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Recognition of dextran-superparamagnetic iron oxide nanoparticle conjugates (Feridex) via macrophage scavenger receptor charged domains

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Abstract

Dextran-coated superparamagnetic iron oxide nanoparticles (dextran-SPIO conjugates) offer the attractive possibility of enhancing MRI imaging sensitivity so that small or diffuse lesions can be detected. However, systemically injected SPIO are rapidly removed by macrophages. We engineered embryonic cells (HEK293T) to express major macrophage scavenger receptor (SR) subtypes including SR-AI, MARCO, and endothelial receptor collectin-12. These SRs possess a positively charged collagen-like (CL) domain and they promoted SPIO uptake, while the charge neutral lipoprotein receptor SR-BI did not. *In silico* modeling indicated a positive net charge on the CL domain, and a net negative charge on the cysteine-rich (CR) domain of MARCO and SR-AI. *In vitro* experiments revealed that CR domain deletion in SR-AI boosted uptake of SPIO 3-fold, while deletion of MARCO's CR domain abolished this uptake. These data suggest that future studies might productively focus on the validation and further exploration of SR charge fields in SPIO recognition.

Introduction

Magnetic resonance imaging (MRI) is a modality that has long attracted considerable interest for early disease detection and staging. However, to be useful for small, indistinct lesions MRI often requires enhancement (1). FDA approved superparamagnetic iron oxide (SPIO) contrast agents are the most detectable and effective medium for enhancing contrast

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Supporting Information The methods for receptor cloning and expression by PCR are described in the supporting information section. In addition we provide and explain data that documents the expression of the receptor subtypes in HEK293T cells. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

in MRI acquisitions (2–6). A major difficulty with current SPIO formulations, *viz.*, dextran coated SPIO nanoparticles, is that they are avidly taken up by macrophages, which results in premature clearance, reduced effectiveness of imaging, and toxicity (7–10). The mechanism of macrophage SPIO uptake is incompletely characterized, and we along with others (11–13) have sought to further elucidate this process to identify possible inhibitory strategies.

Much remains to be learned about the key specifics of particle uptake by macrophages, although it is known that uptake is opsonin-independent for many types of nanoparticles, including gold, silica (14–16), polystyrene (17) and liposomes (18). SPIO-dextran uptake has recently been shown by us to be complement and IgG-independent in mouse knockout models (19). Although several classes of macrophage receptors could conceivably mediate SPIO recognition, multiple studies have collectively provided convincing data that SPIO uptake is coordinated by scavenger receptors (SRs) (11, 12).

What remains to be elucidated is the specific mechanism of SPIO recognition by SRs. This is important as it may provide an opportunity for selectively inhibiting or manipulating SPIO nanoparticle uptake by macrophages. Current evidence reveals that often it is polyanionic pathogen-associated molecular patterns that are eliminated by SRs (12, 15, 17, 20–22). Moreover, the major macrophage SR AI subtype (SR-AI) recognizes negatively charged surfaces via a positively charged collagen-like domain (CL-domain) (12, 17, 20, 23). Therefore we hypothesized that SPIO-dextran nanoparticles, which are weakly anionic, are recognized by the CL-domain of SRs. We further hypothesized that this mode of recognition might be differentially modulated between various SR subtypes.

Our hypotheses were addressed using *in silico* modeling of the SR domain charge field, together with a defined system in which human embryonic kidney cells (HEK293T) were engineered to individually express the major macrophage SR subtypes SR-AI, MARCO, SR-PSOX, SR-BI, and the primary endothelial SR collectin-12 (CL-P1). The experiments indicated that the net positively charged collagen-like domain mediated SPIO uptake by SR-AI, and that deletion of a net negatively charged cysteine rich (CR) domain adjacent to the CL domain differentially affected SPIO binding to SR-AI versus MARCO.

The results of this modeling and *in vitro* study provide an essential step for followup investigations of SR mediated macrophage nanoparticle recognition, and a comprehensive validation of various SR domains according to the fine structure of charge fields. This will lead to strategies for (i) inhibiting nanoparticle clearance, (ii) minimizing undesired labeling of macrophages, and (iii) targeting specific subpopulations of macrophages,

Experimental Procedures

SPIO-Dextran Nanoparticle Preparation and Physical Characterization

Preparation and storage of nanoparticles—Commercial Feridex I.V.® nanoparticles were used for this study and were obtained from a commercial source on our behalf by the UCSD Department of Radiology. Feridex consists of a suspension of SPIO-dextran composites. Each composite is 50–160 nm across and contains multiple SPIO particles approximately 5–6 nm in diameter embedded in a meshwork of linear dextran (10 kDa, T-10). Particles were resuspended in PBS at 1–2 mg (Fe)/ml, filtered through a 0.2 µm membrane filter, and stored at 4°C.

Nanoparticle size determination—The size distribution and z (zeta)-potential of diluted aliquots of the nanoparticle suspension was measured with a Zetasizer Nano (Malvern, UK). To determine any effects of adherent plasma proteins on nanoparticle size, SPIO was mixed

with citrated mouse plasma (1:3 volume ratio), incubated for 10 minutes, and applied to a MINI magnetic column (Miltenyi Biotech), then eluted and sized.

Nanoparticle structure determination—Nanoparticle structure was confirmed using transmission electron microscopy; 5 μ l of 0.5 mg/ml of SPIO-dextran in double distilled water was applied to Formvar/carbon coated grids (Ted Pella, Redding, CA). After drying grids were viewed using a JEOL 1200EX II (JEOL, Peabody, MA) transmission electron microscope at 75 keV and photographed using a Gatan digital camera (Gatan, Pleasanton, CA).

Scavenger Receptor Gene Cloning, Amplification, and Expression in HEK293T cells

In order to provide a general characterization of SPIO recognition according to major SR subtypes, we transfected HEK293T cells using equal amounts of constructs coding for the following receptors: SR-AI which is expressed on macrophages and monocytes (24); MARCO which is a Macrophage Receptor with Collagenous structure expressed on macrophages resident in the lung alveoli, in the spleen, and in the liver (Kupffer cells) (25); lectin SR Collectin-12 (collectin placenta 1 or CL-P1, expressed on endothelial cells) (26); chemokine SR for phosphatidylserine and oxidized lipoproteins (PSOX/CXCL16, expressed on dendritic cells and atherogenic macrophages (27)); and ubiquitous lipoprotein receptor SR-BI (28). The details of cloning, amplification and expression are given below.

SR MARCO—A pCMV6-AC plasmid carrying full-length human macrophage receptor with collagenous structure (MARCO) as transfection-ready DNA (Catalog SC319619) was purchased from OriGene (Rockville, MD). Human MARCO with the truncated cysteine domain and MARCO with charged collagen were designed with flanking BamHI and XhoI restriction sites and synthesized by Epoch Life Sciences (Missouri City, TX); see supplement for sequences of the construct and primers. The inserts were cloned into pCDNA3.1+(Zeo).

SR-AI (CR domain +) and SR-AII (CR domain -)—Full-length mouse cDNA of SR-AI (splicing variant A, NM_001113326) was amplified from mouse liver mRNA by RT-PCR and cloned into a pCDNA 3.1+ plasmid using the BamHI and XhoI restriction sites. pCMV-SPORT6 plasmid encoding mouse scavenger receptor SR-AII (splicing variant B; without the cysteine-rich domain) was obtained from ATCC (Catalog MGC-6140), PCR amplified, and cloned into pCDNA 3.1. All inserts were verified by forward and reverse sequencing. Full details for the primers are provided in the Supplemental Methods.

SR-BI and Collectin-12 (endothelial)—A transfection-ready clone encoding human macrophage scavenger receptor SR-BI was obtained from ATCC in pCMV-SPORT6 cassette. A transfection-ready human Collectin Placenta 1 (CL-P1) or Collectin-12 in pCMV₆-XL4 cassette was obtained from OriGene.

Transfection of HEK293T cells—HEK293T (ATCC) were transiently transfected using Lipofectamine 2000 (Invitrogen) with 0.5 μ g of receptor plasmids or empty vector pCDNA 3.1 (Invitrogen) per 1×10^6 cells in 24 well plates. The mRNA expression of SR-AI, MARCO, CL-P1 and SR-BI was compared via quantitative PCR as described in Supplemental Methods.

Determination of receptor expression on the HEK293T cell surface—

Comparisons of SPIO binding between receptor subtypes required equal SR densities on the cell surface to be valid, or a measure of relative density by which to normalize binding data between receptor subtypes. Our assessment of receptor expression did not rely on one type

of measure alone, and was determined using three different approaches for cross validation. First, we used immunostaining with MARCO rabbit anti-human polyclonal AP9891a, SR-BI rabbit anti-human monoclonal AJ1734a (both from Abgent, San Diego, CA), rat-anti mouse SR-AI (R&D). The second approach involved Western blotting using rabbit anti-human MARCO (Abgent) and rat anti-mouse SR-AI (AbD Serotec MAB 1322). Cells were transfected with SR-AI and MARCO and fractionated with Mem-PER membrane isolation kit (Thermo). The quality of fractionation was determined with anti-HSP90 antibody (Cell Signaling Technology, Danvers, MA) as a cytoplasmic marker and anti-alpha1 sodium-potassium ATPase antibody (Abcam, Cambridge, MA) as a membrane marker. The level of SR-AI and MARCO in each fraction was compared with western blotting using the above-described antibodies. The final measure of cell receptor expression was based on real time PCR of the receptor transcripts (details in the Supplemental data).

SPIO uptake and binding experiments

Nanoparticle uptake experiments in receptor-transfected HEK293T cells and in J774A.1 cells were performed similarly.

Uptake measured by optical absorbance—Cells in 24 well plates were incubated with 0.1 mg/ml SPIO-dextran nanoparticles for 2 h in complete medium at 37° C. For the ligand inhibition experiment, polyinosinic acid, dextran sulfate 500-kDa, fucoidan, dextran or gelatin (all from Sigma) were incubated with J774A.1 cells for 15 min prior to the addition of nanoparticles. SPIO uptake was quantified by adding 200 µl of QuantiChrom Iron Assay reagent (BioAssay Systems), overnight incubation, and measurements of relative absorbance (570 nm). For cell binding experiments, SPIO was added to cells at 4°C at 3-fold higher concentration (0.3mg/ml) for 15min, washed and assayed as above.

Microscopy-based measurements of SPIO uptake—Cells were seeded into 8-well chamber slides (NalgeNunc) and incubated for 2 h with 0.1 mg/ml SPIO, washed, fixed with 4% formaldehyde, and stained with Prussian blue dye (29) to visualize iron inside the cells.

SPIO binding to type I collagen—To determine whether SPIO does in fact bind to collagen, which comprises the CL domain of SRs, microwell 96-well plates (Costar) were coated with either calf skin type I collagen (100 µg/ml 0.1 M acetic acid/PBS; Sigma, C3511) or BSA/PBS (controls) and blocked with 1% BSA/PBS. SPIO solution (3, 10, 30 or 100 µg/ml of Fe in PBS) was added and bound iron quantified using the QuantiChrom Iron Assay as described previously.

In silico modeling of SR domains

We sought to predict whether SR domains might possess charge differences that form the basis of differential recognition between SR receptor subtypes. Therefore, modeling of the SR-AI and MARCO electrostatic charge fields was performed using the homology module of Insight II software (Accelrys, San Diego) on the San Diego supercomputer at UCSD. Collagen-like domains were modeled based on the type I collagen (pdb ID 3HQV). The cysteine-rich domain models were based on the crystal structure of the monomeric cysteine-rich domain of mouse MARCO (pdb ID 2OY3). Equipotential maps of receptor charges were plotted using the NAMD program (30). For human SR-BI, the charge was estimated as the number of charged ionizable residues (positive, ARG and LYS (+1); negative, GLU and ASP (-1)) per 10 residues at pH 7.5.

Results

SPIO-Dextran Nanoparticle Physical Characteristics

SPIO Nanoparticle Size and Structure—We used the commercial SPIO MRI contrast agent Feridex I.V., which is essentially a SPIO-dextran nanoparticle, to study SPIO recognition by macrophage SRs. The measured diameter of the SPIO dextran particles (composites) was between 80–150 nm (average 112 nm), and zeta potential was slightly negative, −13 mV (Fig. 1A–B). TEM confirmed our expectation that the particles consisted of several 5 nm cores of crystalline iron oxide embedded in a meshwork of dextran. The clusters exhibited a worm-like shape, although irregular aggregates were also visible (Fig. 1C).

SPIO Uptake by SRs

SPIO uptake by macrophages is mediated via SRs and blocked by charged polymers—Previously reported SR-dependent recognition/uptake of Feridex by J774A.1 macrophages was confirmed by inhibition experiments using various SR ligands (Fig. 2). Addition of the polyanionic scavenger receptor ligands fucoidan (10 µg/ml) or dextran sulfate (3 µg/ml) to J774A.1 macrophages prior to the addition of SPIO produced up to 80% inhibition of SPIO uptake (P value=0.0003 for dextran sulfate). The addition of positively charged gelatin (hydrolyzed collagen) at 1 mg/ml also inhibited the uptake, whereas branched 20kDa dextran at 1 mg/ml had no effect. (data not shown) The suppression of Feridex uptake by known SR polyanionic ligands confirms that in J774A.1 macrophages recognition is mostly SR-dependent, and inhibition by both positively and negatively charged polymers provides a preliminary indication that charge interactions may play an important role in the uptake.

Charged SRs exhibited greater SPIO uptake—Transiently-transfected MARCO, SR-BI, CL-PI showed similar levels of mRNA expression based on quantitative PCR, while SR-AI showed about 50% lower mRNA expression (Supplemental Fig. S1A–B). For SR-AI, SR-BI and MARCO, we also verified the expression with western blotting and confirmed membrane expression with immunostaining (see Supplemental Fig. S2 and data below).

According to the Prussian blue staining and iron quantification (Fig. 3A–B), SR-AI, CLP1, SR-PSOX and MARCO, which have positively charged SRs, significantly promoted the binding and uptake of SPIO by HEK293T cells (5–20-fold increase compared to vector-transfected), while the charge neutral SR-BI did not show any uptake despite being expressed on the cell surface (Supplemental Fig. S2). According to charge calculations, the SR-BI extracellular domain has a neutral charge of +0.05 and no charged domains were detected in the sequence.

SPIO binds to type I collagen—SR-AI, MARCO and CL-P1 each possess a positively charged CL domain (Fig. 4A) and we sought to determine if the SPIO-dextran nanoparticle could bind collagen alone. Collagen type I has a cationic heparin-binding site with affinity of 150nM (31, 32). We tested the binding of Feridex to a collagen type I-coated plate. There was a concentration-dependent binding that was completely inhibited by addition of polyanionic dextran sulfate 500kDa to the plate prior to the addition of nanoparticles (Fig. 4B, Supplemental Fig. S3).

SR Domain Effects on SPIO Binding and Uptake by MARCO and SR-AI

MARCO and SR-AI exhibited different uptake—SPIO uptake by Prussian blue (Fig. 5A) showed major differences between SR-AI and MARCO (quantitatively 8-fold difference, Fig. 3A). We tested whether this difference is due to the unequal cell surface

expression. Immunostaining of the cell surface receptors (Fig. 5A bottom panel) and western blot analysis of the membrane fraction (Fig. 5B) showed that both receptors are expressed on the cell surface at the approximately same level. The binding of SPIO to receptor-transfected cells showed about 5-fold higher binding to SR-AI than to MARCO (Fig. 5C).

Predicted charge differences between SRs—We sought to determine whether differences between SR-AI and MARCO are due to differences in SR domains. As an initial strategy we modeled charge, which is the most obvious, but not necessarily the only, possible basis for any differential domain effects on SPIO binding. After predicting possible differences between the domains, according to charge, we were prompted to follow up with uptake experiments in which the CR domain was either added or deleted.

Using well-validated and widely used structural modeling approaches and software (see Methods), 3D crystal structure-based models of the extracellular region and its electrostatic profile were built for human SR-AI and for murine MARCO (Fig. 6). The crystal structure of human MARCO is not available, but murine and human MARCO share >70% sequence identity (33) and 96% charge amino acid identity (Supplemental data). According to the 3D equipotential (from +1.8 to −1.8) surface charge map for both SRs (Fig. 6), the CL domain positive charge field extends well outside the protein backbone. In contrast the C-terminal CR domain of both SRs had a negative charge field (Fig. 6). Interestingly the net positive charge field of MARCO was lower than SR-AI, suggesting the possibility of differential effects on recognition by the CR domain.

CL/CR domain effects on SPIO uptake in SR subtypes—Functional tests were made to determine in vitro whether the CL versus the CR domain have differential effects on SR - SPIO binding. SR-AI and MARCO were compared since they are structurally very similar and we made the working assumption that uptake differences between them would be more influenced by the CL and CR domains rather than overall SR structure.

We prepared shortened isoforms of SR-AI (known as SR-AII) and MARCO in which the entire CR domain was deleted (Fig. 7A). In addition, we prepared a chimeric MARCO/SR-AI receptor by fusing a highly charged collagen fragment of SR-AI to the C-terminal part of MARCO's CL domain (Fig. 7A). This charged collagen fragment is known to mediate the binding of acetylated LDL to SR-AI and to inhibit ligand binding (23). The expression of the constructs on the cell surface was verified with western blotting and immunostaining (Fig. 7B–C, Supplemental Fig. S4).

The deletion of the CR domain in SR-AI boosted SPIO uptake almost 3-fold (Fig. 8A). The cell binding studies performed at 4°C showed similar effect of CR domain deletion on SR-AI mediated uptake (Fig. 8B). Deletion of MARCO's CR domain abolished the uptake (Fig. 8C), whereas fusion of the SR-AI collagen fragment to MARCO increased MARCO uptake 2-fold (Fig. 8C). These results suggest that the CL and CR domains on different SRs may produce a different net effect, and future experiments will examine in greater detail the mechanisms of iron nanoparticle recognition in the context of domain charge fields in different SRs.

Discussion

SPIO nanoparticle-based contrast media for MRI are recognized and scavenged by macrophages. This clearance of circulating contrast agent reduces the amount of MRI label available for target tissue contrast enhancement. Moreover interpretation of MRI volumes becomes complicated because the scavenging macrophages end up being labeled and detected. This obstacle to effective MR imaging may potentially be addressed by selective

macrophage SR inhibitors, but currently available polyanionic SR antagonists are not selective and are toxic. The present study was intended to contribute to our understanding of SPIO recognition to provide a basis for further work leading to selective inhibitors of SR mediated SPIO recognition.

Our data confirmed previous observations that that SPIO uptake by macrophages is mediated by SRs (11, 12), and demonstrated that physical differences in collagen-like (CL) and cysteine-rich (CR) domains, very likely charge related, may provide a basis for differential recognition and uptake of SPIO and other nanoparticles according to SR subtype. These results provide an initial point of departure more detailed investigations of SR recognition and specificity, and the precise contribution of charge field fine structure.

Our test platform was the embryonic kidney cell (HEK293T) expression system and we were able to acquire consistent results and successfully confirm previous reported findings for SRs and SPIO. SPIO nanoparticles were recognized by two major and well defined SRs, SR-AI and MARCO, by the SR Collectin-12, which is expressed on endothelial cells (26), and by SR-PSOX, which is mainly expressed on dendritic cells and macrophages in atherosclerotic lesions.

According to our *in vitro* uptake data, and in agreement with our hypothesis that the CL domain plays a key role in SPIO uptake, the CL domain appears to involve mediate recognition of SPIO nanoparticles by SR-AI. We found that the common feature of tested receptors that bound and internalized SPIO-dextran was the presence of a positively charged CL domain, and in agreement with this observation it has been reported that the positively charged fragment of the SR-AI CL domain mediates the binding of polyanionic ligands and negatively charged, acetylated LDL (23).

Importantly, there was a distinct difference in the uptake mechanism between SR-AI and MARCO despite their similar overall structures. Differences in recognition between these two receptor subtypes is suggested by previous reports indicating that MARCO recognizes other anionic nanoparticles, and recognizes larger iron oxide particulates^{4,22}. There was no evidence of iron aggregation, which might favor MARCO, in our experiments, and transiently transfected MARCO in fact had lower uptake than SR-AI, despite the two having similar levels of cell surface expression. The reason for this difference is not clear but could be related to the difference in charge properties of the receptor domains. Deletion of the CR domain from SR-AI boosted uptake, and a similar effect was observed by Doi et al. (23) for the uptake of acetylated lipoprotein. However, deletion of MARCO's CR domain abolished uptake, indicating a differential effect of the CR domain depending on SR subtype.

Our *in silico* models show a net negative charge for the CR domain, and other studies have reported that MARCO's CR domain has a fine structure comprised of positive and negative subdomains (34). This could influence nanoparticle recognition in subtle ways. Bacteria and nanoparticle uptake studies of MARCO and SR-AI have uncovered different SR specificities (25, 34, 35), and deletion of the MARCO CR domain abolishes the uptake of bacteria (34). Since MARCO may recognize SPIO-dextran and other particles through a somewhat different binding mechanism than SR-AI, there is a need for future, specific *in vitro* experiments that would be conceptually linked with what is known about SR behavior and with further, detailed modeling of the fine structure of charge domains and charge interactions between closely spaced receptors. For example, SRs have been reported to form clusters on the cell surface (36), and such receptor clustering could have important implications on local charge fields and on SPIO binding.

In conclusion, we demonstrated that a variety of SRs are able to promote the uptake of dextran-coated SPIO (Feridex) *in vitro*, with the positively charged CL domain largely responsible for uptake by SR-AI. The precise role of the CR domain and charge fields in SPIO uptake by different SR subtypes remains to be elucidated. Specific further investigations suggested by the present study may provide a basis for defined strategies by which to selectively inhibit macrophage uptake of SPIO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Physical characterization of Feridex (SPIO nanoparticles)

(A) SPIO nanoparticle size (intensity) distribution. Particles were processed as described in Methods;

(B) Zeta potential of particles;

(C), TEM of SPIO nanoparticle (Feridex), magnification: 60,000 $\times$. Size bar = 100 μ m. Inset, cropped area showing a representative nanoparticle consisting of a cluster of electron-dense magnetite-maghemite crystals (white arrow on the main image).

Fig. 2. SR ligand tests confirm SPIO uptake mediated by SRs

SPIO nanoparticle uptake by the macrophages in the presence of various inhibitors (n=3–5). Addition of polyanionic SR ligands fucoïdan (10 μ g/ml), and dextran sulfate (3 μ g/ml) to J774A.1 macrophages prior to SPIO resulted in 65% – 76% inhibition of uptake (non-parametric Mann-Whitney test, p value=0.0003, n=3). The uptake was also inhibited by 1mg/ml gelatin. These data suggest that the mechanism of SPIO nanoparticle uptake is SR-dependent and may be based on electrical charge. Pretreatment of J774A.1 cells with 0.1 mg/ml trypsin reduced the uptake by 80% confirming a protein receptor is needed.

Figure 3. SPIO nanoparticle uptake of transiently expressed macrophage SRs

HEK293T cells were transiently transfected with the same amounts of receptor-coding plasmids and then incubated with 0.1 mg/ml SPIO (Feridex) in 10% FBS-supplemented medium for 2 h.

(A) Uptake of SPIO by cells transfected with SRs quantified with QuantiChrom™ Iron Assay Kit (see Methods), p-value 0.0007, non-paired t-test, n=5; Only receptors with charged domains show uptake.

(B) Example images of cells were stained with Prussian Blue and Nuclear Fast Red and the images were taken at low magnification (4× objective). A representative experiment out of five is shown. Size bar = 200 μm.

Figure 4. SPIO binding to collagen-coated surface

(A) Schematic representation of SRs based on previously published literature {Taylor, 2005 #37;Ohtani, 2001 #599;Shimaoka, 2004 #544}. SR-AI, MARCO and CL-P1 contain collagen-like domain (stem) in the extracellular part.

(B) In order to mimic the binding to cationic collagenous receptors, microplate wells were coated with charged collagen type I, and SPIO nanoparticles were incubated in PBS as described in Methods. The binding was only to collagen-coated wells (open bars) and not to BSA coated wells (solid bars). The binding was inhibited by 10 µg/ml dextran sulfate (striped bars).

Figure 5. Comparison of SPIO uptake by SR-AI and MARCO

(A) Upper panel: SPIO uptake by receptor-transfected HEK293T cells. The experiment was performed as in Fig. 3B. Cropped areas are shown. Size bar=50μm. Lower panel: Staining of the receptors on the cell surface (described in Methods).

(B) Western blotting of SR-AI and MARCO after cell fractionation. Both cytoplasmic and membrane fractions are positive for the receptors. Majority of the receptor is in intracellular compartment. HSP90 and Na⁺, K⁺ ATPase staining confirms fractionation efficiency.

(C) SPIO binding to cells at 4°C shows the difference in binding efficiency between SR-AI and MARCO.

Figure 6. Molecular models of SRs and their electrostatic profiles

Electrostatic profiles of mouse MARCO and mouse SR-AI receptors are shown as equipotential surfaces for the values of +1.8 (blue) or -1.8 (red) charges. Note the higher positive charge density of SR-AI and MARCO CL-domain per unit length versus the overall negative charge of the C-terminal CR-domain (red color). Insert: Ribbon presentation of the 3D models of MARCO and SR-AI receptors used for charge profile reconstruction. Different colors denote the three subunits of each receptor.

Figure 7. Modifications and expression of SR-AI and MARCO

(A) CR DOMAIN of SR-AI was deleted. The charged collagen sequence from SR-AI was fused to C-terminal portion of MARCO's CL DOMAIN. CR DOMAIN of MARCO was deleted.

(B) SRs were expressed on HEK293T cells and the levels of expression were verified by western blotting.

(C) To demonstrate equal efficiency of transfection, the cells were stained with antibodies against the SRs. High magnification images of MARCO receptor staining in the membrane are provided in Supplemental data.

Fig. 8. CL-CR domains influence SR mediated SPIO uptake *in vitro*

The uptake of the constructs shown in Fig. 6 was quantified with an iron assay.

(A) SR-AI and SR-AII mediated uptake. Deletion of SR-AI CR DOMAIN increases the SPIO uptake 3-fold (non-parametric Mann-Whitney test, p-value 0.0026, n=3).

(B) Binding of SPIO to SR-AI and SR-AII transfected HEK293T cells at 4°C. Cells were grown, transfected in 24 well plates and incubated with 0.3 mg/ml SPIO at 4°C for 15 min. Results of a triplicate measurement is shown.

(C) MARCO-mediated uptake. Addition of the charged CL to MARCO increased the uptake two-fold, (non-parametric Mann-Whitney test, p-value 0.013, n=5) but the uptake was still much lower than that of SR-AI cells. Deletion of the CR DOMAIN in MARCO completely blocked SPIO uptake.