage of ferritin subunits conjugated to scFv (two bands near the 49 kDa marker correspond to scFv-conjugates of ferritin heavy and light chain). These data suggest a maximum of approximately 9 scFv per FNP, beyond which, further targeting ligands cannot be conjugated.

Figure 1 shows the results of the second analytic method, i.e., radioisotope HPLC tracings of the FNPs following conjugation with ^{125}I -labeled scFv or Ab. At a starting molar ratio of 10:1 Ab:FNP, 39% of the ^{125}I -Ab eluted off the column between 14–17 min, corresponding to FNP conjugates. This indicates a ratio of ~3.9 Ab per FNP. This is most likely a slight underestimate, as we excluded any FNPs conjugates eluting between 17–18 min, in order to avoid overlap with unconjugated Ab. Likewise, 72.7% of ^{125}I -scFv eluted from the column between 14–18 min, corresponding to a ratio of 7.3 scFv per FNP. In summary, the two analytical methods give fairly similar results with regards to the number of each type of ligand incorporated per conjugate. Moreover, the results indicate that FNP are able to accommodate a larger number of smaller (~30 kDa) scFv ligands than Ab (~150 kDa) per particle. One reasonable explanation for this data would be steric hindrance, which could limit the maximum number of ligands that can be conjugated to the FNP surface.

As shown in Figure 2, FNP conjugates were characterized via TEM and dynamic light scattering (DLS). TEM revealed that FNP conjugates retain the characteristic core-shell structure of ferritin. DLS, on the other hand, revealed an increase in the hydrodynamic diameter following conjugation. Whereas FNP alone had a mean diameter of 10.6 ± 0.8 nm, mean diameters of 25.3 ± 0.5 nm vs. 20.9 ± 0.4 nm were observed for Ab/FNP and scFv/FNP, respectively. The polydispersity index (PDI) indicated a similar level of monodispersity for IgG/FNP, as compared to unconjugated FNP (0.23 ± 0.02 vs. 0.20 ± 0.01 , respectively).

Binding of targeted FNPs to cells

To target ferritin to endothelial cells, FNPs were conjugated with monoclonal antibodies (Ab) specific for mouse ICAM-1 or scFv specific for mouse PECAM-1. Binding was first tested using REN-ICAM and REN-PECAM cells, which stably express mouse ICAM-1 and PECAM-1, respectively. Concurrent use of wild-type REN cells, a human mesothelial cell line that has no endogenous expression of mouse ICAM-1 or PECAM-1, allowed assessment of non-specific binding of particles.⁴⁴ Figure 3a–c shows that anti-ICAM-1 Ab/FNP, anti-ICAM-1 scFv/FNP, and anti-PECAM-1 scFv/FNP all bind specifically to transfected REN cells expressing their target ligand, with minimal binding to REN wild-type controls.

While REN cells represent a convenient system for testing binding and specificity, their expression of stably transfected genes (e.g., ICAM-1, PECAM-1) does not necessarily reflect that seen on endothelial cells (ECs). As such, we next tested binding of targeted FNPs to primary human ECs. This also enabled us to test our hypothesis that conjugation of targeting ligand to FNPs would decrease cell binding by ferritin-specific receptors. Indeed, Figure 3d shows a significant difference between unconjugated FNPs and IgG/FNP in binding to HUVECs. While the mechanism of FNP binding in this experiment is not clear, HUVECs are known to express TfR1 and the data suggest that IgG conjugation suppresses interaction of FNPs with a saturable surface receptor. Figure 3e–f shows binding of anti-ICAM Ab/FNP and anti-PECAM scFv/FNP to HUVEC. In the case of ICAM-targeted particles, the cells were preactivated with TNF to induce ICAM-1 expression. Together, these experimental results support the hypothesis that targeted FNPs demonstrate both “artificial” specific binding to endothelial cell adhesion molecules and reduced cell binding via natural ferritin binding mechanisms.

Targeted delivery of ferritin nanocarriers to pulmonary endothelium *in vivo*

We next studied the biodistribution of Ab/FNP and scFv/FNP following intravenous injection in mice. FNPs were ^{125}I -labeled prior to conjugation, such that radiotracing only reflected distribution of particles and not any detached or shed targeting ligand. Figures 4–5 and S3 show the distribution of anti-ICAM Ab/FNP, anti-PECAM Ab/FNP, and anti-PECAM scFv/FNP respectively, with IgG/FNP as controls. In each case, selective pulmonary uptake was observed, with 160.9 ± 6.5 % ID/g (anti-ICAM Ab/FNP), 27.7 ± 2.8 % ID/g (anti-PECAM scFv/FNP), and 85.4 ± 2.1 % ID/g (anti-PECAM Ab/FNP). IgG/FNP conjugates mainly accumulate in liver and spleen. The localization ratio (LR), defined as the ratio of %ID/g of a given organ to that in the blood, is shown for both ICAM-1 and PECAM-1-targeted FNPs. Likewise, the immunospecificity index (ISI), or the ratio of the LR of the targeted particle to that of untargeted (IgG) control, is shown in each figure inset.

Table 1 provides comparison of the pulmonary targeting of ICAM-1 and PECAM-1 directed Ab/FNP with free ligand and multiple forms of larger (> 100 nm) Ab-decorated particles. While all ICAM-1 targeting agents demonstrate selective lung uptake (as compared to relevant controls), the Ab/FNPs have the highest pulmonary accumulation. The lung immunospecificity index (ISI) of Ab/FNPs is also the highest, although the greater variance in the localization ratio of the IgG/PVPs vs. IgG/FNPs (6.7 ± 2.8 vs. 2.8 ± 0.2) limits comparison of the ISI of these different carriers. In contrast, anti-PECAM-1 Ab/FNPs are again far superior to free Ab, but demonstrate similar pulmonary biodistribution and specificity as the larger carriers.

Discussion

Endothelial activation and dysfunction are a part of the pathogenesis of numerous lung diseases, including the acute respiratory distress syndrome (ARDS), pulmonary hypertension, pulmonary embolus, and graft dysfunction after lung transplantation.⁴⁸ One of the barriers to improving outcome in these diseases is adequate and selective delivery of therapeutics to lung endothelial cells.^{49, 50} Currently, the only pulmonary delivery systems

in clinical use involve passive delivery via the inhalational route (e.g., metered-dose inhalers), a limited approach with respect to endothelial delivery and one which is typically problematic in the setting of disease, when the affected areas of the lung are less likely to have normal ventilation. Of note, novel methodology may ultimately overcome these limitations; for example, via electroporation-mediated gene delivery to the lung.⁵¹ This strategy appears to be a safe means of transferring genetic material to healthy lungs, and may be efficacious even in setting of acute and severe lung disease.^{52, 53}

As an alternative to inhalational or intratracheal delivery, however, we and several other groups have developed methods of targeting intravenously administered agents to the pulmonary endothelium, utilizing antibodies to the cell adhesion molecules ICAM-1 and PECAM-1, amongst other targets.⁵⁴ Studies conducted in a variety of pre-clinical animal models have established immunotargeting as a means of increasing both the pulmonary delivery and protective effects of a number of different therapeutics, including antioxidant enzymes,^{55–59} antithrombotic agents,^{42, 44, 60} and small-molecule drugs.^{45, 61, 62} Despite this substantial body of work, it remains unclear what biophysical characteristics are optimal for the delivery of targeted drug carriers to the pulmonary endothelium. A few recent studies have begun to explore this question, demonstrating that elongated, rod-like particles seem to have longer intravascular circulation time and greater ligand-dependent binding than spherical ones.^{63, 64} Similarly, a few studies have explored the impact of nanoparticle size on immunotargeting, although most of these efforts have been limited to the 100–5000 nm range.^{65, 66} Difficulty formulating uniform, polymeric nanoparticles less than 50 nm in diameter has limited characterization of vascular targeting in this size range.

We report here a novel class of endothelial targeting agent based on modification of horse spleen ferritin nanoparticles (FNPs). Ferritin, a highly symmetric, self-assembling protein with endogenous storage, scavenging, and transport functions, is in many ways ideally suited to drug delivery applications.^{67–69} As a component of the serum under physiologic conditions, FNPs are inimitably biocompatible and would be expected to have minimal immunogenicity. FNPs are also quite stable to proteolysis, chemical denaturation and heat, withstanding temperatures of up to 80–100 °C and allowing production in microbial systems at relatively low cost.^{23, 24, 70} FNPs have evolved an interior compartment for iron storage, and apoferritin can be loaded with therapeutic molecules, radiometals, or imaging contrast agents.^{22, 28, 30, 71, 72} While large changes in pH are required to disassemble most FNPs, alternate forms have been synthesized recombinantly to enable disassembly and loading of drug, contrast agent, or even other nanoparticles with less harsh conditions.^{73–75} In this vascular targeting study, however, the primary interest in FNPs was their uniform 12-nm diameter, which places them at the lower end of the 10–50 nm size range which has been so difficult to study with other types of nanocarriers.

In this study, we demonstrate the feasibility of vascular targeting of FNPs, using a straightforward approach of chemical conjugation of antibodies or antibody fragments directed to endothelial surface antigens. Given the small size of FNPs, it was initially unclear how many targeting ligands could be accommodated on the surface of each particle. Two separate methods confirm that our current formulation is indeed multivalent, displaying 4–5 mAbs and 7–9 scFvs per ferritin nanoparticle. These results are in line with a prior study

from Falvo et al, which showed an average of 3 mAbs could be conjugated to each ferritin.²⁶ The fact that a greater number of scFvs could be conjugated to each FNP suggests that steric hindrance plays a role in determining the maximum avidity of these nanoparticles. To some extent, this also calls into question the strategy of direct fusion of large proteins, such as scFv or fluorescent proteins, to the ferritin heavy or light chain.^{32, 76, 77} Certainly, experimental evidence is critical to confirm that these recombinant “fusion” FNPs assemble into a symmetric 24-subunit complex, akin to the native protein.

Beyond simply demonstrating effective endothelial targeting of FNPs, our results have several intriguing implications. First, significant differences were observed between native FNPs and IgG-conjugated FNPs, in terms of their binding to ECs in culture. While the mechanism is not entirely clear, FNP binding appears to be saturable and receptor mediated, suggesting that conjugated immunoglobulin may block uptake by these endogenous ferritin receptors. From the standpoint of vascular targeting, the implication would be that conjugation of antibodies not only directs FNPs to target cells, but also masks them from uptake by cells in the liver and other organs with high expression of ferritin receptors. Better understanding of ferritin uptake and processing could help to resolve persistent questions about the endogenous function of this protein, its potential role in disease, and its potential as a drug delivery vehicle.^{78–80} Moreover, the finding could have interesting implications in developing stealth technologies for protein-based carriers, especially given mounting concerns about immune responses to PEGylated nanoparticles.^{81–84}

The biodistribution of ICAM-1 and PECAM-1 targeted FNPs raises another series of interesting questions. While it is no surprise that ICAM-targeted FNPs, each of which carries multiple mAbs, demonstrate superior lung targeting as compared to isolated anti-ICAM antibody, it is interesting that they also outperform large nanocarriers, like liposomes and polymeric NPs, which typically bear hundreds of targeting antibodies per particle. There are several potential explanations for this finding. Larger nanoparticles are cleared faster by the reticuloendothelial system (RES), and although no significant difference is seen in the blood concentration at the 30-min time point, it is certainly possible that the smaller size of FNPs affords them more cycles through the pulmonary circulation and opportunities to bind the lung endothelium. Likewise, it is also possible that the localization of ICAM-1 on the endothelial surface allows greater accessibility to 20-nm targeted FNPs than 150-nm liposomes or polyvinylphenol particles (PVPs). In fact, the roughly equal lung distribution and specificity of PECAM-targeted nanocarriers would tend to support this latter hypothesis. Taken together, the ICAM and PECAM results suggest that the “optimal size” of targeted nanocarrier is likely to depend not only on the target organ or cell type, but also the choice of surface molecule and even the epitope recognized by the targeting ligand. These intriguing questions warrant further study. The ability to target well-characterized, uniform, 10–50 nm particles to the vascular endothelium will prove useful in these investigations.

Conclusion

Herein we have demonstrated targeting of horse spleen ferritin nanoparticles (FNPs) to the endothelial surface molecules, PECAM-1 and ICAM-1. Antibody and scFv conjugated ferritin was slightly larger than native FNPs, but remained in the 10–50 nm range, with

relatively uniform size. Conjugation of targeting ligands enabled specific binding to target antigen and also seemed to suppress binding to endogenous cell receptors for ferritin. Pulmonary uptake of endothelial targeted, but not untargeted, FNPs was observed *in vivo*. Comparison to free antibody suggests that FNPs are superior for targeting most vascular determinants, which require multivalent engagement. Likewise, comparison to other antibody-bearing NPs suggest that FNPs are favored for targeting of specific endothelial surface molecules, which may be inaccessible to larger carriers. Immunotargeted FNPs are an important tool for further investigation of these intriguing observations, and may prove a translational platform for vascular delivery of imaging agents or therapeutic cargo.

Materials and Methods

Reagents

Horse spleen ferritin was purchased from Sigma-Aldrich (St Louis, MO). Cu, Zn superoxide dismutase, from bovine liver, was from Calbiochem (La Jolla, CA). N-succinimidyl S-acetylthioacetate (SATA), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), and Iodogen were purchased from Pierce Biotechnology (Rockford, IL). Radioactive isotope ^{125}I was purchased from Perkin-Elmer (Wellesley, MA). Whole molecule rat IgG was from MP Biomedicals (Solon, OH). Anti-mouse-PECAM-1 scFv and anti-mouse-ICAM-1 mAb were obtained from BioLegend (San Diego, CA). Anti-human-PECAM monoclonal antibody was provided by Dr. Marian Nakada (Centocor; Malvern, PA).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were maintained in EGM-2 MV medium (Lonza, Walkersville, MD) with 15% fetal bovine serum supplemented with, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies, Carlsbad, CA). REN cells (human mesothelioma) were grown to confluence in RPMI 1640 medium with 10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies, Carlsbad, CA).

Preparation of targeted ferritin nanoparticles

Heterobifunctional crosslinkers N-succinimidyl S-acetylthioacetate (SATA, Pierce Biotechnology, Rockford, IL) and succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce Biotechnology, Rockford, IL) were used for conjugation of antibody to ferritin. SATA was added at 1:5 molar ratio of antibody to SATA for 30 min at rt. SATA was then deprotected using 10% 0.5 M hydroxylamine for 2 h at rt to expose the reactive sulfhydryl groups. SMCC was added at 1:150 molar ratio of 24mer ferritin to SMCC for introduction of maleimide reactive groups onto ferritin outer surface. Ferritin-antibody conjugation was carried out at 1:10 molar ratio for 1 h at rt. The unreacted components after each step were removed by Quick Spin Protein Columns (G-25 Sephadex, Roche Applied Science, Indianapolis, IN). The nanocarriers were purified by size-exclusion high-performance liquid chromatography (SEC-HPLC) using BioSep SEC-s3000 column (Phenomenex, Torrance, CA). The samples were purified by isocratic method with 100% PBS pH 7.4 to remove free antibody.

Characterization of targeted ferritin nanoparticles

The ferritin conjugation was analyzed by western blot. Samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 4–15% gradient gel (Mini-PROTEAN® TGX™ Gel, Bio-Rad, Hercules, CA). Gels were transferred to PVDF membrane (Millipore, Billerica, MA). Membrane was blocked for 1 h with 3% non-fat dry milk in TBS-T (100 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Tween 20). The membrane was incubated with the corresponding primary and secondary antibodies. The bands were quantified using Bio-Rad GS-800 Densitometer (Bio-Rad, Hercules, CA). HPLC analysis was carried out on BioSep SEC-s3000 column using isocratic method with 100% PBS, pH 7.4.

Size and zeta potential analysis of the ferritin nanocarriers was performed using Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Size measurements were carried out in PBS buffer at 25 °C with disposable capillary cuvettes. The samples were filtered with 0.2 µm filter and centrifuged at 16K × g for 15 min. Non-invasive back scatter system (NIBS) with scattering angle of 173° was used for size measurement. Interpretation of size distribution was performed by number distribution. Zeta potential measurements were carried out in PBS pH 7.4 using disposable folded capillary cell.

Ferritin nanocarriers were analyzed with Technai12 or JOEL1010 transmission electron microscope (TEM). Carbon-coated 200-mesh copper grids were placed on a drop of the sample for 2 min, then washed with Milli-Q water. The grids were stained with 2% uranyl acetate. The stain was wicked off using a filter paper and the grids were dried. Grids were analyzed at acceleration voltage of 120K.

Ferritin radiolabeling

Ferritin was radiolabeled with Na¹²⁵I using Pierce Iodination Beads. The reaction was carried out for 15 min at rt. Free ¹²⁵I was removed by Quick Spin Protein Columns (G-25 Sephadex, Roche Applied Science, Indianapolis, IN).

Binding of targeted FNPs

HUVEC, REN wild type, REN-PECAM and REN-ICAM were plated on 24 well plates (Corning Inc., Corning, NY) and grown to confluence (10⁵ cells/cm²). The radiolabeled ferritin nanocarriers were added to the corresponding cells and incubated for 1 h at rt. After 1 h, the cells were washed three times with ice-cold Hank's Balanced Salt Solution (HBSS, Corning Cellgro, Manassas, VA), and then lysed with lysis buffer (1% Triton X-100, 1 M NaOH). The lysates were measured using Wallac 1470 Wizard™ gamma counter (Gaithersburg, MD). The bound radioactive ferritin nanocarriers were plotted against the corresponding total amount added.

Preparation of anti-ICAM Ab/immunoliposomes

Liposomes were prepared as previously described (62), with modification to incorporate 1.25 mol% maleimide PEG₂₀₀₀-DSPE. Liposomes were extruded through 200 µm polycarbonate filters (Avanti Polar Lipids, Alabaster, AL). Anti-ICAM Ab and/or IgG were modified with SATA to introduce sulfhydryl groups, allowing conjugation to maleimide-

functionalized liposomes. A 6-fold excess of SATA was added for 30 min at rt to achieve ~ 1 reactive group per antibody. Ab-SATA was deprotected using hydroxylamine (50 mM) and added to PEGylated liposomes at a concentration calculated to conjugate approximately 200 Ab molecules/liposome, assuming an efficiency of 75–80% (62). A fraction ~10 mol- of ^{125}I labeled IgG-SATA (also deprotected as described) was included in each reaction, for radiotracing particles in biodistribution and cell binding studies. Unreacted components were removed at each step using Zeba desalting columns (Thermo Scientific, Rockford, IL).

Biodistribution of targeted nanocarriers *in vivo*

Animal experiments were carried out according to protocol approved by Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. 1 μg of radiolabeled ferritin nanocarriers was injected IV via internal jugular vein of C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). After 30 min, blood was collected and the organs were harvested. Radioactivity of the samples was determined with Wallac 1470 WizardTM gamma counter. The weight of the samples and their radioactivity were used to calculate percent injected dose per gram (%ID/g).

Statistical analysis

Experimental data were analyzed using student t-test with Bonferroni correction. Differences were deemed significant at $p < 0.008$ for relative loading (%Id/gr) graphs and at $p < 0.01$ for localization ratios.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FNP	ferritin nanoparticle
EC	endothelial cell
ICAM-1	intercellular adhesion molecule-1
PECAM-1	platelet-endothelial cell adhesion molecule-1

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Figure 1.

Quantitative analysis of number of radiolabeled whole antibody and scFv moieties conjugated using HPLC. HPLC radiotracer of a) radiolabeled-IgG conjugated to ferritin. The IgG¹¹²⁵/FNP conjugate peak is indicated with number (1) and an overlay of (2) HPLC absorbance trace of IgG (red color). Green colored dashed line indicates the cut-off line used for calculation of areas under the curve (AUC) for conjugate vs. free antibody peaks. Area under the curve (AUC) for b) conjugated vs. free antibody. HPLC radiotracer of c) radiolabeled-scFv conjugated to ferritin. The scFv¹¹²⁵/FNP conjugate peak indicated is with number (1) and an overlay of (2) HPLC absorbance trace of scFv (red color). Area under the curve (AUC) for c) conjugated vs. free scFv.

Figure 2.

TEM images and size analysis of FNPs. TEM images of a) FNP, b) anti-ICAM Ab/FNP, c) anti-PECAMscFv/FNP, Scale bar: 100 nm (a, b), and 50 nm (c). Particle size analysis (size distribution by number) obtained by DLS for d) FNP, anti-ICAM Ab/FNP, and anti-PECAMscFv/FNP. Illustration of e) FNP, f) whole antibody/FNP and g) scFv antibody fragment/FNP conjugates. Size and polydispersity index (PDI) for h) FNP and IgG/FNP. DLS frequency curve for i) FNP and IgG/FNP.

Figure 3.

Binding of targeted FNPs to ICAM positive, PECAM positive, and negative REN cells, as well as to HUVECs. FNP was ^{125}I -labelled prior to antibody conjugation. Cells were grown to confluence and incubated with ferritin nanoparticles for 1 h at 37 °C. Bound radiolabeled nanoparticles were measured by gamma counter. Binding of a) anti-ICAM Ab/FNP and c) anti-ICAMscFv/FNP to REN and REN-ICAM cells. Binding of b) anti-PECAMscFv/FNP to REN and REN-PECAM cells. Binding of d) free ferritin was examined on HUVECs against IgG/FNP. Ferritin binding to HUVECs was evaluated using e) human anti-ICAM Ab/FNP and f) human anti-PECAM Ab/FNP.

Figure 4.

Targeting of FNPs to ICAM-1. a) Biodistribution of ^{125}I -labeled anti-ICAM mAb and IgG FNP in mice at 30 min. Tissue uptake is indicated as mean $\pm$ SEM (n=3). b) Localization ratio of selected organs. Significant differences determined by t-test with Bonferroni correction to account for multiple comparisons.

Figure 5.

Targeting of FNP to PECAM-1. a) Biodistribution of ^{125}I -labeled anti-PECAM mAb and IgG FNP in mice at 30 min. Tissue uptake is indicated as mean $\pm$ SEM (n=3). b) Localization ratio of selected organs. Significant differences determined by t-test with Bonferroni correction to account for multiple comparisons.

Table 1

a) Lung biodistribution, b) localization ratio, and c) Immunospecificity index of various targeted vs. non-targeted agents: mAb - isolated monoclonal antibody, PVP - 100-nm polyvinylphenol particles, liposomes – 150-nm PEGylated immunoliposomes, FNP - ferritin nanoparticles. Anti-PECAM liposome data reproduced from;⁴⁵ anti-IgG and anti-PECAM PVP data reproduced from;⁴⁶ anti-ICAM PVP data reproduced from.⁴⁷

Lung Biodistribution (% ID/g)				
	Free mAb	PVP	Liposomes	FNP
IgG	7.2 ± 1.3	10.6 ± 3.3	8.5 ± 0.5	14.5 ± 1.8
ICAM	52.5 ± 8.9	126.9 ± 14.8	95.4 ± 13.0	160.9 ± 6.5
PECAM	40.9 ± 9.6	88.5 ± 7.6	110.8 ± 5.8	85.4 ± 2.1

Localization Ratio				
	Free mAb	PVP	Liposomes	FNP
IgG	0.24 ± 0.03	6.7 ± 2.8	0.81 ± 0.32	2.8 ± 0.2
ICAM	4.2 ± 0.6	121.2 ± 26.8	6.6 ± 1.5	49.9 ± 10.8
PECAM	2.5 ± 0.4	62.8 ± 11.9	11.2 ± 0.9	26.9 ± 1.2

Immunospecificity Index (ISI)				
	Free Ab	PVP	Liposomes	FNP
ICAM	17.9	18.0	8.1	26.7
PECAM	10.6	9.3	13.9	10.8