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Co-coating of receptor-targeted drug nanocarriers with anti-phagocytic moieties enhances specific tissue uptake versus non-specific phagocytic clearance

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ABSTRACT

Nanocarriers (NCs) help improve the performance of therapeutics, but their removal by phagocytes in the liver, spleen, tissues, etc. diminishes this potential. Although NC functionalization with polyethylene glycol (PEG) lowers interaction with phagocytes, it also reduces interactions with tissue cells. Coating NCs with CD47, a protein expressed by body cells to avoid phagocytic removal, offers an alternative. Previous studies showed that coating CD47 on non-targeted NCs reduces phagocytosis, but whether this alters binding and endocytosis of actively-targeted NCs remains unknown. To evaluate this, we used polymer NCs targeted to ICAM-1, a receptor overexpressed in many diseases. Co-coating of CD47 on anti-ICAM NCs reduced macrophage phagocytosis by ~50% for up to 24 h, while increasing endothelial-cell targeting by ~87% over control anti-ICAM/IgG NCs. Anti-ICAM/CD47 NCs were endocytosed via the CAM-mediated pathway with efficiency similar (0.99-fold) to anti-ICAM/IgG NCs. Comparable outcomes were observed for NCs targeted to PECAM-1 or transferrin receptor, suggesting broad applicability. When injected in mice, anti-ICAM/CD47 NCs reduced liver and spleen uptake by ~30–50% and increased lung targeting by ~2-fold (~10-fold over IgG NCs). Therefore, co-coating NCs with CD47 and targeting moieties reduces macrophage phagocytosis and improves targeted uptake. This strategy may significantly improve the efficacy of targeted drug NCs.

1. Introduction

Nanomaterials whose composition and functionalization are compatible with biological systems represent promising tools to access and interact with cells and tissues [1]. An example is that of biomaterials explored for delivery of pharmaceuticals: a myriad of drug nanocarriers (NCs) exist which offer valuable control over drug solubility, stability, release, etc., improving the therapeutic outcome [2–6]. However, the site-specific targeting of these systems has proven difficult to control since natural clearance mechanisms often eliminate them prematurely, hindering their ability to access the intended body sites [2,7,8]. For instance, many drug NCs use passive extravasation across capillary pores or gaps to reach tissues, such as the case for numerous NCs explored for cancer therapies [5,7,8]. In other cases, they are designed to target endothelial-surface receptors involved in active transport between the bloodstream and the underlining tissue [9,10], as for those intended to cross the blood-brain barrier [11,12]. In both cases, NC removal via renal filtration (if sufficiently small) or the mononuclear phagocyte system in the liver and spleen (most commonly) is in detriment of their accumulation in the intended tissue [7,8,13]. In addition, their removal by tissue resident macrophages also hinders their ability to reach the intended cells [14]. Coupling of drug NCs to affinity molecules such as antibodies, peptides,

Abbreviations: Amil, amiloride; BSA, bovine serum albumin; CAM, cellular adhesion molecule; Fil, filipin; HUVECS, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; ID, injected dose; ID/g, injected dose per gram of tissue; IgG, immunoglobulin G; MDC, monodansylcadaverine; NCs, nanocarriers; PECAM-1, platelet-endothelial cell adhesion molecule 1; TNFα, tumor necrosis factor alpha; TfR, transferrin receptor; PLGA, poly(D,L-lactide-*co*-glycolic) acid.

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aptamers, etc. helps them bind and enter target cells, but this does not preclude non-specific clearance [9,15]. In fact, some of these affinity moieties may enhance clearance, e.g., through Fc domain of targeting antibodies which can bind to Fc receptors (FcR) on phagocytic cells, nucleic acid-based interaction with scavenger receptors, etc. [14].

In order to improve this aspect, grafting of polyethylene glycol (PEG) onto drug NCs is a common strategy to lower interactions with phagocytes and other elements involved in clearance, including opsonins, the complement, etc. [8,13] However, while undoubtedly valuable, PEG has been associated with generation of anti-PEG antibodies, which defeats this purpose, and its presence also reduces intended targeting interactions [9,16]. Alternative options to lower phagocytic uptake of nanomaterials include the use of high aspect ratio shapes to promote circulation in the blood midstream [17] and reduce phagocytic uptake, which depends on the length and stiffness of the device, the angle of contact with phagocytes, etc [18]. Nevertheless, high aspect ratio objects often exceed the dimensions permissible for active vesicular transport across the endothelium or into tissue cells, and how these parameters affect specific routes is unclear [9]. Therefore, there is significant room for new options which may offer improvement.

An attractive alternative is that of coupling CD47 on the surface of drug NCs. CD47 is a transmembrane glycoprotein expressed on most cells, which plays a key role on the non-phagocytic versus phagocytic balance, among other functions [19]. As an example, red blood cells (RBCs) continuously pass through the hepatosplenic system without being removed, despite the fact that RBCs come in contact with phagocytic cells in these organs [20]. This anti-phagocytic effect results from the interaction between the extracellular domain of CD47 expressed on RBCs and the extracellular domain of signal regulatory protein α (SIRPα) expressed on macrophages [19,21]. Binding of CD47 to SIRPα recruits tyrosine phosphatase SHP-1 to the phagocyte plasmalemma, which then dephosphorylates substrates involved in the phagocytic process [20,22]. Particularly, phosphorylation of myosin-IIA is required for reorganization/contraction of cytoskeletal acto-myosin elements involved in phagocytic engulfment; hence, myosin-IIA dephosphorylation impairs this process [23]. Thereafter, RBCs detach from phagocytes, which is speculated to depend upon *trans*-endocytosis of the CD47-SIRPα complex and/or actin polymerization into protrusions that “push” the RBC away [24,25]. With time, CD47 becomes oxidized and suffers conformational changes on the RBC surface, losing this anti-phagocytic function and resulting in phagocytic removal of senescent RBCs in the spleen [26]. This is not limited to CD47 expressed on RBCs: CD47 is rather ubiquitously expressed and, because of this, it acts as a marker of the self and exerts such anti-phagocytic function generically. Such is the case for interactions of phagocytes with CD47-expressing platelets, lymphocytes, tumor cells, hematopoietic stem cells, etc. [20] Owing to this, CD47 function is being explored for diverse treatments, e.g., through disruption of CD47-SIRPα interaction between phagocytes and cancer cells or modulation of tissue graft rejection [27,28].

In the context of drug delivery [29], murine macrophage cell cultures incubated in the presence of soluble CD47 were observed to exert lower phagocytosis of colloidal drug carriers added thereafter [30]. In addition, either the ectodomain of CD47 or specific peptides derived from its SIRPα-binding region have been coupled to the surface of micro- or nano-devices, which has then been shown to reduce phagocytosis and clearance of said devices both in cell cultures and laboratory animals [14,23,31]. A similar CD47-grafting strategy was applied to the surface of biomaterials used in implants, where it helped reduce neutrophil and macrophage attachment [28]. Therefore, CD47 coating is an attractive alternative to lower phagocytic clearance of drug NCs.

Nevertheless, previous pioneer works had focused on devices which were not actively targeted against cell-surface receptors meant to achieve specific binding and induction of endocytosis by target cells. Co-coating of CD47 and affinity moieties used for targeting, e.g., antibodies, could impact the anti-phagocytic activity of CD47 and/or the induction of specific endocytic transport by the intended cells. For instance, presence of targeting antibodies on CD47-coated NCs could exacerbate FcR-mediated signaling toward phagocytosis by macrophages, dendritic cells, etc., since this process finely depends on the balance between pro-phagocytic and anti-phagocytic signals [14]. In some other cases, phagocytic cells themselves express common targets selected for drug delivery, such as transferrin receptor, folate receptor, etc. [32,33] Hence, said specific interaction of NCs with macrophages are likely to drive uptake via non-phagocytic routes, e.g., clathrin or caveolar-like endocytosis associated to these receptors [12,34]. Additionally, although CD47 exerts its anti-phagocytic signal on macrophages by binding to SIRPα, which is not expressed on most tissue cells, CD47 can also bind to other surface proteins, including thrombospondin, integrins, etc. [20] Since these molecules are present on other body cells, CD47 may negatively impact the intended endocytosis induced by targeting moieties on NCs. Therefore, both the effect of targeting moieties on CD47 anti-phagocytic function, and the effect of CD47 on the endocytic function of targeting moieties is unknown and unpredictable.

The goal of this study was to examine whether co-coating of CD47 with targeting moieties on the surface of drug NCs would provide reduced phagocytosis by macrophages involved in clearance while still enabling specific binding and uptake by target cells. Our results in cell culture and *in vivo* support this hypothesis.

2. Methods

Antibodies and Reagents. Monoclonal antibodies recognizing human or murine intercellular adhesion molecule 1 (ICAM-1), a cell-surface receptor overexpressed in many diseases [35], were from hybridomas from American Type Culture Collection (Manassas, VA). Recombinant human or murine CD47, tagged with polyhistidine or human Fc, were from LD Biopharma Inc. (San Diego, CA) and Creative Biomart (Shirley, NY), respectively. Antibodies to thrombospondin-1 (TSP-1) and integrin $\alpha_v\beta_3$ were from ThermoFisher Scientific (Waltham, MA) and R&D systems (Minneapolis, MN) respectively. Non-specific murine IgG and secondary antibodies (Texas Red- and Alexa Fluor 350-labeled goat anti-mouse IgG) were from Jackson Immuno Research (West Grove, PA). Bovine serum albumin (BSA), herein referred to as albumin, was from Fisher Scientific (Kerrville, TX). Green fluorescent Fluorespbrite®-labeled polystyrene beads of 100 nm or 1 μm in diameter were from Polysciences Inc. (Warrington, PA). PLGA (Poly (D,L-lactide-*co*-glycolic acid)) was from Lakeshore Biomaterials (Birmingham, AL). ^{125}Iodine (^{125}I) and ^{131}Iodine (^{131}I) were from Perkin-Elmer (Waltham, MA) and Pierce iodination tubes were from Thermo Scientific (Rockford, IL). Media and supplements for cell culture were from Cellgro (Manassas, VA), Gibco BRL (Grand Island, NY), EMD Millipore Corporation (Billerica, MA) or Sigma Aldrich (St. Louis, MO). All other reagents were from Sigma (St. Louis, MO), unless otherwise noted.

Preparation of coated micro- and nano-particles. PLGA NCs were prepared by nanoprecipitation and solvent evaporation, as in our previous publications [36]. An organic phase of acetone containing 19 mg/mL PLGA (50:50 copolymer ratio; 32 kDa average molecular weight) and 1 mg/mL FITC was added under agitation into an aqueous phase, and the emulsion was stirred for 16 h to allow evaporation of the organic solvent. The resulting NC suspension was filtered, dialyzed, and concentrated in a rotary