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Polymer Brain-Nanotherapeutics for Multipronged Inhibition of Microglial α -Synuclein Aggregation, Activation, and Neurotoxicity

Neal K. Bennett¹, Rebecca Chmielowski¹, Dalia S. Abdelhamid², Jonathan J. Faig², Nicola Francis^{1,3}, Jean Baum², Zhiping P. Pang³, Kathryn E. Uhrich², and Prabhas V. Moghe^{1,4,*}

¹Department of Biomedical Engineering, Rutgers University, 599 Taylor Road, Piscataway, NJ 08854

²Department of Chemistry & Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854

³Department of Neuroscience and Cell Biology, Child Health Institute of New Jersey, Rutgers Robert Wood Johnson Medical School, 89 French Street, New Brunswick, NJ 08854

⁴Department of Chemical & Biochemical Engineering, Rutgers University, 98 Brett Road, Piscataway, NJ 08854

Abstract

Neuroinflammation, a common neuropathologic feature of neurodegenerative disorders including Parkinson disease (PD), is frequently exacerbated by microglial activation. The extracellular protein α -synuclein (ASYN), whose aggregation is characteristic of PD, remains a key therapeutic target, but the control of synuclein trafficking and aggregation within microglia has been challenging. First, we established that microglial internalization of monomeric ASYN was mediated by scavenger receptors (SR), CD36 and SRA1, and was rapidly accompanied by the formation of ASYN oligomers. Next, we designed a nanotechnology approach to regulate SR-mediated intracellular ASYN trafficking within microglia. We synthesized mucic acid-derivatized sugar-based amphiphilic molecules (AM) with optimal stereochemistry, rigidity, and charge for enhanced dual binding affinity to SRs and fabricated serum-stable nanoparticles via flash nanoprecipitation comprising hydrophobe cores and amphiphile shells. Treatment of microglia with AM nanoparticles decreased monomeric ASYN internalization and intracellular ASYN oligomer formation. We then engineered composite deactivating NPs with dual character, namely shell-based SR-binding amphiphiles, and core-based antioxidant poly (ferrulic acid), to investigate concerted inhibition of oxidative activation. In ASYN-challenged microglia treated with NPs, we observed decreased ASYN-mediated acute microglial activation and diminished microglial neurotoxicity caused by exposure to aggregated ASYN. When the composite NPs were

*Corresponding Author: Prabhas Moghe, Distinguished Professor of Biomedical Engineering, 599 Taylor Road, Piscataway, NJ 08854, USA, Ph: 908-230-0147, moghe@rutgers.edu.

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administered *in vivo* within the substantia nigra of fibrillar ASYN-challenged wild type mice, there was marked attenuation of activated microglia. Overall, SR-targeting AM nanotechnology represents a novel paradigm in alleviating microglial activation in the context of synucleinopathies like PD and other neurodegenerative diseases.

Graphical Abstract

Keywords

Neurodegenerative Diseases; Parkinson's Disease; Synuclein; Microglia; Scavenger Receptor; Brain Health; Nanotechnology

1. Introduction

Aggregated and increased levels of extracellular α -synuclein (ASYN) are characteristic of Parkinson's disease (PD). As the primary cell type to rapidly internalize and degrade ASYN, microglia are a natural target for regulating ASYN trafficking and associated neuroinflammation¹. While microglia play a role in the physiological clearing of ASYN, disruptions to the lysosomal clearance pathways caused by overabundance of ASYN² or by common mutations associated with idiopathic and familial PD³ result in dramatic increases in extracellularly released ASYN⁴. Elevated levels of extracellular ASYN could in turn contribute to the interneuronal spreading of pathological ASYN species, particularly since neuron-to-glia transmitted ASYN has been observed to form pathogenic aggregates⁵. A considerable body of work also indicates that intercellularly transmitted ASYN from neuron-to-glia triggers microglial activation⁵⁻⁶. The result of chronic and excessive ASYN exposure and microglial activation is the secretion of neurotoxic substances including reactive oxygen species⁷. Sustained microglial activation has a disproportionate influence on dopaminergic (DA) neuronal loss, given the relative abundance (4.5 fold microglia relative to neurons)⁸ of microglia within the substantia nigra (SN), a prominent site of DA neuronal cell death, and is exacerbated by the inherently low antioxidant capacity of DA neurons. As DA neurons die, further ASYN monomers and aggregates are released into the extracellular space, propagating a cycle of microgliosis and neurotoxicity. Preventing initial ASYN-mediated microglial activation and ASYN aggregation during PD pathogenesis are two key precursor events that could halt the progressive loss of DA neurons.

A number of factors that are known to enhance the intracellular ASYN aggregation, including low local pH⁹, high calcium concentrations¹⁰, restricted space and high macromolecule concentrations¹¹, are all characteristic of the vesicular space and account for why ASYN within intracellular vesicles has been observed to be especially prone to aggregation¹². In order to reduce intracellular ASYN aggregation, it would then be desirable to identify and disrupt receptor-mediated internalization by microglia. Multiple

membrane receptors, including toll-like receptors ¹³, scavenger receptors ^{6c, 14}, and integrin MAC1 ¹⁵ have been implicated in microglial interactions with pathological proteins and activation in neurodegenerative disease. While TLR4 ¹⁶, TLR2 ^{13b}, and MAC1 ¹⁷ have been implicated as receptors that interact with ASYN, scavenger receptors have remained relatively unexplored. SRA1 and CD36 have been noted to mediate microglial interaction and activation by amyloid β ^{14b, 18}, which forms similar fibrillar structures as ASYN ¹⁹. We hypothesized that scavenger receptors are a key target for regulating ASYN trafficking and reducing intracellular ASYN aggregation.

Research has been done previously to develop synthetic compounds that mimic the charge and hydrophobicity of scavenger receptor-binding ligands, including using sulfatide derivatives ²⁰ and modified synthetic phospholipids ²¹. Using amphiphilic macromolecules (AMs) as biomimetic synthetic ligands based on sugar-based backbone, aliphatic side chains, and a hydrophilic poly(ethylene glycol) (PEG) tail ²², new structure-activity relations indicated that optimal hydrophobicity, stereochemistry, and charge promote binding affinity to scavenger receptors SRA1 and CD36 ²³. Due to their amphiphilic nature, these molecules can be complexed around hydrophobic core solutes via kinetic flash nanoprecipitation techniques, forming nanoparticles (NP) with the potential for drug encapsulation and demonstrated resistance to AM release in serum-rich environments ²⁴. In this study, we advance microglial scavenger receptor-mediated internalization of ASYN as a therapeutic target in the pathway of ASYN aggregate formation and propagation.

The overall paradigm we investigated was the possible role of scavenger receptor-binding NPs in modulating the internalization of ASYN in microglia and the subsequent intracellular accumulation of aggregated forms of ASYN (Figure 1). Our central hypothesis is that by targeting SR-expressing microglia, the AM NPs will counteract the microglial-mediated conversion of ASYN to higher order oligomers and fibrillar aggregates, whose transmission to neurons is a major risk factor in neuroinflammatory pathways. Given the acute role of microglia in elevating oxidative damage to neurons, we also engineered composite deactivating NPs (**CODE NPs**) via a concerted approach to introduce antioxidant polymers via NPs. In the process of microglial activation, intracellular reactive oxygen species (ROS) production is known to be a critical regulator ²⁵. While ASYN phagocytosis alone has been shown to result in microglial production of intracellular ROS ^{6b}, internalized exogenous ASYN can result in ROS production through other means as well, including by disruptions in mitochondrial function ²⁶. While antioxidant therapies have shown promise in PD ²⁷, their efficacy may be limited by factors including stability and ability to localize to relevant brain regions and cell types. Our research group has previously created polymeric and diacid forms of the antioxidant ferulic acid, which have demonstrated sustained antioxidant release with little bioactive decomposition ²⁸. We hypothesize that the scavenger receptor-based presentation of these stabilized antioxidants will concertedly ameliorate ASYN-induced microglial activation (Figure 1). We demonstrate these phenomena using a series of NPs with graded SR-binding in cultured microglia and *in vivo* following intracranial injections of NPs after administration of ASYN. Such CODE NPs are the first generation therapeutic candidates to manage ASYN aggregation/transmission (long-term) and ameliorate oxidative stresses (short-term) within neurodegenerative pathophysiology.

2. Results

2.1 Scavenger Receptors as Co-ordinated Binding Targets for α -Synuclein

We confirmed the binding ability of two of the key scavenger receptors in microglia, SRA1 and CD36, for monomeric ASYN using a ligand-receptor binding assay. Monomeric ASYN was observed to bind to both SRA1 and CD36, with dissociation constant values of 177 and 101 nM, respectively (Figure 2A), assuming a 1:1 binding stoichiometry. The fluorescence of the saturation plateau of monomeric ASYN binding to SRA1 was observed to be roughly twice that of CD36 (Supplementary Figure 1), which may suggest a binding stoichiometry of monomeric ASYN to SRA1 of greater than 1:1, however, this could be a result of differences in available ASYN antibody binding sites between the ASYN-SRA1 and ASYN-CD36 complexes. The ASYN-receptor interactions were also observed in a cellular context; when rat primary microglia were plated onto non-adhesive surfaces coated with monomeric ASYN, cellular attachment was significantly improved compared to nonadhesive surfaces alone, and attachment was significantly decreased in the presence of SRA1 or CD36 targeting antibodies (Figure 2B). Rat primary microglia populations isolated from rat pup cortices by mechanical agitation and used in these and later experiments were found to be highly pure, with few non-microglial cells present (Supplementary Figure 2).

Next, we investigated whether scavenger receptors mediated ASYN internalization. Quantitative fluorescence intensity from internalized Dy594-labelled synuclein was significantly decreased following the antibody-based blocking of individual scavenger receptors (Figure 2C-2D). Monomeric ASYN incubated with primary microglia, followed by Triton-X permeabilization, fixation, and staining for ASYN showed minimal observable fluorescence (Supplementary Figure 3), further suggesting that the fluorescence observed was primarily intracellular rather than cell-surface bound. Based on these results, we proposed a nanoparticle (NP) therapeutic with multiple scavenger-receptor binding capability as a design approach to regulate synuclein trafficking while enabling microglia-specific delivery of therapeutic molecules.

2.2 Amphiphilic Macromolecules (AMs) Lower α -Synuclein Uptake Competitively and Attenuate α -Synuclein Oligomerization in Microglia

Next, we investigated the molecules from the scavenger receptor-binding AM library, which most compete with ASYN uptake. Rat primary microglia were co-incubated with Dy594-labeled synuclein and NPs comprised of different shell based AM molecules. Based on this selected screening, we observed that the molecule M12-P5 (abbreviated as 1cM) (Figure 3A) within the shell layer of NPs elicited the greatest reduction in the uptake of synuclein (Figure 3B). All NPs were used at the equivalent concentrations, with identical polystyrene cores (to present “biologically inactive cores”), and all had comparable shell molecule mass concentrations. As an additional validation, the NPs were evaluated as substrates for competitive inhibition of microglial binding to ASYN; here, 1cM shell NPs significantly decreased microglial adhesion to ASYN coated surfaces, similar to the effects of scavenger receptor antibodies (Figure 3C).

Next, we explored the role of NPs to modulate the ASYN content of microglia. Treatment with scavenger receptor-targeting NPs was observed to lower the presence of intracellular ASYN aggregates, particularly oligomers, in monomeric ASYN treated primary microglia (Figure 3D, full gel in Supplementary Figure 4A). Oligomeric forms of ASYN were not detected in the extracellular media containing ASYN incubated for 24 hours at 37°C (Supplementary Figure 4B), so the oligomers were cell-associated. Further, this data indicates that the formation of intracellular ASYN oligomers in microglia results from high internalization of monomeric ASYN, and that multi-receptor regulation of trafficking decreased intracellular ASYN aggregation.

The key shell chemistry of the AM with meso stereochemistry M₁₂PEG (abbreviated as 1cM, to indicate the anionic carboxylic acid-functionalized mucic acid backbone) has been observed to bind scavenger receptors SRA1 and CD36²⁹. Thus, the scavenger receptor-targeting behavior of 1cM can confer preferential targeting to microglia, which highly express scavenger receptors³⁰. To test this, NPs comprised of Alexa Fluor 680-conjugated 1cM shell and polystyrene core were incubated for 24 hours with mixed population of primary rat microglia and SH-SY5Y human neuroblastoma cells, labeled with CellTracker Green and Red, respectively. Microglia in culture were visualized to contain both CellTracker dyes, presumably due to phagocytosis of cell debris. Fluorescently labeled NPs were observed to localize primarily to microglia in mixed culture (Supplementary Figure 5). This SR-based targeting of microglia by NPs enables control of synuclein trafficking as well as delivery of additional pharmacologic factors that could further decrease microglial activation, as designed next.

2.3 Composite-Deactivating (CODE) NPs with Synuclein-Regulating Shell and Antioxidative Core Ameliorate Activation and Neurotoxicity

Microglia are known to increase production of ROS in early stages of activation^{6b}, and antioxidant therapies have shown promise in PD²⁷. Therefore, we first investigated effects on activation in primary rat microglia incubated with NPs made with different ferulic acid-derived core molecules. These molecules were selected from a limited screen of additional ferulic acid-derived molecules and other antioxidants (Supplementary Figure 6). We observed that antioxidant core selection affected microglial activation response.

Antioxidant ferulic acid adipic polymer (PFAA) significantly decreased intracellular ROS production in microglia exposed to monomeric ASYN, while its non-polymeric form, ferulic acid (adipic) diacid (FAA acid) did not (Figure 4). These differences in detected intracellular ROS production are likely not due to changes in microglial viability (Supplementary Figure 7A) or microglial proliferation or aggregation (Supplementary Figure 7B). Notably, the addition of PFAA in the core of NPs did not lower the ability of the AMs to decrease the presence of intracellular ASYN oligomers in monomeric ASYN treated microglia (Supplementary Figure 4A). Further, the addition of non-nanoparticle-based administration of PFAA in suspension did not affect monomeric ASYN-mediated ROS production, and did not reduce microglial neurotoxicity (data not shown), indicating the unique role of nanoparticle-based targeting and activity following cellular uptake.

It is well documented that aggregated forms of ASYN, particularly oligomers, cause increased microglial activation^{13b}, neuronal damage^{6d, 31} and neurotoxicity³². Fibrillar ASYN and Lewy bodies are reported to be generally more structurally stable and have decreased toxicity³³ compared to oligomers, while fibrillization-impaired, oligomer-producing mutant mice displayed significantly more neurotoxicity^{32a}. Therefore, it has been proposed that stabilization of the fibrillization pathway, encouraging Lewy body or fibrillar sequestration of oligomers could be of therapeutic interest³⁴. For this reason, we examined the ability of scavenger-targeting NPs to moderate the trafficking of higher order forms of ASYN. Primary microglia co-treated with NPs and 71 µg/mL ASYN fibrils had a dramatic effect on reducing fibrillar ASYN binding or internalization, compared to untreated or scavenger receptor antibody-treated controls (Figure 5A). Extracellular staining of microglia incubated with the same mass concentration of ASYN monomers (71 µg/mL or approximately 5 µM) is depicted as well for comparison. ASYN fibrillization was monitored using Thioflavin-T fluorescence, and bound or internalized ASYN was confirmed to be in the form of Triton-X insoluble fibrils (Supplementary Figure 3). Antioxidant-loaded NPs were observed to similarly decrease fibrillar ASYN-mediated microglial activation, quantified by intracellular ROS production (Figure 5B). In these experiments, it should be noted that the antioxidant-loaded NPs had a more significant reduction in fibrillar ASYN-mediated intracellular ROS production compared to monomeric ASYN-mediated intracellular ROS production.

A conditioned media experiment based on BV2 immortalized microglia and SH-SY5Y neuroblastoma was used to recapitulate an advanced PD state. BV2 microglia have been observed to behave similarly to activated microglia³⁵, while SH-SY5Y cells have been commonly used as dopaminergic model cells in neurotoxicity studies³⁶. BV2 microglia were treated with SR-targeting antibodies or antioxidant loaded nanoparticles, or non-SR targeting and non-antioxidant loaded NPs for 24 hours, followed by co-treatment with either 20 µM monomeric ASYN³⁷ or the same mass of fibrillar ASYN. SH-SY5Y cells were treated with media conditioned by the ASYN- or LPS-stimulated microglial cells for 24 hours. The SR-targeted, antioxidant loaded particles significantly reduced neurotoxicity from fibrillar ASYN but not monomer-mediated microglial activation (Figure 5C). Treatment of BV2 microglia with non-NP based administration of PFAA did not appear to counteract activation caused by monomeric or fibrillar ASYN (Supplementary Figure 8A). Further, 24 hour treatment with residual fibrillar ASYN in conditioned media was not a significant contributor to toxicity in SH-SY5Y cells (Supplementary Figure 8B).

Finally, we investigated whether SR-targeting, antioxidant-delivering CODE NPs could reduce microglial activation in response to aggregated ASYN species *in vivo*. 1cM shell PFAA core NPs were co-injected with ASYN fibrils into mouse substantia nigra pars compacta (SNpc), and resulting activated microglia were quantified, compared to mice with co-injected PSPEG shell PS core NPs (non-SR-targeting, non-antioxidant core NPs) and ASYN fibrils, and ASYN fibrils alone. 1cM shell PFAA core NPs significantly decreased the presence of activated microglia, visualized by staining for ED1, in the vicinity of co-injected fibrillar ASYN, relative to mice that received either PSPEG shell PS core NPs or no NP treatment (Figure 6A–B, Supplementary Figure 9). No fibrillar ASYN or activated

microglial recruitment was observed in sham-injected mouse hemispheres (Supplementary Figure 10).

3. Discussion

While a number of promising strategies are currently in use to ameliorate the motor deficits that occur in PD, there have been limited efforts to develop therapies addressing the progressive nature of the disease due to gradual loss of SN DA neurons. Despite improvements in symptoms, synucleinopathies, which involves in neuroinflammatory activation of microglial cells, may continue to propagate and causes cell toxicity. We hypothesized that microglia, a key cell in the trafficking of ASYN, would be a valuable cellular target in the brain in order to address nanotherapeutics to counteract persistent ASYN-mediated microglial activation and ASYN aggregation.

In this study, we demonstrated via several complementary experimental methods that monomeric ASYN associates with and is internalized by microglial scavenger receptors SRA1 and CD36. Others have examined the role of CD36 as a mediator of microglial activation via ASYN. Su et. al. have demonstrated that human ASYN overexpressing mice have increased levels of activated microglia^{6c}. Further, they have isolated microglia from CD36-deficient mice and found attenuated activation response to ASYN. Despite these efforts and the knowledge of other receptors such as Toll-like receptor^{13b} or macrophage antigen-1¹⁷ that mediate microglial activation by ASYN, there has been little validation on other scavenger receptors and few efforts developing therapeutics targeting multiple receptors responsible for microglial interaction with ASYN.

Our studies offer valuable insight into the nature of interactions between monomeric ASYN and scavenger receptors. M12-P5 (1cM), which has a mucic acid backbone and four aliphatic chains and relatively higher lipophilic character, more significantly reduced monomeric ASYN uptake by microglia, compared to AMs with a tartaric acid backbone and two aliphatic chains, molecules 16 and 17. Both T12P5meso and 1cM have acidic end groups, and both significantly reduced monomeric ASYN internalization by microglia, which may also suggest that cationic domains on scavenger receptors mediate interactions with ASYN. Since T12P5meso significantly reduced monomeric ASYN internalization while T12P5L did not, these findings suggest that stereochemistry significantly impacts the ability of AMs to competitively bind scavenger receptors. Further study into the nature of interactions between ASYN and scavenger receptors, including stoichiometry and immediate downstream effects, may be valuable areas of investigation.

Our studies indicate that intracellular oligomerization of ASYN may be facilitated by internalized receptors. The observation that both scavenger receptor-targeting antibodies and AMs decreased the presence of intracellular ASYN oligomers suggests that scavenger receptors may play a role in the initiation of oligomerization. Our data reveals that while a multiple scavenger receptor-binding AM decreases internalized monomeric ASYN to similar levels as using multiple scavenger receptor-binding antibodies, NPs formed from these AMs have a more dramatic effect on decreasing the formation of intracellular ASYN oligomers. Key differences between these AMs and scavenger receptor antibodies include charge

properties, lipophilicity, and binding sites on scavenger receptors. It is possible that AMs and antibodies may block binding sites for extracellular ASYN, but due to these differences, only the AMs appear to block epitopes on the internalized scavenger receptors that contributes to intracellular ASYN oligomerization. It has recently been discovered that amyloid- β stimulates activation of microglia through a heterotrimer of CD36, TLR4, and TLR6³⁸. Some similar receptor complex may play a role in the recognition and binding of ASYN, and even in intracellular oligomerization. The charge, lipophilic, or binding character of AMs may also likely prevent the formation of complexes of multiple ASYN-binding receptors to a greater degree than scavenger receptor-binding antibodies.

Multipronged scavenger receptor-targeting NPs displayed a number of key effects on aggregated ASYN trafficking. NPs were observed to have more of an effect than antibody treatments in reducing extracellular bound or internalized ASYN fibrils. Others have claimed that fibrils are a less toxic form of ASYN compared to oligomers³³, and that a possible therapeutic strategy may be to encourage sequestration of ASYN in higher order fibrillar forms to decrease the presence of oligomeric species³⁴. By reducing the binding of fibrillar ASYN to the surface of microglia, these NP treatments may encourage the increased fibrillization of ASYN in the extracellular space. A possible mechanism for 1cM NPs achieving these effects may be due to the multimeric or charged nature of the NPs, such that they are able to influence a larger region of the cell surface, including other cell surface receptors, besides the individual scavenger receptor to which a single 1cM molecule may bind. As mentioned previously, the NPs may also be able to prevent the formation of multi-receptor complexes, which may play a role in the binding of ASYN fibrils. Further investigation may be warranted to elucidate the nature of interactions between multimeric NPs, cell surface receptors, and multimeric receptor substrates.

A key advantage of the NP formulation was the ability to target delivery of hydrophobic core molecules to microglia. Previous attempts to regulate inflammation in models of PD through pharmacological inhibition of microglia using myeloperoxidase inhibitors, minocycline, or nonsteroidal anti-inflammatory agents have shown inconsistent results^{7, 39}. However, it may be that activation pathways specific to PD or ASYN must be targeted for enhanced therapeutic effect. We additionally observed that different antioxidant molecules had differential efficacy at reducing monomeric ASYN-mediated microglial activation. Some of the differences in efficacy may be attributable to antioxidant stability as well, as we have observed that the chemical structure of PFAA allows for an extremely slow release of ferulic acid over months of storage at 2–8 degrees Celsius (data not shown). The ferulic acid diacid core NPs were observed to be much larger in size (~600 nm) compared to the antioxidant polymer NPs (~200 nm), which could result in different interactions with microglial scavenger receptors, and different stability between formulations. Finally, we demonstrated that the targeted delivery of a ferulic acid-derived antioxidant was able to decrease the presence of activated microglia in the vicinity of fibrillar ASYN *in vivo*. Further studies could investigate the efficacy *in vivo* of this NP formulation in more closely disease-relevant conditions, including the ability to decrease microglial activation in response to other pathogenic forms of ASYN like oligomers, or the ability to decrease longer term, more chronic microglial activation in response to ASYN. This approach could form a welcome supplement to other promising disease-modifying strategies including cell replacement

therapies⁴⁰ by possibly slowing either neurotoxic microglial effects or propagation of synucleinopathy to grafted tissue. While our animal studies demonstrated acute efficacy with intranigral stereotactic drug administration, systemic administration may be more desirable, as there is greater ease of repeat administration and less risk of infection and less need for costly, technically demanding surgical procedures⁴¹. Future studies may focus on incorporation of chemical modifications to our AMs, such as quaternary ammonium groups⁴², that may facilitate crossing of the blood-brain barrier, while hopefully retaining microglia- and scavenger receptor-targeting ability.

In summary, scavenger receptor targeted nanoparticles can be designed to concertedly regulate two critical phenomena underlying synucleinopathies, the intracellular conversion of α -synuclein to oligomers upon microglial clearance, which may be a key route to export and transmission of synuclein aggregates, leading to chronic neurotoxicity; and the acute management of hyperoxidative damage caused by microglia to neurons.

4. Materials and Methods

Cell culture

Rat primary microglia were kindly provided by the lab of Dr. David Shreiber in the form of mixed glial populations. Microglia were isolated from mixed glial populations by mechanical dissociation, using a rotating shaker at 200 rpm for 30 minutes, followed by centrifugation of cell media. For all experiments, primary microglia were plated at a density of 50,000 cells per square centimeter and cultured in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals). BV2 microglia were kindly provided by Drs. Bin Liu (University of Florida) and Jason Richardson (Northeast Ohio Medical University). BV2 microglia and SH-SY5Y (ATCC) were also both cultured in DMEM/F12 supplemented with 10% fetal bovine serum.

Pull-down assay

Histidine-tagged recombinant Human SRA1 protein (R&D) and CD36 (Sino Biological) were prepared to a final concentration of 20 $\mu\text{g/mL}$ in PBS supplemented with 5 mM imidazole (Sigma). Nickel coated plates (Fisher Scientific) were washed three times with PBS, followed by addition of 50 μl (1 μg) of scavenger receptor protein solutions to each well and incubation for 1 hour at room temperature with 300 rpm agitation on a rotary plate shaker. In order to prevent non-specific binding of ASYN to the nickel plate⁴³, the plates were incubated with 5% bovine serum albumin (BSA) (Sigma) for 1 hour at room temperature with 300 rpm agitation. After three washes with PBS, ASYN (rPeptide) was added in PBS at seven concentrations from 3.33 μM to 0 μM into wells either coated or uncoated with scavenger receptor proteins and blocked with BSA, and incubated overnight at room temperature with 300 rpm agitation. The plate was washed three times with PBS, followed by incubation with a primary antibody against ASYN in PBS, and incubated for one hour at room temperature with 300 rpm agitation. After three washes with PBS, the plate was incubated with a fluorescent secondary antibody in PBS overnight at room temperature with 300 rpm agitation. After three washes with PBS, the fluorescence intensity in each well was measured using a Tecan Infinite M200 Pro microplate reader. Fluorescence

in wells blocked with BSA and uncoated with scavenger receptors was subtracted from corresponding wells that were coated with scavenger receptors.

Amphiphilic molecule synthesis

Macromolecules 1cM and M12 were synthesized as previously detailed and characterized using established techniques including ¹H NMR-spectroscopy, gel permeation chromatography, differential scanning calorimetry, and dynamic light scattering ⁴⁴. The critical micelle concentration, size, and charge data has been published in the literature ⁴⁵.

Antioxidant molecule synthesis

Ferulic acid-based derivatives (Acid FAA and FADG) were synthesized and characterized following previously developed methods ²⁸. Upon Acid FAA and FADG isolation, polymers were synthesized using a modified solution polymerization method ⁴⁶. In brief, ferulic acid-based derivative (1 equiv. Acid FAA or Acid FADG) was suspended in anhydrous dichloromethane (DCM) under argon. Triethylamine (4.4 equiv) was added, to acquire a pale yellow solution, and the reaction cooled to 0 °C. Triphosgene (1 equiv, dissolved in anhydrous DCM to acquire a 15 % w/v solution) was added drop-wise over 30 minutes. After stirring 4 h, the reaction was quenched with 1 N hydrochloric acid (50 mL) and allowed to stir for 15 minutes. The organic layer was then passed through a MgSO₄-packed gravity filter, collected in a round bottom flask, concentrated *in vacuo* to ~10 mL, and precipitated over 400 mL chilled diethyl ether. Pure polymer was isolated via vacuum filtration and dried under vacuum overnight. PFAA possessed a molecular weight of 44 kDa, a polydispersity index of 1.3, and a T_g of 84 °C. PFADG had a molecular weight of 26.2 kDa, a polydispersity index of 1.2, and a T_g of 102 °C. Further chemical analyses of these polymers is discussed by Ouimet *et al* ²⁸.

Nanoparticle fabrication

NPs were prepared via flash nanoprecipitation, characterized by dynamic light scattering to determine particle size and polydispersity, and tested for serum stability as described previously ^{22b, 24, 47}. In brief, a confined impinging jet mixer was utilized to mix 250 µL of an aqueous stream with 250 µL of 50/50 v/v% mixture of tetrahydrofuran (THF) (Sigma) and DMSO (Sigma) stream containing 40 mg/mL NP shell molecule, and 20 mg/mL hydrophobic core molecule. Upon mixing, the exit stream was immediately introduced into 4.5 mL of phosphate buffered saline (PBS) (PBS:THF of 9:1) and subsequently dialyzed against PBS to remove residual THF. Five percent of the mass of shell molecule was fluorescently labeled with Alexa Fluor 680 Succinimidyl Ester (Thermo Fischer) in order to facilitate detection *in vitro* and to determine sites of NP retention *in vivo*.

ASYN internalization

Rat-derived primary microglia were pre-incubated with scavenger receptor-targeting treatments, including either 10 µg/mL SRA1 (R&D) and CD36 (Abcam) antibodies or NPs for 24 hours, followed by 24 hour co-incubation with 5 µM monomeric ASYN (rPeptide), ⁴⁸ fluorescently labeled with DyLight 594 nm (Dy594) microscale antibody labeling kit (Thermo Fischer) or 5 µM monomer-equivalent fibrillar ASYN. For fibrillar ASYN studies,

cells were fixed prior to characterization by immunocytochemistry. For monomeric ASYN studies using live cells, extracellular non-internalized synuclein fluorescence quenched by adding 0.5 mg/mL trypan blue (VWR) for 30 minutes, followed by two washes with PBS⁴⁹. Live cells were imaged on a Leica SP2 confocal microscope using a 40 x dry objective. Intracellular fluorescence quantification was performed using NIH-ImageJ software (<http://rsb.info.nih.gov/ij/>), by measuring mean grey value in cells segmented by applying the same fluorescence thresholds to all collected images. For untreated cell controls, fluorescence images were segmented based on cell boundaries visualized in brightfield images.

Cellular adhesion assay

A 96-well non-tissue culture treated plate was coated with nonadherent matrix, 50 µg/mL collagen IV (Sigma) in distilled water at 37 degrees Celsius for 1 hour, washed once, and air-dried, followed by addition of 1 µg monomeric ASYN (rPeptide) and air-drying³⁰. 50,000 cells per well were plated in 100 µL media supplemented with 1 mg/mL BSA (Sigma) and placed in an incubator for 2 hours, either with or without the presence of scavenger receptor-binding competitors, including antibodies or NPs. The plate was then washed three times with PBS and the number of cells was determined using Alamar Blue assay (Life Technologies) and comparison to a standard curve of adherent cells detected using Alamar Blue.

iROS detection

Rat-derived primary microglia were pre-incubated with scavenger receptor-targeting treatments or NPs for 24 hours, followed by co-incubation with monomeric ASYN (rPeptide). After 24 hours of co-incubation, iROS production was quantified using a 2',7' - dichlorofluorescein diacetate (DCFDA) detection assay kit (Abcam). In brief, cells were washed with 10% FBS supplemented assay buffer, and if applicable, incubated with 50 µM tert-butyl hydrogen peroxide for 2 hours as a positive iROS production control. Cells were then stained with a 40 µM DCFDA solution in supplemented assay buffer for 30 minutes^{6b}, followed by 2 washes using supplemented assay buffer, followed by fluorescence measurement at 485 nm and 535 nm emission on a Tecan Infinite M200 Pro microplate reader. Fluorescence readings of supplemented assay buffer alone were subtracted from all measurements, and DCFDA fluorescence was normalized to untreated cell controls.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma) for 30 minutes at room temperature, or for studies using fibrillar ASYN, 4% paraformaldehyde, 4% sucrose (Sigma), and 1% Triton X-100 (Sigma) to solubilize monomeric ASYN. Cells were simultaneously blocked and permeabilized in blocking buffer containing PBS supplemented with 1% BSA, 5% normal goat serum (MP Biomedicals), and 0.1% Triton X-100 for 1 hour at room temperature. Primary antibodies were incubated in blocking buffer at 4 degrees Celsius overnight, followed by three 15 minute washes with PBS. Afterwards, cells were incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488, 594, or 647 from Life Technologies) in blocking buffer for 1 hour at room temperature, followed by three 15 minute washes with PBS. Cells were counter-stained with either BOBO-1 (Thermo Fischer) or 4'6-Diamidino-2-phenylindole dihydrochloride (Sigma) to visualize cell nuclei. For

studies where only extracellular or cell-surface species were imaged, Triton-X-100 was omitted from blocking buffer. Stained samples were imaged on a Leica SP2 laser scanning confocal microscope.

ASYN fibrillization

Fibrillization was carried out using 100 μ L of 1 mg/mL monomeric ASYN (rPeptide) in a 96-well plate (BD Falcon), with a 5 mm glass bead (Sigma) for nucleation, and agitated at 300 rpm at 37 degrees Celsius for 50 hours minimum on a Southwest Science Multi-Therm plate shaker⁵⁰. Plates were sealed using parafilm to minimize evaporation, and final volumes were corrected to initial volumes using sterile distilled water. Fibrillization was verified by sampling ASYN before and after fibrillization, by incubating 2 μ M ASYN solution in PBS with 40 μ M Thioflavin-T (Sigma) for 10 minutes at room temperature, followed by measuring fluorescence excitation and emission at 450 and 485 nm on a Tecan Infinite M200 Pro microplate reader.

Western Blotting

Intracellular ASYN was collected by incubating cells with RIPA lysis buffer and protease and phosphatase inhibitor cocktails (Pierce). Samples were mixed with Bolt LDS Sample Buffer and Bolt Sample Reducing Agent (Thermo Fischer), and heated for 10min at 70 degrees Celsius, then loaded onto a Bolt 4–12% Bis-Tris Plus gel (Thermo Fischer) and run for approximately 60 minutes at 150 V. Samples were transferred onto methanol-activated polyvinyl difluoride (PVDF) membrane (Thermo Fischer) in methanol-free transfer buffer (Pierce) at 300 mA for 90 minutes at 4 degrees Celsius. The PVDF membrane was then blocked in PBS with 5% by weight milk (Santa Cruz Biotechnology) and 0.05% by volume Tween-20 (Sigma) for 1 hour with agitation. Primary antibodies were added in blocking buffer at 4 degrees Celsius overnight without agitation. PVDF membranes were then washed three times for 10 minutes in PBS with 0.05% by volume Tween-20, followed by incubation with secondary antibodies in blocking buffer for 1 hour at room temperature. PVDF membranes were then washed three times for 10 minutes in PBS with 0.05% by volume Tween-20, followed by developing using incubation with ECL blotting solution (Pierce) for 3 minutes. PVDF membranes were then developed using Kodak Biomax Carestream light film (Sigma).

Neurotoxicity Assay

BV2 immortalized microglia and SH-SY5Y neuroblastoma were used for neurotoxicity experiments. BV2 microglia were plated at 50,000 cells per square centimeter, and SH-SY5Y were plated separately at 100,000 cells per square centimeter. Microglia were treated with SR-targeting antibodies or antioxidant loaded nanoparticles, or non-SR targeting and no-antioxidant loaded NPs for 24 hours, followed by co-treatment with either 1 μ g/mL LPS (Sigma), 20 μ M monomeric ASYN, which has been demonstrated to activate BV2 cells³⁷ or the same mass of fibrillar ASYN. SH-SY5Y cells were treated with media conditioned by the ASYN- or LPS-stimulated microglial cells for 24 hours. Cytotoxicity in response to stimulated microglia conditioned media was quantified in SH-SY5Y populations was quantified using a lactate dehydrogenase assay (Promega), normalized to a positive control of totally lysed SH-SY5Y cells, and to untreated BV2-conditioned SH-SY5Y cells.

Animal studies

All animal experiments were carried out according to the Rutgers University Policy on Animal Welfare and were approved by the Institutional Animal Care and Use Committee (IACUC) at Rutgers University Robert Wood Johnson Medical School. Wildtype C57BL/6 mice (Jackson Laboratory) were anesthetized with isoflurane (induction at 4% and maintained at 0.5–1% inhalation). ASYN fibrils were concentrated to 6 mg/mL using a 3 kDa MWCO Amicon centrifuge filter (Millipore). To maintain the same mass ratio between ASYN and NP shell molecule used in most previous experiments described, NP stock was concentrated to a final shell concentration of 5.6 mg/mL, using the same Amicon centrifuge filters. 1 μ L total injection volume was injected into the substantia nigra pars compacta for each mouse, either fibrillar ASYN alone (0.5 μ L 6 mg/mL fibrillar ASYN and 0.5 μ L saline), fibrillar ASYN and 1cM shell and PFAA core NPs (0.5 μ L 6 mg/mL fibrillar ASYN and 0.5 μ L NP), or fibrillar ASYN and PSPEG shell and PS core NPs (0.5 μ L 6 mg/mL fibrillar ASYN and 0.5 μ L NP), with 1 μ L saline injected on the opposite hemisphere using a 1 μ L gastight Hamilton syringe. Bilateral injections were made at the following coordinates (in mm): AP, -2.9 (from bregma); ML, ± 1.3 ; DV, -4.5 (from dura). Mice were sacrificed and processed for immunohistochemistry 48 hours after injection. Quantification of microglial recruitment and activation was performed by staining 50 μ m sections of brain slices. Four representative brain slices were quantified per animal.

Statistical analysis

All data are presented as mean $\pm$ s.d. Statistical significance is evaluated by single-factor ANOVA, with $P < 0.05$ considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the new nanotechnology paradigm for scavenger receptor-mediated management of α-synuclein uptake and aggregation to counteract neurotoxicity in neurodegenerative diseases like PD

We envision nanoparticles comprised of synthetic scavenger receptor-binding amphiphilic molecules to reduce both intracellular ASYN aggregation and microglial activation by regulating microglial interactions with monomeric and aggregated ASYN and delivering anti-inflammatory agents along these pathways, ultimately decreasing microglial-mediated neurotoxicity and synucleinopathy propagation.

Figure 2. Alpha-synuclein (ASYN) associates with scavenger receptors (SR)

A) Monomeric ASYN bound to histidine-tagged CD36 and SRA1 immobilized on a nickel-coated assay plate in a concentration dependent manner, with saturated binding at the two highest concentrations. N=3. B) Microglia seeded onto collagen IV and monomeric ASYN coated surfaces in the presence of SR-targeting antibodies had significantly reduced adherent cell numbers relative to microglia seeded onto collagen IV and monomeric ASYN coated surfaces without the addition of antibodies. Uncoated tissue culture plastic was used as a positive growth control, and collagen IV alone coated surfaces as a negative growth control. N=3, **P<0.01 by 1-way ANOVA. C) Live-cell fluorescence imaging and D) quantification of intracellular fluorescence of microglia cultured for 24 hours with SR-targeting antibodies, followed by 24 hour co-treatment with fluorescently labeled monomeric ASYN and either CD36 or SRA1 antibodies. Each SR-targeting antibody separately and together significantly reduced intracellular fluorescence, relative to microglia treated with both antibody isotype controls. Scale bar = 50 μ m. N=3, **P < 0.01, ***P<0.005 by 1-way ANOVA.

Figure 3. Amphiphilic molecule (AM) library screening reveals candidate molecules for regulating ASYN trafficking

A) The amphiphilic molecules tested in nanoparticle (NP) shell included polystyrene-PEG as well as M12P5 (abbreviated as 1cM), T12P5L and T12P5meso, who have similar sugar backbones with aliphatic branches and hydrophilic PEG chains. B) Rat primary microglia were pre-incubated with NPs comprised of candidate AM molecules in the shell and polystyrene (PS) in the core, followed by co-incubation of microglia with NPs and fluorescently labelled monomeric ASYN. Intracellular fluorescence for each condition was normalized to background fluorescence of untreated microglia and to monomeric ASYN-

only treated microglia. Microglial treatment with two AM shell NPs resulted in a significant decrease in intracellular fluorescent ASYN, with 1cM having a more significant effect relative to microglia treated with non SR-targeting polystyrene-polyethylene glycol (PSPEG) shell NPs. N=3, **P < 0.01, ***P<0.001, by 1-way ANOVA. C) Microglia seeded onto surfaces coated with collagen IV and monomeric ASYN in the presence of SR-targeting 1cM shell NPs had significantly reduced adherent cell numbers relative to microglia seeded onto surfaces coated with collagen IV and monomeric ASYN without the addition of NPs. Uncoated tissue culture plastic was used as a positive growth control, and collagen IV alone coated surfaces as a negative growth control. N=3, ***P<0.001 by 1-way ANOVA. D) Primary rat microglia treated with monomeric ASYN and SR-targeting NPs or antibodies were lysed after 24 hours in order to examine intracellular forms of ASYN. Oligomeric ASYN were detected within monomeric ASYN-treated microglia, and the presence of these species was decreased in SR antibody-treated microglia, and about absent in 1cM NP treated microglia. Monomeric ASYN is still internalized in the presence of SR-targeting antibodies.

Figure 4. Antioxidant delivery counters ASYN-mediated microglial activation

A) Microglia were treated with NPs comprised of 1cM shell and different antioxidant cores, including ferulic-acid derived molecules, and non-antioxidant control cores, the mucid acid backbone of 1cM, and polystyrene. B) After 24 hour co-treatment with NPs and monomeric ASYN on microglia, intracellular reactive oxygen species (iROS) was detected using DCFDA dye. Only the polymeric form of ferulic acid was found to significantly reduce monomeric ASYN-mediated increases in iROS. PFAA was used in subsequent studies, and 1cM shell/PFAA core NPs have been identified as a NP formulation capable of reducing ASYN monomer uptake and ASYN-induced microglial activation. N=3, ****P<0.0001 by 1-way ANOVA.

Figure 5. CODE NPs reduce ASYN-induced microglial neurotoxicity and intracellular formation of ASYN oligomers

A) Primary rat microglia were incubated with either SR-targeting antibodies or SR-targeting NPs, followed by co-incubation with SR-targeting molecules and monomeric ASYN or the same mass of fibrillar ASYN. Microglial cell surface-bound ASYN was visualized by immunofluorescence staining for ASYN (red) and microglial cell marker ED1 (green) and nuclear stain BOBO-1 in the absence of permeabilization agents. Scalebar = 200 μ m. B) Primary rat microglia were incubated with either SR-targeting Abs or NPs prior to co-incubation with monomeric ASYN or the same mass of fibrillar ASYN. Microglial activation in response to ASYN was quantified by measuring iROS relative to untreated microglia. ASYN monomers, fibrils, and tert-butyl hydroperoxide (TBHP) all caused high degrees of microglial activation. SR-targeting antioxidant containing NPs were found to reduce fibrillar ASYN-mediated microglial activation, while SR-targeting antibody treatments did not, relative to microglia treated with fibrillar ASYN alone. N=3, *P<0.05, **P<0.01 by 1-way ANOVA. C) BV2 microglia were treated with SR-targeting antibodies or antioxidant loaded nanoparticles, or non-SR targeting and no-antioxidant loaded NPs for 24 hours, followed by co-treatment with either 20 μ M monomeric ASYN or the same mass of fibrillar ASYN. SH-SY5Y cells were treated with media conditioned by the ASYN- or LPS-stimulated microglial cells. Cytotoxicity in response to stimulated microglia conditioned media was quantified in SH-SY5Y populations. Thus, the SR-targeted, antioxidant loaded CODE particles significantly reduce neurotoxicity from fibrillar ASYN but not monomer-mediated microglial activation. N=3, ***P<0.001 by 1-way ANOVA.

Figure 6. CODE NPs reduce microglial recruitment and activation in response to aggregated ASYN in vivo

A) Wildtype mice were injected in the substantia nigra pars compacta (SNpc) one hemisphere with 1 μ L saline and 1 μ L total injection volume of either 3 μ g fibrillar ASYN and saline, fibrillar ASYN with 1cM shell PFAA core NPs, or fibrillar ASYN with PSPEG shell PS core NPs. After two days, animals were perfused and brains were collected, sliced, and stained for ASYN (green), activated microglia marker ED1 (magenta), iNOS (red), and given nuclear stain BOBO-1 (blue). Three representative slices are depicted. Scale bar = 100 μ m. B) Four representative 50 μ m-thick brain slices were quantified per animal, and the number of ED1+ cells in the vicinity of ASYN staining were counted. There was a

significant decrease in the number of ED1+ cells located in the vicinity of ASYN fibrils injected into the SNpc. N=3 mice per condition, *P<0.05, **P<0.01 by 1-way ANOVA.

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